BIOCHEMICAL STUDIES ON DENITRIFICATION IN *PSEUDOMONAS DENITRIFICANS*

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SUMMARY

1. *Pseudomonas denitrificans* (A.T.C.C. 13876) grew aerobically with or without nitrate, and anaerobically with nitrate. It did not grow anaerobically in the absence of nitrate or with nitrite as the sole electron acceptor. Sodium nitrite up to a concentration of 2 gm/l had no effect on the aerobic growth rate.

2. The respiratory particles from oxygen-grown cells contained flavin, ubiquinone, and cytochromes *b*, *c*, and *a*. Respiratory particles from cells grown anaerobically on nitrate contained a 2-fold higher level of flavin, 70 per cent more cytochrome *b*, and 20 per cent more cytochrome *c*. No cytochrome *a* was detected in anaerobically grown cells. The ubiquinone level was unchanged.

3. Formate dehydrogenase activity in anaerobically grown cells was increased 8-fold over the level present in aerobically grown cells. Formate-nitrate reductase and $NADH_2$-nitrate reductase levels were increased 9-fold and 6-fold, respectively, by growing the cells anaerobically on nitrate. The succino-oxidase system which was highly active in aerobic cells was undetectable in nitrate-grown cells. Succinate was not active as an electron donor for nitrate reduction.

4. Inhibitors of the electron transport chain such as sodium amytal, rotenone, and piericidin A inhibited $NADH_2$ oxidation by both nitrate and oxygen. Antimycin A and 2-heptyl-4-hydroxyquinoline-N-oxide inhibited electron transfer from $NADH_2$ to oxygen, but not to nitrate.

5. Both $NADH_2$ and formate nitrate reductases were associated with the membrane-bound electron transport system. Nitrate was stoichiometrically reduced to nitrite. Inhibitor studies suggested the participation of flavin, ubiquinone, sulfhydryl groups, cytochrome b, and a metal (possibly molybdenum) in the electron transfer chain with nitrate as terminal acceptor.

6. Oxygen strongly inhibited formate-nitrate reductase, but had little effect on $NADH_2$-nitrate reductase. Nitrate inhibited electron transfer from $NADH_2$ to oxygen with a concomitant stoichiometric reduction of nitrate.

7. Chlorate and bromate were competitive inhibitors of nitrate reductase. Chlorate acted as an acceptor for the formate dehydrogenase system. Similarities in K_s and K_m values for chlorate with both the particulate and solubilized nitrate reductases, the competitive nature of chlorate inhibition, and similar patterns of inhibitions

of electron transfer with nitrate or chlorate as the acceptor suggest that chlorate acts as an alternative substrate for nitrate reductase.

8. Nitrate reductase was solubilised by deoxycholate treatment and purified about 60-fold. The purified enzyme which had a cytochrome *b* type absorption spectrum utilized reduced dyes such as benzyl viologen as electron donor, but was no longer active with the natural donors, formate and $NADH_2$. Cytochrome *c* reduced with ascorbate-TMPD did not serve as an electron donor. Metal chelating agents, especially those that bind molybdenum (dithiol and KCNS), and the -SH reagent PCMB were highly inhibitory.

9. Nitrate was stoichiometrically reduced to nitrite by both particulate and solubilised enzymes. The K_m for nitrate was 6.7×10^{-4} M with the solubilised enzyme.

10. Nitrite reductase was localised in the 144,000 x *g* supernatant fraction (144-S). Over 95 per cent of the total activity of the cell homogenates with either $NADH_2$ or reduced benzyl viologen as electron donor was found in the supernatant fraction.

11. The soluble nitrite reductase was purified 160-fold. The enzyme utilized reduced benzyl or methyl viologen,

leucomethylene blue, or reduced FMN as hydrogen donors. $NADH_2$ and $NADPH_2$ were active as electron donors only in the presence of a flavin co-factor. Riboflavin, FMN, or FAD was equally effective. Maximal activity with any of the three flavins occurred at about 10^{-4} M. Michaelis constants were: FMN, 1.7×10^{-5} M; FAD, 1.1×10^{-5} M; riboflavin, 2.0×10^{-5} M.

12. Nitrite was reduced stoichiometrically to nitric oxide. K_m for nitrite was 10^{-4} M with $NADH_2$ as the electron donor.

13. Inhibitor studies indicated the participation of a metal(s) and sulfhydryl groups in the reduction of nitrite. Nitrite reduction was also inhibited by aromatic nitro compounds. The inhibitory effect of 2,4-dinitrophenol was due to its chemical reduction by reduced flavin at the expense of the enzymic reduction of nitrite. The 2,4-dinitrophenol was reduced chemically by $FMNH_2$ to 2-amino-4-nitrophenol.

DECLARATION

I hereby declare that the work presented in this thesis has been performed by myself, except where otherwise stated in the text, and that it has not been submitted in any previous application for a degree.

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Professor D.J.D. Nicholas for his guidance and advice. I am also grateful to Dr. A. Snowball and Miss J. Durban for assistance in the final proof reading of this thesis, and to my husband, Dr. J.C. Radcliffe for his patience and encouragement. The financial assistance of a Commonwealth Postgraduate Award is gratefully acknowledged.

PREFACE

Part of the work described in this thesis has already been published:

BARBARA C. RADCLIFFE and D.J.D. NICHOLAS. Some Properties of a Nitrite Reductase from *Pseudomonas denitrificans*. Biochim. Biophys. Acta, **132** (1968) 545-554.

INTRODUCTION

The element nitrogen can occur in a variety of oxidation states ranging from +5 to -3 as shown in Table I. Nitrogen gas, nitrate, and ammonia are the forms most commonly found in nature.

Nitrate is widely distributed in soil, concentrated in geological nitrate deposits, and in rain and sea water. Nitrite occurs in association with nitrate, but in lesser amounts. Molecular nitrogen is the chief constituent of the earth's atmosphere which contains 78.10 per cent nitrogen by volume. Both hydroxylamine and hydrazine are labile compounds which are toxic to many forms of life and are not found in appreciable quantities in nature. Ammonia occurs in the atmosphere, water, soils, and muds as a product of the decay of organic matter.

Compounds of nitrogen are utilized by living cells in a variety of reactions that can be formulated into a simplified cyclic scheme as shown in Figure 1. This cycle maintains a suitable distribution of nitrogen for the maintenance of life and is equal in importance to the photosynthetic process which provides a fixed carbon source for plants and animals.

The processes of nitrogen fixation, nitrification,

TABLE I.

OXIDATION STATES OF NITROGEN

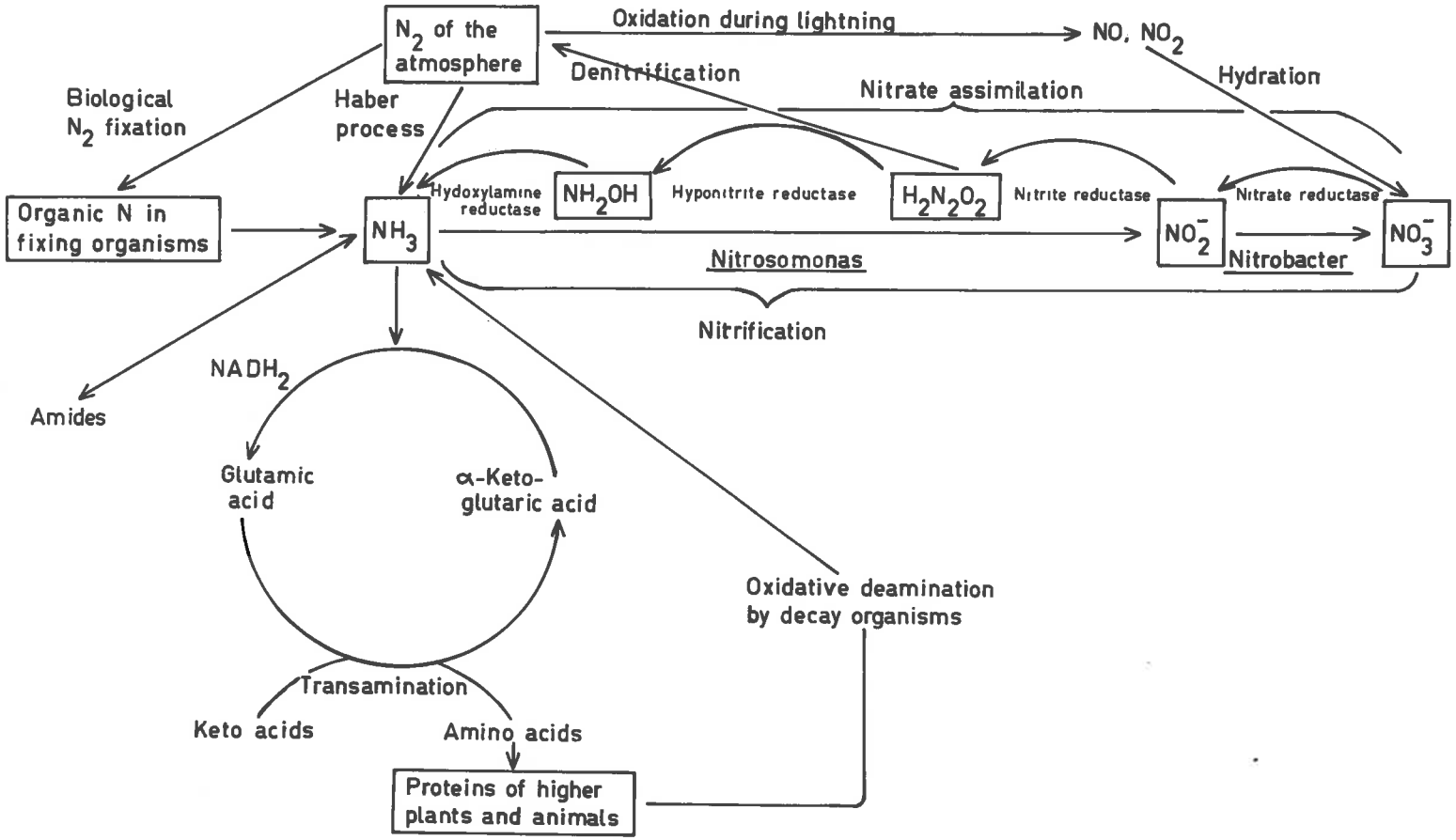
Oxidation-reduction state of nitrogen	Compound	Hydrate
+5	N_2O_5 (Nitrogen pentoxide)	HNO_3 (Nitric acid)
+4	NO_2 (Nitrogen dioxide)	-
+3	N_2O_3 (Nitrogen trioxide)	HNO_2 (Nitrous acid)
+2	NO (Nitric oxide)	-
	$(NOH)_2$ (Nitroxyl)	-
	$N_2O_3^{2-}$ (Nitro-hydroxylamate)	-
+1	N_2O (Nitrous acid)	$H_2N_2O_2$ (Hyponitrous acid)
0	$NO_2{\cdot}NH_2$ (Nitramido or imidonitric acid)	-
	$NH(OH)_2$ (Dihydroxy-ammonia)	-
-1	NH_2OH (Hydroxylamine)	-
-2	H_2N-NH_2 (Hydrazine)	-
-3	NH_3 (Ammonia)	-

FIGURE 1.

THE NITROGEN CYCLE

Schematic representation of the reactions involved in the nitrogen cycle (Conn and Stumpf, 1963).

THE NITROGEN CYCLE



nitrate assimilation, and denitrification are carried out by a variety of microorganisms which are affected in different ways by variations in environmental factors. Thus the information in Figure 1. can be somewhat misleading unless one keeps in mind that it gives no indication of the reaction rates of the various steps mediated by microorganisms.

I. METABOLISM OF NITRATE.

Most higher plants and some microorganisms metabolize nitrate, the most abundant form of nitrogen in the soil, and reduce it to ammonia which is then utilized in the formation of amino acids, proteins, nucleic acids, and other cell constituents. This process is known as nitrate assimilation, and provides nitrogen compounds of a type suitable for utilization by those animals and other organisms that are incapable of using inorganic nitrogen compounds as a sole source of nitrogen.

Other microorganisms utilize nitrate as the terminal electron acceptor in place of free oxygen. A number of workers have suggested classifications for the various types of nitrate reduction. As early as 1904, Jensen proposed five categories based on the products of the reaction.

In some cases nitrate acts as a non-essential acceptor that is reduced only as far as nitrite which accumulates in the medium. Verhoeven (1956) called this type of metabolism "incidental dissimilatory nitrate reduction", and differentiated it from "true dissimilatory nitrate reduction" as exhibited by some other microorganisms which reduce nitrate as an acceptor essential for anaerobic growth to nitrite or to a mixture of nitrogen gas and nitric and nitrous oxides.

Pewson and Nicholas (1961b) questioned whether any real distinction could be drawn between "incidental" and "true" dissimilatory nitrate reduction, and grouped both types under the heading "nitrate dissimilation or respiration". Denitrification is regarded by these workers as a special case of nitrate respiration, defined as the production of nitrogen gas or its oxides from either nitrate or nitrite.

Some organisms are capable of both assimilatory and dissimilatory nitrate reduction. Micrococcus denitrificans, for example, assimilates nitrate under both aerobic and anaerobic conditions, but dissimilates nitrate under anaerobic conditions only (Chang and Morris, 1962). The presence of ammonium ions partially inhibits the transformation of nitrate into cell nitrogen, but has no effect

on nitrate reductase activity.

A third type of nitrate metabolism occurs in strict anaerobes such as Clostridium welchii in which nitrate is reduced to nitrite without the participation of cytochromes. This process is known as nitrate fermentation.

A. Assimilatory Nitrate Reduction.

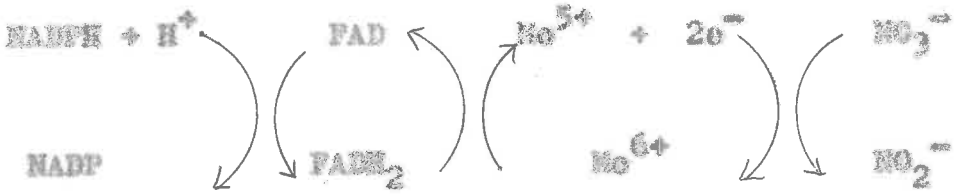
Nitrate assimilation requires an eight-electron reduction of nitrogen from the +5 to the -3 oxidation state. It has been suggested that this process occurs in a series of two-electron steps (Nicholas, 1963).

The assimilatory-type of nitrate reductase is widely distributed in both higher plants and in microorganisms. In higher plants it has been reported to occur in cauliflower (Candela, Fischer, and Hewitt, 1957), corn seedlings (Hageman and Flesher, 1960), tomato roots (Vaidyanathan and Street, 1957), bean cotyledons (Egami et al., 1957), and rice seedlings (Tang and Wu, 1957). In microorganisms assimilatory nitrate reductase has been found in the yeast Hansenula anomala (Silver, 1957), in nitrate grown Azotobacter (Taniguchi and Okunchi, 1960), Neurospora crassa (Nason and Evans, 1953), and in the green alga Ankistrodesmus braunii (Kessler, 1956).

1. Neurospora Nitrate Reductase.

The Neurospora enzyme may be taken as a typical example of the assimilatory-type of nitrate reductase and is described below.

Nason and Evans (1953) purified a soluble assimilatory nitrate reductase 70-fold from the mycelia of N. crassa and showed that it was a sulfhydryl-metallo-flavoprotein. The preferred electron donor for the reduction of nitrate to nitrite was $NADPH_2$. The enzyme was shown to be adaptive in character and required the presence of nitrate or nitrite for its formation. Molybdenum was established as the functional metal component of the enzyme (Nicholas and Nason, 1954). It has been postulated that the metal functions in the form of a phosphomolybdate complex (Nicholas and Scevin, 1956; Minsky and McElroy, 1958). The following sequence for electron transfer has been suggested:



2. Nitrate Reductases From Higher Plants.

Paneque et al. (1965) purified a nitrate reductase from spinach 130-fold. They showed that two proteins were involved in the transfer of electrons from $NADPH_2$ to nitrate;

the first reduced FMN by $NADPH_2$, and the second transferred electrons from $FMNH_2$ to nitrate. Reduced FMN, FAD, benzyl viologen, and methyl viologen were effective donors for the system. They concluded that $NAD(P)H_2$-nitrate-oxido-reductase must be considered as a mixture of two different proteins, $NAD(P)H_2$-FMN-oxido-reductase and $FMNH_2(FADH_2)$-nitrate-oxido-reductase.

Schrader, et al. (1968) concluded however from studies with extracts from leaves of maize, marrow, or spinach that nitrate reductase was a single moiety with the ability to utilize either $NADH_2$ or $FMNH_2$. Higher concentrations of $FMNH_2$ than of $NADH_2$ were required for optimal activity suggesting that $NADH_2$ is the electron donor in vivo.

Ingle, Joy, and Hageman (1966), in studies of the induction of the nitrate assimilatory system in radish cotyledons, showed that nitrate reductase was induced by nitrate, and to a lesser extent by nitrite and ammonia.

Stoy (1956) found that photoreduced flavin could act as an effective electron donor for nitrate reductase from wheat leaves.

Anaerobic conditions are not necessary for the formation or activity of this type of nitrate reductase, and cytochromes are not involved in the electron transfer

pathway. Assimilatory nitrate reductases are generally soluble enzymes, and are formed in the presence of nitrate as an inducer.

An exception to the general property of solubility is found in the nitrate reductase from Azotobacter grown on nitrate medium (Taniguchi and Ohmachi, 1960). Although this nitrate reductase system was found in association with large particles, it resembles the Neurospora system in that it is a sulfhydryl-metallo-enzyme with no cytochrome participation occurring in the reduction of nitrate. Nitrite and hydroxylamine reductase activities were found in the soluble fraction.

B. Respiratory Nitrate Reduction.

Respiration that utilizes nitrate in place of oxygen occurs in a number of bacteria when grown under anaerobic conditions. This type of anaerobic metabolism differs significantly from that of obligate anaerobes or facultative anaerobes which utilize fermentation processes that do not involve a cytochrome-containing electron transfer system. In nitrate respiration, cytochrome carriers are involved in a manner similar to aerobic respiration, and oxidative phosphorylation has been shown to be coupled to the transfer

of electrons to nitrate in organisms such as Aerobacter aerogenes, Escherichia coli, Micrococcus denitrificans, and Pseudomonas denitrificans.

Nitrate respiration (dissimilation) may be divided into three types: (a) that in which nitrate is reduced only as far as nitrite which accumulates in the medium; (b) that in which nitrate is reduced to molecular nitrogen or gaseous oxides of nitrogen, nitric and nitrous oxides; and (c) that in which nitrate is reduced to ammonia, which is not assimilated into cellular materials, but accumulates in the medium.

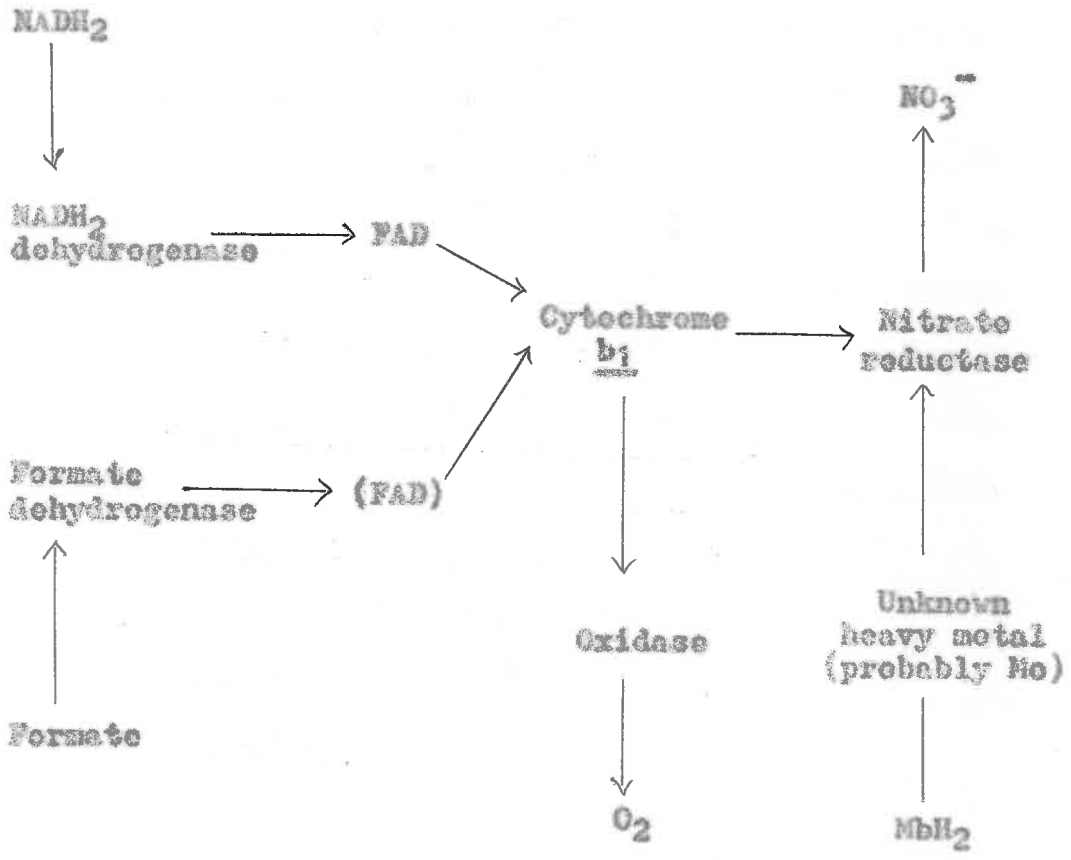
1. Non-denitrifying Respiration.

A typical example of the non-denitrifying type of nitrate respiration occurs in some strains of Esch. coli which, when grown anaerobically in nitrate medium dissimilate nitrate and excrete nitrite into the medium (Taniguchi, 1961).

The electron transport system for the reduction of both nitrate and oxygen is localized in the cell membrane fraction and utilizes $NaBH_4$ or formate as an electron donor. However a soluble factor that stimulated the nitrate reductase activity has been observed (Taniguchi, et al., 1953). The factor could not be replaced by FAD, Fe^{2+}, or

menadione alone or in combination. The mode of electron transfer for this nitrate reducing system is summarised in the following scheme.

Particulate system



This nitrate reductase from Esch. coli was solubilized by alkaline incubation after heat treatment and then purified to homogeneity (Taniguchi and Itagaki, 1960).

The enzyme was shown to contain one mole of molybdenum and 40 moles of iron per mole of enzyme, but no flavin. Reduced dyes or cytochrome b_3, but not reduced pyridine nucleotides or flavin, functioned as electron donors for the purified enzyme. Ota, Yamanaka, and Okunuki (1964) found P/NO_3^- ratios during the reduction of nitrate to nitrite of 0.65 and 1.1 with glutamate and citrate as the respective electron donors.

The Esch. coli enzyme (respiratory type) differs from the Neurospora nitrate reductase (assimilatory type) in that it is initially present in particulate form, contains cytochrome, utilizes cytochrome b_1 as the physiological electron donor and contains iron but no flavin.

2. Denitrification.

The second type of nitrate respiration, denitrification, was first described by Gayon and Dupetit (1886) who showed that bacteria could oxidize an organic substrate with nitrate instead of oxygen, thereby producing nitrogen and sometimes nitrous oxide. Denitrification has been demonstrated in members of the genera Achromobacter, Bacillus, Denitrobacillus, Micrococcus, Pseudomonas, Spirillum and Thiobacillus.

i. Substrate degradation during denitrification.

The following studies have shown that substrate degradation during nitrate respiration differs little from that which occurs with oxygen as the terminal acceptor. Sacks and Barker (1952) showed that in P. denitrificans, succinate is completely reduced to carbon dioxide and water concomitant with the reduction of nitrate to nitrogen. In Pseudomonas aeruginosa glucose is also completely reduced when nitrate is the electron acceptor (Verhoeven and Goos, 1954). Spangler and Gilmour (1966) showed that in Pseudomonas stutzeri, glucose and gluconate were degraded by the same pathways whether cells were grown aerobically or anaerobically with nitrate as the hydrogen acceptor.

Not all aerobically oxidized substrates can however be oxidized by nitrate. P. stutzeri, for example, can utilize benzoic acid and certain aliphatic hydrocarbons aerobically but not anaerobically even in the presence of nitrate (Hansen and Kallio, 1957). This may be explained by the hypothesis that oxidation of these compounds requires an initial oxygenation step in which the participation of molecular oxygen is required.

ii. Effect of oxygen on nitrate respiration.

Since these microorganisms can grow with either

oxygen or nitrate as the terminal hydrogen acceptor it is of some interest to consider the effect of oxygen on the denitrification process.

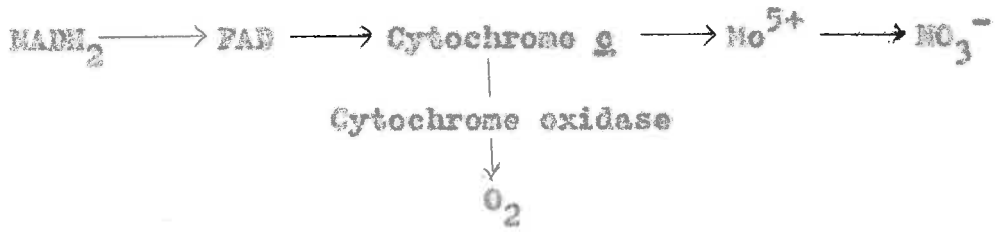
Meiklejohn (1940) reported that in two species of Pseudomonas, denitrification occurred in aerated as well as in anaerobic cultures. Sacks and Barker (1949) showed however that in P. denitrificans, the formation of the nitrate reductase system was suppressed by oxygen, and that performed nitrate reductase was inhibited by oxygen. Schmidt and Kampf (1962) observed that nitrate reduction occurred at a more rapid rate in aerobic than in anaerobic cultures of Pseudomonas fluorescens.

Pichinoty (1965) reported that in both aerobic cultures and in soil, oxygen inhibited bacterial denitrification. The biosynthesis of nitrate, nitrite and nitrous oxide reductases was repressed and nitrite and nitrate reductase activity was inhibited. Skerman and MacRae (1957) also found that in cells of P. denitrificans adapted to nitrate, oxygen completely suppressed nitrate reduction when present at concentrations greater than 0.2 ppm. They concluded that reports of nitrate reduction occurring in apparently aerated cultures were due to some of the cells being deprived of oxygen. In the same organism it was

later observed (Skerman and MacRae, 1961) that when the oxygen supply was inadequate for respiratory requirements, nitrate reductase production continued. The partial pressure of oxygen in the medium was found to be the determining factor in the suppression of nitrate reduction.

In Micrococcus denitrificans (Cheng and Morris, 1962) dissimilatory activity of a growing culture was found to be affected by aeration in at least three ways: (a) adaptive formation of the system was suppressed; (b) if the system was already present, any further synthesis was partially repressed; and (c) the activity of the preformed system was inhibited completely. Activity of the complete system was measured by following the production of nitrogen gas from nitrate. Lam (1969), however, demonstrated that the activity of the nitrate reductase component itself was little affected by oxygen.

In cell-free extracts of Pseudomonas aeruginosa (Fewson and Nicholas, 1961b) inhibition of nitrate reduction by oxygen was facilitated by cytochrome oxidase. The following scheme for electron transfer to alternative hydrogen acceptors was proposed:



In Esch. coli K-12 nitrate reductase synthesis was controlled by two principal growth conditions: (a) it was repressed by oxygen in the presence or absence of nitrate; and (b) enzyme synthesis was initiated by anaerobic conditions even in the absence of nitrate. The rate of nitrate reductase synthesis was, however, increased 20-fold by the addition of nitrate. It was suggested that the system may be under the control of redox-sensitive substance (Showe and DeMoss, 1968).

Noehsner and Wuhrman (1963) in a study of eight strains of denitrifying bacteria found that the effect of oxygen tension was variable, but no strain required complete anaerobiosis for the formation of nitrate reductase.

It is generally considered that the aerobic process is more efficient than nitrate-linked respiration since (a) cells with active nitrate-reducing systems use oxygen, when available, in place of nitrate; (b) denitrification generally occurs only when oxygen is limiting and oxygen

suppresses the production of nitrate reducing systems; and (c) cell yield is usually lower in bacterial cultures grown anaerobically with nitrate than in those grown aerobically.

iii. Oxidative phosphorylation and nitrate respiration.

Oxidative phosphorylation coupled to nitrate reduction has been demonstrated in a number of organisms. Naik and Nicholas (1966) showed that oxidative phosphorylation occurs concomitant with nitrate reduction in cell-free extracts of P. denitrificans and M. denitrificans. In Aerobacter aerogenes, Hadjipetrou and Stouthamer (1965) calculated from molar growth yields that about three moles of ATP were produced per mole of nitrate reduced.

iv. Effect of nitrate on electron transport systems.

In Haemophilus parainfluenzae, White (1962a) found that the relative concentration of the three cytochrome oxidases present in the cells was dependent on growth conditions. In vigorously aerated cultures, cytochrome $\underline{a}$ was the principal oxidase formed. Maximal development of cytochrome a_2 occurred with limited aeration, while the cells grown anaerobically with nitrate showed a marked increase in the concentration of cytochrome a_1. The cytochromes in this organism can be oxidized by nitrate,

but cytochrome a_1 is oxidized to a greater extent than the other two terminal oxidases (White and Smith, 1962).

Wimpenny and Wamsley (1968) compared the changes that occurred in Krebs cycle enzymes in Aerobacter aerogenes and P. aeruginosa when grown on nitrate. In A. aerogenes, aconitase and fumarase activities were undetectable in nitrate-grown cells and nitrate appeared in the growth medium, while in P. aeruginosa, aconitase were unaffected and no nitrite appeared in the medium. Isocitrate dehydrogenase was not affected by nitrate in either organism. In cell-free extracts of P. aeruginosa, aconitase and fumarase were inhibited by both nitrate and nitrite, but more strongly by nitrite.

In Esch. coli, Wimpenny (1968) found that nitrate and nitrite at intermediate concentrations induced the formation of high levels of cytochrome a_{1532} while at higher concentrations of acceptor the cytochrome disappeared. Higher concentrations of cytochrome a_2 were found with oxygen than with nitrate as acceptor. Nitrate and nitrite inhibited activity of aconitase, fumarase, and isocitrate dehydrogenase.

v. Nitrate reductase in Pseudomonas aeruginosa.

Fewson and Nicholas (1961b) described a purified nitrate reductase from P. aeruginosa that reduced nitrate

to nitrite at the expense of $NADPH_2$. The enzyme required FAD, cytochrome *g*, and molybdenum for full activity. The sequence of electron transfer in the purified enzyme was suggested to be:

$$NADH_2 \longrightarrow FAD \longrightarrow \text{cytochrome } g \longrightarrow Mo^{5+} \longrightarrow NO_3^-$$

The reactivation of the enzyme by FAD was competetively inhibited by PCMB; it was thus suggested that sulfhydryl groups were involved in the binding of FAD to the protein.

vi. Nitrate reductase in Bacillus stearothermophilus.

Nitrate reductase in *B. stearothermophilus* catalyzes the anaerobic oxidation of $NADH_2$ and appears to transfer electrons from cytochrome b_1 to nitrate (Downey, 1966). Cytochrome g_1 does not appear to be involved in electron transport to nitrate.

vii. Nitrate reductase in Achromobacter fischeri.

Sadana and McElroy (1957) described a purified nitrate reductase from nitrate-grown *A. fischeri* (*Photobacterium sepia*). This enzyme exhibited a cytochrome-type spectrum when reduced with peaks 550, 520, and 419 nm. Iron and flavin appeared to be involved in nitrate reduction by this enzyme.

viii. Nitrate reductase in Micrococcus denitrificans.

Lam (1969) found that nitrate reductase from M. denitrificans was bound to the cell membrane fraction and utilized either NAH_2 or succinate as an electron donor for the reduction of nitrate to nitrite. While nitrite was the principal product of nitrate reduction by the particulate preparation, small amounts of nitric oxide and nitrous oxide were also formed.

The nitrate reductase proper was solubilized by deoxycholate treatment and was purified 100-fold. In this form the natural electron donors, NAH_2 and succinate, were ineffective, but reduced benzyl or methyl viologen could act as hydrogen donors. Nitrate was reduced stoichiometrically to nitrite. Molybdenum and sulfhydryl groups were required for activity. No cytochrome or flavin could be detected in the purified preparation.

3. Nitrate Fermentation

Egami has postulated the existence of a third category of nitrate reduction which occurs in anaerobic cells without the participation of cytochrome (Takahashi, Taniguchi, and Egami, 1963). This process cannot be classified as nitrate assimilation since nitrite accumulates in the medium and is not reduced further. This type of nitrate

reduction has been studied in Clostridium welchii (Katsura et al., 1954). The enzyme was soluble and $NAMH_2$-dependent. It was inhibited by cyanide, azide, and thiourea, but not by CO in the dark.

II. METABOLISM OF ORGANO-NITRO COMPOUNDS.

It has been suggested that nitrate metabolism might involve coupling the nitrate to organic compounds which could then be reduced to amines. A number of systems metabolising organo-nitro compounds both oxidatively and reductively have been described.

A. Bacteria.

A number of bacterial strains have been reported that metabolize various nitroaryl compounds. For example, Tabak, Chambers and Kabler (1964) isolated Pseudomonas, Flavobacterium, and Achromobacter species capable of reducing o-, m-, and p-nitrophenol, 2,4-dinitrophenol, and 2,4,6-trinitrophenol. Gunderson and Jensen (1956) reported that 2,6-dinitro-p-cresol could serve as the sole source of carbon and nitrogen for a strain of Corynebacterium simplex. Related compounds such as p-nitrophenol, 2,4-dinitrophenol, and 2,4,6-trinitrophenol were also attacked. Compounds such as o-nitrophenol, m-nitrophenol, 2,6-dinitrophenol, p-nitrobenzoic acid, 3,5-dinitrobenzoic

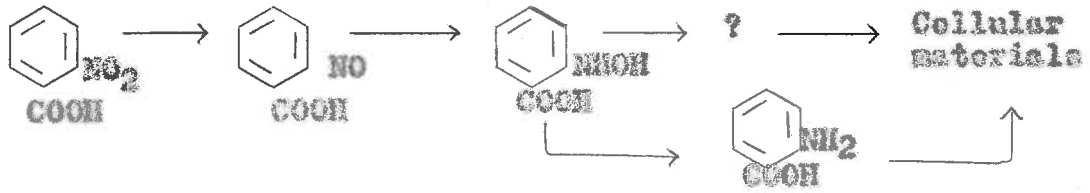
acid, 4,6-dinitro-o-butylphenol and 3,5-dinitrosalicylic acid were not attacked. The authors suggested that the nitro groups must be para to a hydroxyl group for bacterial attack to occur.

Germanier and Wuhrmann (1963) isolated from soil strains of Pseudomonas that could utilise aromatic-nitro compounds as the sole source of carbon and nitrogen. After four days growth under aerobic conditions, the cells had produced ammonia, nitrate and nitrite from o-, m-, and p-nitrophenol, and ammonia and nitrite from m- and p-nitrobenzoic acid.

Two Pseudomonas species isolated from the filter beds of a detoxication plant were capable of utilising m-nitrophenol and p-nitrophenol as their sole source of carbon. Under aerobic conditions with 100 ppm nitrophenol in a liquid medium containing inorganic salts, one gram ion of nitrite was formed per mole of nitrophenol decomposed. Under anaerobic conditions no nitrite was formed. Aminophenols were not intermediates in the degradation of the nitrophenols (Simpson and Evans, 1953).

In Pseudomonas fluorescens, in which p-nitrobenzoic acid can act as the sole source of carbon and nitrogen, the metabolic pathway is through p-aminobenzoic acid which is then oxidized to p-hydroxybenzoic acid and protocatechuic acid (Durham, 1958).

Ke, Gee, and Durham (1959) suggested from simultaneous adaptation studies that p-nitrobenzoic acid is metabolized in a species of Flavobacterium thus:



They maintain that the route through aminonitrophenol is off the main metabolic pathway.

Cartwright and Cain (1959) found that a Nocardia species and a strain of Pseudomonas fluorescens reduced nitrobenzoic acid to aminobenzoic acid. The nitroreductase was non-specific and required $NADH_2$. It was stimulated by FAD, and inhibited by hydroxylamine, nitrite and metal chelating agents. Activity was restored by adding Mg^{2+}, Mn^{2+}, or Fe^{2+}. In Nocardia reduction of the nitro-group probably proceeded through nitrosobenzoic acid and hydroxylaminobenzoic acid, although the enzymes responsible could not be isolated by the usual protein fractionation techniques.

Tewfik and Evans (1966) studied the metabolism of dinitro-o-cresol by a Pseudomonad (N.C.I.B. 9771). The compound was metabolized to 2,3,5-trihydroxytoluene via

3-amino-5-nitro-cresol, 3-methyl-5-nitro-catechol and 3-methyl-5-amino-catechol. The transformation of 3,5-dinitro-p-cresol to 3-amino-5-nitro-cresol demonstrated in cell-free extracts required $NADH_2$, and was stimulated by $FADH_2$, Mn^{2+}, and Fe^{2+}. Arthrobacter simplex employed an initially different pathway, oxidizing dinitro-p-cresol to 3-methyl-5-nitrocatechol. A nitro-reductase could not be demonstrated in this organism, but it did contain a nitrite reductase.

An enzyme system from Neurospora required FAD and sulfhydryl groups and a metal component to reduce p-nitro-benzene to p-nitroaniline, via p-nitroso-nitrobenzene and p-hydroxylamino-nitrobenzene. This reduction may not be due to a specific enzyme. Nitroaryl compounds may merely act as non-specific acceptors like methylene blue and indophenol (Mason, 1956).

Tsukamura (1954) reported that whole cells or cell-free extracts of Mycobacterium avium reduced picric acid to picramic acid. It was observed that either L-amino acid oxidase or an $NADH_2$-cytochrome c reductase catalysed the reaction.

Saz and Slie (1953, 1954) found that dialysed sonicates of Esch. coli formed arylamine from chloramphenicol as well as from a number of other organo-nitro-compounds with

$NADH_2$ or $NADH_2$-malate system as the electron donor.

Cell-free extracts of Streptococcus haemolyticus reduced the nitro-groups of chloromycetin to an amino group. The same extract reduces nitrate to nitrite; this reaction is competetively inhibited by chloromycetin as is the chloromycetin reduction by nitrate. The two reactions were similarly inhibited by a number of compounds and it has been suggested by Egami, Ebata and Sato (1951) that a single system might be responsible for the two reactions.

Gruville and Stern (1935) observed reduction of 2,4-dinitrophenol and dinitro-o-cresol by formic dehydrogenase in the presence of benzyl viologen. Xanthine oxidase also reduced 2,4-, 2,5-, and 2,6-dinitrophenol as well as dinitro-o-cresol. Benzyl viologen was not required for the reaction but stimulated the reaction rate when present. Lactic dehydrogenase also reduced dinitro-o-cresol in the presence of pyocyanine, toxoflavin, or β-anthroquinone sulfonate. Naik and Nicholas (1966) reported that 2,4-dinitrophenol competed with benzyl viologen or methyl viologen for electrons from $NADH_2$-benzyl viologen reductase in Azotobacter vinelandii and suggested that the nitrophenol was reduced to 2-amino-4-nitrophenol.

B. Plants.

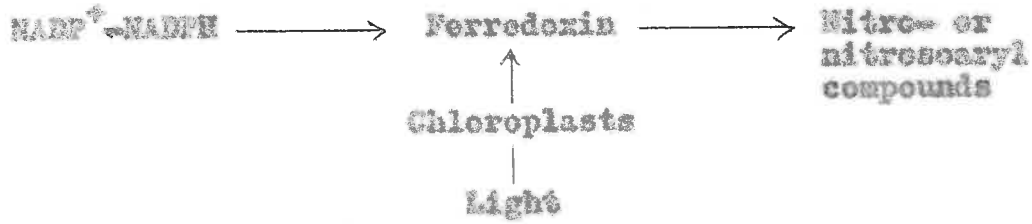
Ahmed and Morris (1967, 1968) observed that nitrite reduction in the green alga Ankistrodesmus braunii was completely inhibited by 10^{-3} M 2,4-dinitrophenol. Nitrate reduction was inhibited by only 65 to 80 per cent by the same concentration of inhibitor in whole cells and was unaffected in cell-free extracts. It was suggested that the effect on the reduction of nitrite was due to the ability of the nitrophenol to compete for reducing power, although unequivocal evidence for this was not obtained.

Under anaerobic conditions chloroplasts in light reduce 2,4-dinitrophenol to aminonitrophenol. The reaction is inhibited by o-phenanthroline, 3-(4-chlorophenyl)-dimethylurea and hydroxylamine, all Hill reaction inhibitors. Vessels (1960) suggested that dinitrophenol serves as a Hill oxidant and can be reduced by illuminated chloroplasts.

Del Campo et al. (1966) described a number of systems capable of reducing nitrophenols. Clostridium ferredoxin and hydrogenase mediated the reduction of dinitrophenol or aminonitrophenol to diaminophenol by hydrogen. Spinach ferredoxin, FMN, or benzyl viologen could substitute for bacterial ferredoxin. Six moles of hydrogen were utilized per mole of dinitrophenol reduced. Illuminated, heat

treated spinach chloroplasts, under anaerobic conditions, reduced dinitrophenol to aminonitrophenol with ascorbate-dichlorophenol-indophenol dye as donor. In this case, ferredoxin was not required. With ferredoxin present, dinitrophenol competed with NADP for the electrons. At a concentration of 1.5 mM, dinitrophenol completely blocked electron flow from water to NADP or ferricyanide.

Wessels (1965) pointed out that it is not necessary to postulate a specific nitro-reductase in order to account for the reduction of nitro-compounds by cells or cell-free extracts. The non-heme iron component of iron-flaveproteins may be involved in nitro-group reduction in the same way as ferredoxin as shown below:



c. Animals.

Parker (1952) reported that 2,4-dinitrophenol was reduced by rat liver homogenates to 4-amino-2-nitrophenol; some 2-amino-4-nitrophenol was also formed (about 10 per cent of the total amine). Maximum rate of reaction occurred at pH 7.0 and 37° in the presence of sodium lactate or sodium succinate.

Kielly (1956) studied the rate of $NADH_2$ oxidation by calf liver xanthine oxidase with a series of electron acceptors including aromatic nitro-compounds. A number of aromatic nitro-compounds were reduced, and it was shown that 2,4-dinitrophenol was reduced to 2-nitro-4-aminophenol. A nitro-group in the *para* position was much more easily reduced than one in the *ortho* position. For example, in 2,5-dinitrophenol which has two nitro-groups *para* to each other, the reduction rate was more rapid than with 2,4-dinitrophenol. With 2,6-dinitrophenol which has no *para* groups, the rate was slower than with either 2,4- or 2,5-dinitrophenol. Intermediates, the nitroso- and hydroxylamino-derivatives are probably much more reactive than the parent compounds as suggested by the rapid reduction of *p*-nitroso-phenol.

Fouts and Brodie (1956) reported that aromatic nitro-reductase activity in mammals was found mainly in the liver. Liver homogenates reduced *p*-nitrobenzoic acid to *p*-amino-benzoic acid with $NADPH_2$ as electron donor. The system was inhibited by oxygen. The reduction rate was stimulated by adding FAD, FMN, or riboflavin; and excess of flavin accelerated normal activity. It was also observed that riboflavin or FAD stimulated nitro-reductase activity in pigeon liver homogenates (Adamson *et al.*, 1965).

III. METABOLISM OF NITRITE.

Nitrite is a highly reactive compound and is biologically toxic. It lies in a key position in nitrate metabolism being the first product on the assimilatory pathway to either hydroxylamine and ammonia or the dissimilatory pathway to nitrogen and its oxides. Nitrite can be reduced by a number of non-enzymic reactions. For example both $NAMH_2$ and ascorbate reduced nitrite under anaerobic conditions with the formation of NO, N_2O, and nitrogen gas in the percentage composition 88, 3, and 9, respectively, with ascorbate, and 75, 10, and 15, respectively, with $NAMH_2$. The reaction with ascorbate takes place at pH values below 6, and with $NAMH_2$ below pH 4. With either ascorbate or $NAMH_2$ greater reduction occurred at lower pH values (Evans and McAuliffe, 1956).

Bar-Akiva and Sternbaum (1966) suggested that nitrite reduction in citrus plants occurred at least partly as the result of a chemical reaction with ascorbic acid.

Nitrite was demonstrated to be the first product of denitrification by Gayon and Dupetit (1886). Quastel, Stephenson and Whetham (1925) found that Bacterium coli, when grown anaerobically on nitrate, produced nitrite. Yamagata (1939) was the first to observe a cell-free nitrite reductase which he demonstrated in extracts of Bacillus

nyocyaneus (P. aeruginosa). The product of nitrite reduction was not determined.

A. Effect of Nitrite on Bacterial Metabolism.

White (1962) reported that nitrite was toxic to the respiratory system of Haemophilus parainfluenzae. Nitrite was highly toxic to the nitrate-reducing enzyme from Thiobacillus denitrificans. Denitrification in the presence of sulfur was inhibited 40 per cent by nitrite concentrations as low as 3.5×10^{-4} M (Baalsrud and Baalsrud, 1954).

In Micrococcus denitrificans, Bovell, (1967) found that nitrite which accumulates during growth with nitrate as terminal oxidant, inhibited growth when hydrogen was the electron donor. This inhibition of growth was attributed to the inhibition of hydrogenase by nitrite. Growth on hydrogen and oxygen was inhibited 74 per cent by 5×10^{-3} M nitrite, and completely inhibited by 10^{-2} M nitrite. On sucrose and oxygen no inhibition occurred at 10^{-2} M nitrite, but complete inhibition occurred at 5×10^{-2} M.

B. Assimilatory Nitrite Reduction

Assimilatory-type nitrite reductases are generally soluble and reduce nitrite to hydroxylamine or ammonia.

1. Bacillus pumilis.

Taniguchi et al. (1953) described a nitrite reductase in cell-free extracts of B. pumilis that utilized leucomethylene blue as electron donor.

2. Neurospora crassa.

Nason, Abraham and Averbach (1954) prepared a 10-fold purified nitrite reductase from N. crassa. The enzyme reduced nitrite at the expense of $NADH_2$ and produced ammonia. Three moles of $NADH_2$ were oxidized per mole nitrite reduced. The enzyme was stimulated by flavin and inhibited by metal chelating agents.

Nicholas, Medina and Jones (1960) purified an assimilatory nitrite reductase from mycelia of N. crassa and showed that it was a flavoprotein containing iron and copper. It was proposed that the copper is involved in the terminal step and that univalent copper reduced nitrite non-enzymically. The divalent copper thus formed is then enzymically reduced by the penultimate electron donor (a cytochrome).

3. Azotobacter agile (A. vinelandii).

Spencer, Takahashi, and Nason (1957) described a soluble nitrite reductase system found in A. agile that reduced nitrite to ammonia at the expense of reduced pyridine nucleotides. The authors concluded that this enzyme plays

a part in nitrate assimilation, but not in nitrogen fixation in this bacterium.

4. Escherichia coli.

Cell-free extracts of Esch. coli strain Bn have been shown to contain at least three nitrite reducing systems (Kemp and Atkinson, 1966). One is specific for $NADPH_2$, one for $NADH_2$, and a third, which apparently contains cytochrome, oxidised flavin or viologen dyes. Little is known of this last system.

The $NADPH_2$-specific enzyme also catalysed the reduction of hydroxylamine, cytochrome c or sulfite (Lazzarini and Atkinson, 1961). Sulfite and nitrite appear to compete for the same reaction site on the enzyme. The enzyme is repressed by cysteine, and is absent from mutant cultures which are unable to use sulfite as the source of sulfur for growth. It was suggested that this system functions *in vivo* as a sulfite reductase (Kemp *et al.*, 1963).

The $NADH_2$-dependent nitrite reductase appears to be responsible for nitrite reduction *in vivo* in Esch. coli Bn (Kemp and Atkinson, 1966). Nitrite is reduced to ammonia. Hydroxylamine, but not sulfite or nitrate, is also reduced by the enzyme.

5. Nitrosomonas europaea.

Wallace and Nicholas (1968) described a soluble nitrite reductase from N. europaea that reduced nitrite to ammonia utilizing $NADPH_2$ or reduced benzyl viologen as the electron donor. Activity with $NADPH_2$ was stimulated by FAD or FMN. Nitrite (^{15}N-labelled) was rapidly incorporated into cell protein.

6. Algae.

Molecular hydrogen can act as hydrogen donor via hydrogenase in Chlorella pyrenoidosa (Stiller, 1966), Ankistrodesmus braunii, and Scenedesmus obliquus (Kessler, 1956). Three moles of hydrogen were utilized per mole of nitrite reduced. The presence of carbon dioxide was required for optimal functioning of the system.

Hattori and Myers (1966) partially purified a soluble nitrite reductase from the blue-green alga Anabaena cylindrica. Nitrite was reduced to ammonia by $NADPH_2$ with ferredoxin, methyl viologen or benzyl viologen, or diquat as electron carriers.

7. Higher plants.

Joy and Hageman (1966) reported in studies on nitrite reductase in Spinacea oleracea and Zea mays that two proteins were involved in nitrite reduction. One was a diaphorase

with ferredoxin-NADP-reductase activity which was required to transfer electrons from $NADPH_2$ to a suitable acceptor which donated electrons to the second protein, nitrite reductase proper. Ferredoxin was the natural donor, and the enzyme also functioned with reduced dyes. Ferredoxins from spinach, maize, or Clostridium were found to be interchangeable.

Ramirez et al. (1966) purified spinach nitrite reductase about 500-fold and freed it from NADP-reductase and nitrate reductase activities. Reduced ferredoxin or methyl viologen were effective as electron donors. The enzyme did not appear to contain flavin.

Betts and Nevitt (1966) found that nitrite reduction in spinach chloroplast grana took place at substantially greater rates than did hydroxylamine reduction. They questioned whether hydroxylamine was an intermediate in the accepted sense in nitrite reduction in higher plants.

A. Dissimilatory Nitrite Reduction.

Dissimilatory-type nitrite reduction is generally associated with cell-membrane fragments. Cytochromes are involved in the transfer of electrons.

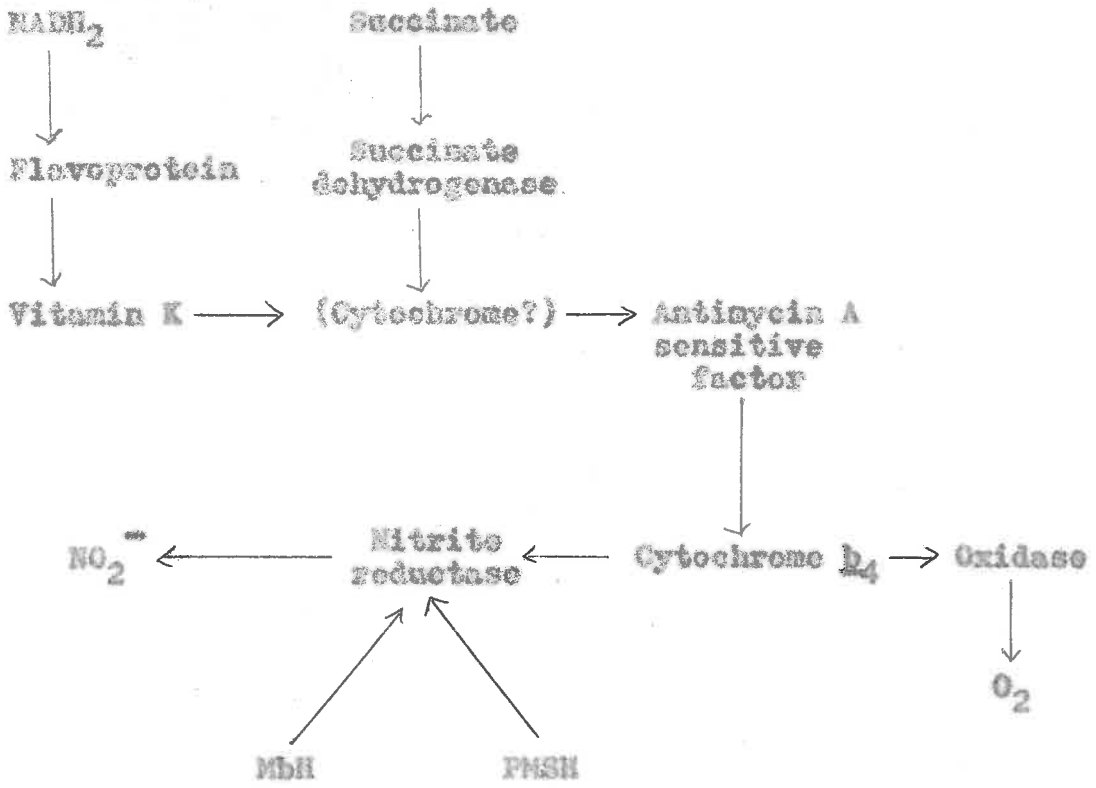
1. Pseudomonas aeruginosa.

Walker and Nicholas (1961) purified a nitrite reductase 600-fold from P. aeruginosa. Nitrite was reduced to nitric oxide with reduced flavin, pyocyanine, methylene blue, or 1,4-naphthoquinone as hydrogen donor. The enzyme had a cytochrome c-type spectrum and an additional absorption maximum between 630 and 635 mμ which was attributed to copper. Iron and copper were required for nitrite reduction and phosphate or sulfate and sulfhydryl groups were necessary for maximal activity.

Yamanaka, Ota, and Okunuki (1960a) reported that Pseudomonas cytochrome oxidase, which is formed in higher concentrations under anaerobic conditions, transfers electrons from reduced Pseudomonas cytochrome c-551 to either oxygen or nitrite (Yamanaka and Okunuki, 1960a, b). The enzyme showed both heme a_2 and a c-type heme spectra but contained no copper. Nitrite was reduced to nitric oxide.

2. Halotolerant Micrococcus.

Asano (1959) described a particulate nitrite reductase from a halotolerant strain of Micrococcus that utilized $NADH_2$, succinate, and reduced phenazine methosulfate of leuco-methylene blue as electron donors. Ferrocytochrome b_4 reduced nitrite in the presence of nitrite reductase. The following scheme of electron transport was postulated:



Nitrite was reduced to nitrogen gas. The reaction was inhibited by hydroxylamine, carbon monoxide in the dark, nitric oxide, Amytal, dicoumarol, antimycin A, and metal chelating agents. Iron and copper were suggested as the metals involved (Asano, 1960).

3. Pseudomonas denitrificans.

In a denitrifying soil bacterium tentatively identified as P. denitrificans, Iwasaki (1960) described a nitrite reducing system using p-phenylene diamine or dimethyl-p-phenylene diamine as electron donor. Nitrogen gas was

produced. Hydroxylamine was also utilized as hydrogen donor; in this case, nitrous oxide was the product. A dismutation-type of reaction was postulated. With hydroxylamine the following reaction could occur:

$$HNO_2 + NH_2OH \longrightarrow N_2O + 2H_2O$$

An unusual cytochrome-like pigment was isolated from the bacterium. The pigment had no distinct α-band or β-band in the reduced state, but instead a broad absorption band between 560 and 540 mμ and a sharp peak at 423 mμ (394 mμ in the oxidized state). This pigment was called cryptocytochrome g, and the fraction containing it was observed to catalyse the above reaction between nitrite and hydroxylamine. It was postulated that this pigment might be the nitrite reductase proper. Cytochrome g_{552} with absorption maxima at 552 mμ, 523 mμ, and 418 mμ in the reduced state, appeared to act as an electron donor for the cryptocytochrome g (Iwasaki, 1960).

The cryptocytochrome pigment was later electrophoretically separated from a non-heme component; the latter contained the nitrite reducing activity in the presence of cytochrome g_1 552, while the cryptocytochrome g itself was devoid of any activity (Suzuki and Mori, 1962). The non-heme component had an absorption maximum in the oxidized state at 394 mμ and plateaux at 750–780 mμ and 460–480 mμ

and a peak at 280 nm. No absorption was detected in the visible region in the reduced state.

Prolonged dialysis against KCN resulted in a loss of activity which was restored by adding 10^{-6} M Cu^{+} or Cu^{2+}, but not by other metals. Activity was inhibited by KCN and sodium diethyldithiocarbamate, but not by PCMB. The nitrite reducing enzyme was thus suggested to be a copper containing protein (Suzuki and Iwasaki, 1962).

4. Nitrosomonas europaea.

Hooper (1968) described a hydroxylamine: nitrite oxido-reductase similar to the above system from N. europaea. Leuco-pyocyanine was utilized as hydrogen donor. For each mole of nitrite utilized, 1 mole of gas (a mixture of nitrous oxide and nitric oxide) was produced and 1.8 mole of hydroxylamine disappeared. The reaction apparently takes place in two steps: (1) hydroxylamine oxidase or hydroxylamine dehydrogenase catalyses the oxidation of hydroxylamine to a compound such as (HNO) with the reduction of a bacterial electron acceptor. (HNO) spontaneously dimerises to $H_2N_2O_2$ which decomposes to form nitrous oxide and water. (NH_2OH + oxidized electron carrier $\rightarrow \frac{1}{2} N_2O$ + 1/2 H_2O + reduced electron carrier). (2) In the second step, nitrite is reduced to N_2O, NO and water by a bacterial electron donor. (HNO_2 + reduced electron carrier $\rightarrow N_2O$ +

NO + H_2O + oxidized electron carrier). This reaction apparently does not occur during the normal processes of nitrification of N. europaea.

5. Micrococcus denitrificans.

In M. denitrificans nitrite reduction has been shown to be coupled to phosphorylation (Naik and Nicholas, 1966). The nitrite reductase in this organism is only loosely associated with the cell-membrane fraction, (Lam, 1969). This nitrite reductase contains cytochrome a and a_3 in one complex. Purified nitrite reductase had cytochrome oxidase activity, but this activity was thought to be of secondary importance *in vivo* since a constitutive cytochrome oxidase not connected with nitrite reductase was present in much greater concentration. Nitrite was reduced to nitric oxide. M. denitrificans grows with nitrite as terminal hydrogen acceptor in place of oxygen or nitrate.

D. Nitrite Reduction in Muscle.

Nitrite reduction by pig muscle minces was observed by Walters and Taylor (1964). This was shown to be due to the muscle itself and not to bacterial contamination. Part of the nitrite metabolized was reduced to nitric oxide. The endogenous respiration of the minces was inhibited by nitrite thereby giving rise to the suggestion that nitrite

competed with other electron acceptors such as oxygen. It was later shown (Walter, Casselden and Taylor, 1967) that nitrite oxidized myoglobin to the metmyoglobin form and also acted anaerobically as a terminal respiratory electron acceptor to ferrocytochrome *c* with the formation of nitrosyl-ferricytochrome *c*. The nitrosyl-groups was transferred from ferricytochrome *c* to metmyoglobin by $NADH_2$-cytochrome *c* reductase action. The nitrosyl metmyoglobin thus formed was reduced to nitrosyl-myoglobin by mitochondrial enzyme systems.

IV. METABOLISM OF NITRIC OXIDE.

There is good evidence that nitric oxide, a gas in which nitrogen is at the +2 oxidation state, is an intermediate in denitrification. It is also possible that nitric oxide or a compound with which it equilibates is involved in the reduction of nitrate to ammonia.

A. Nitric Oxide in Assimilatory Nitrate Reduction.

McNall and Atkinson (1956) isolated a strain of *Esch. coli* that could utilize nitric oxide as its sole source of nitrogen for growth. Steinberg (1956) reported that *Aspergillus niger* and *Nicotiana tabacum* grew on nitrohydroxylaminate ($N_2O_3^{2-}$) which is a hydrate of nitric oxide. A number of other organisms including bacteria

fungi, an alga, and higher plants were assayed for nitric oxide reductase activity (Fewson and Nicholas, 1960). Organisms grown on nitrate as sole source of nitrogen contained a higher level of nitric oxide reductase activity than did those grown on ammonium-nitrogen.

B. Nitric Oxide in Dissimilatory Nitrite Reduction.

Several denitrifying organisms convert nitric oxide to nitrogen gas. Najjar and Allen (1954) found that extracts of both P. stutzeri and Bacillus subtilis reduced nitric oxide to nitrogen gas. Chung and Najjar (1956) in further studies on nitric oxide reduction by P. stutzeri found that the reaction required a reduced pyridine nucleotide as electron donor and FAD or FMN and copper or iron ions for maximal activity.

Baalsrud and Baalsrud (1954) in studies on Thiobacillus denitrificans observed that in the presence of thiosulfate, nitrite was rapidly converted to nitric oxide which was subsequently reduced to nitrogen gas.

Fewson and Nicholas (1960) described a metallo-flavoprotein nitric oxide reductase from P. aeruginosa that had been purified 25-fold. Reduced pyocyanine was active as electron donor, and flavin and iron participation was implicated.

V. METABOLISM OF NITROUS OXIDE.

The role of nitrous oxide as an intermediate in denitrification has been much disputed. Some early workers found that nitrous oxide was a common product of denitrification (Gayon and Dupetit, 1886). Wijler and Delwiche (1954) reported nitrous oxide to be the major product of denitrification under most soil conditions. The fate of N_2O appeared to be pH dependent; above pH 7 nitrous oxide was readily reduced to nitrogen gas while below pH 7 its reduction was strongly inhibited.

Nitrous oxide was the major product of denitrication by Corynebacterium nephridii (Hart, Larson and McCloskey, 1965), and was produced from both nitrate and nitrite. From growth experiments and manometric studies, it was concluded that the organism was unable to further utilise nitrous oxide.

Allen and van Niel (1952) concluded from simultaneous enzyme induction in Pseudomonas stutzeri that nitrous oxide was either an intermediate product or was reversible derived from an intermediate. The observation that nitrous oxide production from nitrite was inhibited at levels of cyanide which allowed the nitrate to be reduced to nitrogen led to the conclusion that nitrous oxide was not a true intermediate in nitrate reduction.

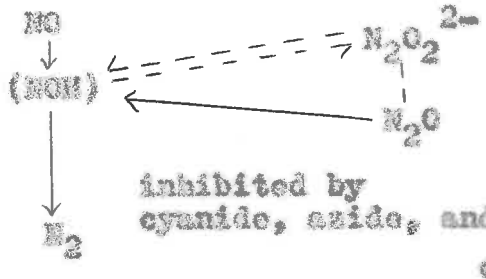
Sacks and Barker (1952) in experiments with P. denitrificans found that nitrous oxide was utilized only after a lag period and that N_2O reduction to nitrogen could be inhibited by levels of azide and dinitrophenol that allowed the reduction of nitrite to nitrogen gas to proceed unimpeded. They also concluded that nitrous oxide was not the normal precursor of nitrogen gas.

Najjar and Allen (1954) reported that extracts of a denitrifying strain of Bacillus subtilis produced an appreciable quantity of nitrous oxide from nitrite. In this organism nitrite was converted to a mixture of 70 per cent nitrogen, 20 per cent nitrous oxide, and 10 per cent nitric oxide.

Delwiche (1959), in studies on the same organism, found that utilization of nitrous oxide occurred after a shorter lag period when cells were grown on low levels of nitrate (10^{-2} M) than on high levels (5×10^{-2} M). He concluded that nitrous oxide production may occur during denitrification under certain conditions, and that its subsequent utilization takes place when other hydrogen acceptors are unavailable.

Pewson and Nicholas 1961c) postulated that these data could be satisfactorily explained by assuming that a compound at the oxidation level of nitroxyl (HOH) is the

actual intermediate in nitrate reduction and that this compound is in equilibrium with hyponitrite ($H_2N_2O_2$) which can decompose to form nitrous oxide. A further adaptive enzyme would be necessary for the utilization of nitrous oxide thus formed. This hypothesis can be presented schematically thus:



Pseudomonas denitrificans is a Gram negative soil bacterium capable of growing aerobically or anaerobically in the presence of nitrate. Nitrate is reduced to nitrogen gas, nitric oxide, and nitrous oxide. Growth does not occur anaerobically in the absence of nitrate. Nitrite cannot substitute for nitrate as the sole electron acceptor.

In this thesis studies on the enzymes involved in the denitrification process in P. denitrificans will be described. The nitrate reductase system is membrane-bound as found in other denitrifying bacteria, but the nitrite reductase is soluble and not associated with the cytochrome system. The significance of these results will be discussed.

MATERIALS AND METHODS.

I. ORGANISM AND CULTURE METHODS.

Pseudomonas denitrificans Bergey *et al.* (A.T.C.C. 13867) was obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A.

A. Maintenance of Stock Cultures.

Routine stock cultures were maintained on nutrient agar slopes incubated aerobically at 37°. Stocks were subcultured every four days and checked periodically for contamination.

Long term stocks were maintained in a dehydrated state on silica gel according to a modification (Grivell and Jackson, unpublished results) of the method of Perkins (1962). A heavy suspension of cells grown on nutrient agar slopes was made up in cold, sterile, double strength basal nutrient medium. An equal volume of 15 per cent (w/v) sterile, reconstituted powdered skim milk ("Bonlac") was then added as a stabilizer, and about 0.5 ml of the resulting mixture transferred aseptically to small, cotton-plugged tubes half-full of 6-18 mesh silica gel (containing no dyes) which had been dry sterilized at 130° for 90 minutes. The tubes were cold at the time of

transfer and were placed in an ice bath after adding the inoculum to avoid excess heating during dehydration. The inoculated tubes were stored at 2° in a tightly sealed jar containing silica gel to ensure continued dehydration.

B. Culture Media.

The medium used for nutrient agar slopes contained per liter: 10 g glucose, 5 g Difco yeast extract, 15 g Difco Bacto-agar, and 6 mg $FeCl_3 \cdot 6\ H_2O$. The ferric chloride was sterilized separately and added aseptically to each tube of molten sterile medium.

The basal nutrient medium consisted of the following macro-nutrients (g/l): 10 KNO_3, 10 glucose, 1 peptone, 1.5 K_2HPO_4, 0.2 $MgSO_4 \cdot 7\ H_2O$, 0.2 NaCl; and micronutrients (mg/l): 2 Fe as $FeSO_4$, 1 Mo as Na_2MoO_4, 0.025 Mn as $MnCl_2$, 0.25 Cu as $CuSO_4$, and 0.25 Zn as $ZnSO_4$. The medium was adjusted to pH 7.0. The glucose was sterilized separately and added aseptically to the rest of the medium after autoclaving to prevent excessive caramelization.

The medium used for aerobic cultures was identical with the basal medium except that the potassium nitrate was omitted.

For metal deficient cultures a special medium modified from that of Daniels (1966) containing glutamic acid in place of peptone was used. It contained the

following nutrients (g/l): 10 KNO_3, 10 glucose, 2 $(NH_4)_2SO_4$, 1 $MgSO_4 \cdot 7\ H_2O$, 0.2 NaCl, 3.3 sodium glutamate and 1.5 K_2HPO_4. After extraction of iron from the macronutrients as described below, one ml of the following micronutrient solution was added per liter of medium. The salt solution consisted of 2 g $MnSO_4 \cdot 7\ H_2O$ and 2 g $ZnSO_4 \cdot 7\ H_2O$ in 100 ml of triple distilled water. To the control flasks 0.02 g of $FeSO_4 \cdot 7\ H_2O$ per liter was also added.

All media were sterilized by autoclaving at 121° and 15 lb.inch^{-2} for times ranging from 15 minutes to 1 hour depending on the volume of the medium used.

C. Purification of Media.

Metal-deficient media were prepared by treating the glutamate medium in one of the following ways.

1. Iron.

The method of Waring and Werkman (1942) was employed for the production of iron-free medium. Sufficient macro nutrients for 10 l of the glutamate medium were dissolved in 2 l of double distilled water and filtered through Whatman No. 42 filter paper into a separatory funnel (the stopcock was not greased, but only wet with distilled water). Fifteen ml of chloroform containing 5 mg/ml

8-hydroxyquinoline was added to the solution in the funnel and shaken vigorously for 1 minute. After allowing this to stand for 5 minutes, 45 ml of chloroform was added and the funnel again shaken. The green chloroform layer was withdrawn after separation. This procedure was repeated 3 times or until no colored iron complexes were formed. The solution was then washed four times with chloroform and excess chloroform removed by heating and shaking.

The triple distilled water used for diluting the concentrated culture solution was checked for purity by the dithizone test as described by Stout and Arnon (1939). Purified dithizone was prepared by placing 10 ml of distilled water into a separatory funnel along with 5 ml chloroform and 3 drops dithizone reagent (1 mg diphenyl-thiocarbazone/ml chloroform) and 6 drops 6 N NH_4OH. The mixture was shaken and allowed to separate, the chloroform layer drawn off and the dithizone contained in the ammoniacal water retained for the subsequent test in the same separatory funnel. This process also removes any metal contaminants from the funnel itself.

For the dithizone test proper 200 ml of water to be tested and 5 ml chloroform were placed in the funnel containing purified dithizone prepared as above, shaken for 1 minute and allowed to settle. A red or purplish

color indicates the presence of metals.

All glassware used in these experiments was acid washed with 6 N nitric acid, rinsed with 1 per cent (w/v) Na_4 EDTA and rinsed 6 times with double distilled water. Culture flasks were not cotton-plugged, but covered with inverted beakers to avoid metal contamination from particles of cotton wool (Nicholas, 1966).

2. Copper.

Copper was removed by the co-precipitation method of Nicholas (1966). The 5-times concentrated solution was adjusted to pH 2.0 with 6 N HCl and 5 ml 20 per cent (w/v) $CuSO_4$ solution added. Hydrogen sulfide from a Kipps' apparatus was passed through a saturated solution of barium hydroxide to remove polysulfides, then bubbled through the medium for 15 minutes. After standing for 15 minutes, the copper sulfide was filtered off and excess hydrogen sulfide removed by boiling and bubbling with nitrogen gas. The medium was readjusted to pH 7.0 with HCl diluted with triple distilled water.

D. Growth of Batch Cultures.

Batch anaerobic cultures were grown in 10 l carboy of the basal nutrient medium. Inocula from agar slopes were first subcultured in 250 ml flasks of the medium and

then transferred to 1 liter cultures to provide inocula for the bulk cultures. The 10 l cultures were bubbled with oxygen-free nitrogen at 200 ml/min to ensure anaerobiosis and allowed to grow for 18 hours at 37°.

Aerobic cultures were grown in 5 l batches in the aerobic medium and aerated vigorously with air (500 ml/min) that had been passed through a sterile cotton wool filter.

For the production of iron-deficient cells, the bacteria were subcultured several times in iron-free medium until decreased growth was observed. These iron-deficient cells were then used as the inoculum for a larger batch cultures.

E. Harvesting of Cultures.

The cells were collected during late logarithmic phase of growth at 0-2° in a Sorvall RC-2 refrigerated centrifuge fitted with a continuous-flow head. The cells were washed three to six times with cold (2°) 0.85 per cent (w/v) saline solution until all nitrite was removed and then stored at -17°.

II. PREPARATION OF CELL-FREE EXTRACTS.

Cell-free extracts were prepared as described below from the washed cell pastes. All procedures were carried

out at 2°.

A. Disruption of Cells.

Three methods were used for the breakage of washed cells.

1. Freezing and Thawing.

The repeated freezing and thawing technique was carried out with the cell paste suspended in buffer as described above. The suspension was placed in a polyethylene centrifuge tube and immersed in a mixture of dry ice and ethanol. After freezing it was rapidly thawed by holding the tube in warm water. This procedure was repeated six times.

2. French Pressure Cell.

The washed cells were suspended in 2 volumes of 0.05 M Tris-HCl buffer (pH 7.4) and crushed in a French pressure cell (American Instrument Company, Silver Springs, Maryland, U.S.A.) at 20,000 lb·inch^{-2}.

3. Ultrasonication.

The cell paste was suspended as above and placed in a double-walled glass cell. Ice water was circulated through the outside chamber of the cell to maintain the temperature at 2°. The cells were subjected to ultrasonic

vibration at 20 Kc/sec for 2 to 5 minutes with an M.S.E. Mullard Ultrasonic Probe.

B. Crude Extracts

Deoxyribonuclease (1 mg/100 ml) and $MgCl_2$ (10^{-4} M) were added to the homogenate prepared by one of the above methods and the mixture allowed to stand at room temperature for 15 minutes to react with any DNA present thereby decreasing the viscosity of the preparation. The homogenate was then centrifuged in the cold at 10,000 x g for 20 minutes to sediment any unbroken cells and large cell debris. The resulting supernatant fraction is referred to hereafter as the crude extract.

C. Preparation of Azotobacter Particles.

Azotobacter vinelandii (O) was grown in a nitrogen-free medium containing (g/1): Sucrose, 50; KH_2PO_4, 0.1; $MgSO_4 \cdot 7\ H_2O$, 0.4; sodium citrate, 0.2; calcium acetate, 0.2; NaCl, 0.1; $FeSO_4 \cdot 7\ H_2O$, 0.01; $Na_2MoO_4 \cdot 2\ H_2O$, 0.001 (Nicholas et al., 1960). The cells, collected in a Sorvall centrifuge fitted with a continuous-flow head, were suspended in 3 volumes of 0.01 M phosphate buffer (pH 7.5) and crushed in a French pressure cell (7,000 lb·inch^{-2}) in the cold. The particulate fraction sedimenting between 100,000 and 144,000 x g which contained the $NADH_2$-benzyl

viologen reductase activity was used (Naik and Nicholas, 1966). Each preparation was tested before use to ensure that it was devoid of nitrate and nitrite reducing activity.

III. ENZYME ASSAYS.

A. Nitrate Reductase.

The following assay methods were used for determinating nitrate reducing activity. Zero time and boiled enzyme controls were always included in each experiment. All assays were carried out in 0.1 M potassium phosphate buffer unless otherwise indicated.

1. $NADH_2$-Nitrate Reductase.

$NADH_2$-nitrate reductase activity was assayed anaerobically by following the appearance of nitrite in the following standard reaction mixture: 1 µmole $NADH_2$, 0.5 µmole KNO_3, an appropriate amount of cell-free extract and 0.1 M phosphate buffer (pH 6.5) to a final volume of 1.0 ml. After rigorously evacuating the contents with a vacuum pump carefully to avoid excessive frothing, the tubes were equilibrated at 30° for 5 minutes. The reaction was started by tipping the nitrate from the side-arm of the Thunberg tube into the rest of the reactants. The reaction was stopped by exposing the reaction mixture to air and precipitating the protein with 0.1 ml 1 M zinc

acetate and 1.9 ml 95 per cent (v/v) ethanol. Nitrite was determined in suitable aliquots of the supernatant solution left after centrifuging at 3,000 x g for 5 minutes as described on page 60.

2. Formate-Nitrate Reductase.

Formate-nitrate reductase activity was determined either as for the $NADH_2$ assay in Thunberg tubes with the substitution of 1 µmole of sodium formate for the $NADH_2$ or manometrically.

For the manometric assay the reaction was followed in Warburg manometer vessels at 30° with the following reactants: 1 to 5 µmoles sodium formate, 1 to 5 µmole KNO_3, an appropriate amount of cell-free extract, 2.0 ml 0.1 M phosphate buffer (pH 6.5) and distilled water to a final volume of 3.0 ml. The evolution of CO_2 with time was followed and the total amount of nitrite formed determined.

3. Benzyl Viologen-Nitrate Reductase.

The activity of a solubilised nitrate reductase was determined using reduced benzyl viologen as the electron donor. The reaction mixture contained: 0.5 µmole KNO_3, 1 µmole oxidized benzyl viologen, 1 µmole $NADH_2$, 0.05 ml Azotobacter particles, 0.01-0.05 ml solubilized nitrate

reductase, and 0.1 M phosphate buffer (pH 7.5) to a total volume of 1 ml. The $NaBH_4$ was added to the reaction mixture last, immediately before evacuating the Thunberg tubes, to minimise loss due to the active $NaBH_4$-oxidase in the Azotobacter particles. The reaction was initiated by tipping the nitrate from the side arm of the Thunberg tube into the rest of the reactants and terminated by exposing to air and precipitating with zinc acetate and ethanol as described previously.

4. Nitrate Reductase Assay with Ascorbate-TMPD as Electron Donor

Ascorbate was coupled to the dye tetramethyl-phenylene-diamine (TMPD) which reduces the native cytochrome c for the assay of nitrate reductase activity with reduced cytochrome c as electron donor. The reaction mixture contained 0.75 µmole sodium ascorbate, 1.5 µmole TMPD, 0.5 µmole KNO_3, 20 µmoles $MgCl_2$, 0.05 ml cell extract, and 0.1 M phosphate buffer (pH 7.5) to a final volume of 1 ml. The sodium ascorbate was prepared by neutralising ascorbic acid to pH 6.0-6.5 immediately before use.

B. Formate Dehydrogenase.

Formate dehydrogenase activity was assayed manometrically using the following reaction mixture: 5 µmoles sodium formate, 5 µmoles methylene blue or other electron

acceptor, 2.0 ml 0.1 M phosphate buffer (pH 7.0), 0.05 to 0.20 ml cell-free extract; and distilled water to 3.0 ml total volume. The evolution of CO_2 was followed at 30° under an atmosphere of nitrogen.

C. Formate-Pyruvate Exchange Assay.

This assay is based on the exchange between ^{14}C-labelled formate and pyruvate:

$$CH_3COCOO^- + H^{14}COO \longrightarrow CH_3CO^{14}COO + HCOO^-$$

This reaction is closely related to the phosphoroclastic reaction. The reaction mixture was slightly modified from that of Wood (1966) and consisted of 50 µmoles sodium pyruvate, 50 µmoles sodium formate-^{14}C, (0.067 uC), 50 µmoles sodium phosphate buffer (pH 7.2), 0.1 ml of a reducing solution consisting of 0.01 M $FeSO_4$ and 0.03 M 2,3-dimercaptopropanol (BAL), 0.1 ml cells or cell extract; 15 mg yeast extract (when using whole cells) and water to a final volume of 1.0 ml. The reaction was carried out in Thunberg tubes under an atmosphere of nitrogen. Cells for this assay were suspended in 0.85 per cent (w/v) NaCl instead of Tris-HCl.

After incubation at 30° for 90 minutes the reaction was stopped by adding 0.5 ml 5 N H_2SO_4 and 2.5 ml water. Protein was removed by centrifuging at 3,000 x g for 10 minutes. To 2 ml of the resulting supernatant was added

0.2 ml of 1.5 M 2,4-dinitrophenylhydrazine in 18 N H_2SO_4, and the pyruvate-2,4-dinitrophenylhydrazone recovered on a tared Whatman glass fiber filter disk by vacuum filtration. The product was washed twice with water and dried and weighed before counting in a Beckman Lowbeta gas flow counter.

B. Nitrite Reductase Assays.

The activity of nitrite reductase in the cell extracts was assayed by the following four methods.

1. $NADH_2$-FMN Nitrite Reductase.

The reaction mixture consisted of 1 µmole $NaNO_2$, 0.1 µmole FMN, 0.05-0.1 ml enzyme preparation, 1 µmole $NADH_2$, and 0.1 M phosphate buffer (pH 6.0) to a final volume of 1 ml. The reaction was carried out in Thunberg tubes that were rigorously evacuated and allowed to equilibrate at 30° for 5 minutes before tipping the nitrate into the rest of the reaction mixture to initiate the reaction. After an appropriate incubation period, the reaction was stopped by opening the tubes to air and adding 0.1 ml 1 M zinc acetate and 1.9 ml redistilled 95 per cent (v/v) ethanol to precipitate any residual $NADH_2$ that might interfere with the chemical determination of nitrite (Medina and Nicholas, 1957). Nitrite was determined in 0.1 ml

portions of the supernatant solution left after centrifuging at 3,000 x g for 5 minutes.

2. Benzyl Viologen-Nitrite Reductase.

The reaction mixture for this assay contained 2 μmoles $NADH_2$, 0.05 ml *Azotobacter* particles, 0.01-0.05 ml enzyme, 1 μmole $NaNO_2$, 1 μmole oxidised benzyl viologen, and 0.1 M phosphate buffer (pH 7.0) to a final volume of 1.0 ml. The $NADH_2$ was added to the reaction mixture last, immediately before evacuating the Thunberg tube, to minimise loss due to the active $NADH_2$ oxidase activity of the *Azotobacter* particles. The incubation procedure was the same as described above.

3. Reduced FMN-Nitrite Reductase.

For this assay the following reactants were employed: 0.1 μmole FMN, 0.5 μmole $NaNO_2$, 0.2 ml enzyme preparation, 0.2 mg $Na_2S_2O_4$, and 0.1 M phosphate buffer (pH 6.0) to a total volume of 1 ml. The reaction was begun by tipping the sodium dithionite into the rest of the reactants from the side-arm of the evacuated Thunberg tube. The incubation procedure was as described above.

4. Manometric Assay.

The reaction was carried out in standard Warburg manometer flasks fitted with double side-arms. The

following reactants were used: Main compartment: 5 µmoles $NADH_2$, 0.2 µmole FMN, and 0.2 ml enzyme, contained in 2.4 ml of 0.1 M phosphate buffer (pH 6.0); Side-arm 1: 5 µmole $NaNO_2$; Side-arm 2: 0.2 ml alkaline sulfite solution, 0.2 ml alkaline permanganate solution, or 0.2 ml water; Center well: 0.2 ml 20 per cent (w/v) KOH. The flasks were flushed for 40 minutes with oxygen-free nitrogen gas. The reaction, initiated by adding the $NaNO_2$ from side-arm 1, was allowed to continue for 2 hours or until gas production had ceased. Gas production was followed during the course of the reaction and residual nitrite determined at the end of the incubation period.

E. $NADH_2$-Oxidase Activity.

$NADH_2$-oxidase in cell extracts was measured either by following $NADH_2$ oxidation or oxygen uptake.

1. Spectrophotometric Method.

$NADH_2$-oxidase activity in the extracts was determined by following the decrease in absorption at 340 nm in a Unicam SP 700 recording spectrophotometer at 25°. The reaction mixture consisted of 0.01 ml of cell-extract in 0.1 M phosphate buffer (pH 7.5) in a final volume of 2.9 ml in a 1 cm cuvette. The reaction was begun by adding 0.5 µmole $NADH_2$ to the mixture in the cuvette.

2. Oxygen Uptake Method.

Oxygen uptake was determined with a Beckman Oxygen Analyzer (Model 39065) complete with an adaptor box (96260). The cell-free extract was diluted appropriately with 0.1 M phosphate buffer (pH 7.5) and placed in the reaction vessel which was thermostatically controlled at 30°. The reaction was initiated by injecting 2 µmoles of $NADH_2$ through the substrate inlet port with a hypodermic needle. The absolute quantity of oxygen present was determined by measuring the amount of $NADH_2$ needed to exhaust all the oxygen in the reaction vessel in the presence of the $NADH_2$-oxidase preparation from P. denitrificans.

F. Succinate Oxidase.

Succinate oxidase activity was measured by oxygen uptake using the same technique as that for $NADH_2$ oxidase with 2 µmole sodium succinate as substrate in place of $NADH_2$.

IV. PURIFICATION OF ENZYMES.

A. Solubilization of Nitrate Reductase.

Further purification of the nitrate reductase enzyme itself required a separation of the enzyme from the membrane-bound respiratory system. Three methods were used in the attempt to solubilize the enzyme.

1. Cold Acetone Treatment.

Twenty volumes of cold acetone (-30°) were added to a suspension of particles (10 mg protein/ml) in the cold, (-17°) with stirring. The mixture was stirred for 3-6 minutes and the particles collected rapidly by vacuum filtration and dried under reduced pressure in a vacuum dessicator in the cold. The resulting acetone powder was dissolved in 0.1 M Tris-HCl (pH 7.4) and assayed for nitrate reductase and formate dehydrogenase activity.

2. Alkaline Incubation After Heat Treatment.

A suspension of particles (10 mg protein/ml) was adjusted to pH 8.5 and heated to 60° for 5 minutes, then cooled rapidly and incubated at 2° for 4 hours. The supernatant and residue left after centrifuging at 144,000 x g for 2 hours were tested for nitrate reductase and formate dehydrogenase activity.

3. Deoxycholate Treatment.

The particles (20 mg protein/ml) in 0.1 M Tris-HCl were adjusted to pH 8.0 and 1 mg sodium deoxycholate per mg protein was added. The mixture was incubated at 30° for 30 minutes, and then centrifuged at 225,000 x g for 2 hours to give a clear red supernatant fraction and a pellet. These fractions were assayed for enzymic activity.

B. Purification of Nitrite Reductase.

Nitrite reductase was purified by means of differential centrifugation and ammonium sulfate fractionation as follows. The crude cell extract was prepared as described previously, and centrifuged in a Beckman Spinco Model L centrifuge at 144,000 x g for 2 hours to give a supernatant and a particulate fraction. The supernatant fraction contained most of the nitrite reducing activity that was present in the crude extract. This fraction was diluted with an equal volume of 0.1 M phosphate buffer (pH 6.0) and ammonium sulfate added with stirring in the cold (2^{0}) until a saturation of 75 per cent was reached. The solution was allowed to stand for 30 minutes and the precipitate centrifuged off and discarded. The residual solution was treated with more ammonium sulfate until 80 per cent saturation was reached. The precipitate, centrifuged after the solution had stood for 30 minutes, was dissolved in 0.1 M phosphate buffer (pH 6.0) and dialysed for 12 hours against the same buffer.

V. CHEMICAL AND BIOLOGICAL DETERMINATIONS.

A. Nitrite.

Nitrite concentration was determined in aliquots containing from 0 to 50 µmoles of nitrite, by the method of Medina and Nicholas (1957). Each sample was diluted

to 5 ml with distilled water and 0.5 ml 1 per cent (w/v) sulfanilamide in 1 M HCl and 0.5 ml 0.02 per cent (w/v) N-(1-naphthyl)-ethylene diamine dihydrochloride in water added. Absorbance at 520 nm was read in a Hilger colorimeter after 5 minutes and the nitrite concentration determined from a standard calibration graph.

B. Nitric Oxide.

1. Alkaline Permanganate Method.

Nitric oxide was estimated by the method of Anderson (1965). Alkaline permanganate solution (125 mM $KMnO_4$ in 1 M KOH) absorbs nitric oxide and oxidises it to nitrite. The alkaline permanganate solution was included in one side-arm of a double armed Warburg manometer vessel. After the reaction had taken place samples of the alkaline permanganate solution were removed from the side arm with a curved pasteur pipette. A 0.2 ml sample was mixed with 3 ml of alkaline arsenite solution (1.3 per cent (w/v) $NaAsO_2$ in 0.67 M KOH), 0.8 ml water and 1.0 ml 0.25 M KH_2PO_4 adjusted to pH 11 with KOH. After 10 minutes the precipitate (mainly MnO_2) was removed by centrifuging at 3,000 x g for 10 minutes, and an appropriate sample removed for the determination of nitrite as described previously.

For standardization of the above procedure, a known amount of nitric oxide was generated by the reaction of a

fixed amount of nitrite with excess acidified potassium iodide:

$$HNO_2 + KI \longrightarrow NO + 1/2\ I_2 + H_2O$$

This reaction was carried out in a double-armed Warburg flask at 30° under an atmosphere of nitrogen. The reaction mixture consisted of: in the main compartment—1 m-equivalent H_2SO_4 and 0.2 μmoles KI in 2.3 ml of water; in side arm 1—0.2 ml $NaNO_2$ solution of varying concentrations; in side arm 2—0.4 ml alkaline permanganate solution. After flushing the flask with oxygen-free nitrogen the nitrite was tipped into the acidified potassium iodide solution and allowed to react for 60 minutes with shaking to ensure good gas exchange. The alkaline permanganate solution was then assayed for nitrite as described above. A recovery of 85 per cent of the theoretical yield was obtained by this method.

2. Alkaline Sulfite Solution.

Alkaline sulfite solution, which also acts as an absorbent for nitric oxide, was prepared by the method of Treadwell and Hall (1942). In 100 ml of distilled water, 20 gm Na_2SO_3 and 2 gm KOH, were dissolved. Nitric oxide reacts with this solution to form sodium N-nitrosohydroxylamine-N-sulfonate ($Na_2N_2O_2SO_3$).

C. NAD and $NADH_2$.

The fluorometric method of Lowry, Roberts, and Kapphahn (1957) was used to measure NAD. The concentration of $NADH_2$ was determined from its absorbance at 340 nm assuming an extinction co-efficient of 6.22×10^{-3} mole cm^{-1}.

D. Ubiquinone Extraction and Assay.

The ubiquinone content of cell-extracts was determined by the method of Humphrey and Redfearn (1960). In a 15 ml conical centrifuge tube, one ml of cell-extract containing 10-40 mg protein was denatured by rapidly adding 4 ml of cold (-17°) methanol in which was dissolved 1 mg/ml pyrogallol. Five ml light petroleum (b.p. 40-60°) was added immediately and the mixture shaken vigorously for 1 minute. The mixture was centrifuged in a M.S.E. bench centrifuge at 2,000 x g for 5 minutes to separate the layers, and the light petroleum layer transferred to another 15 ml centrifuge tube. The denatured cell-extract was extracted again with 3 ml of light petroleum. The combined light petroleum extracts were treated with 2 ml of 95 per cent (v/v) methanol and shaken for 30 seconds. After separation the light petroleum layer was transferred to a small beaker and evaporated under reduced pressure in a vacuum dessicator. The residual lipid was then

redissolved in 3 ml of spectroscopically pure ethanol and the spectrum of the oxidized substance measured in the 230-320 nm region on a Unicam SP800 spectrophotometer. One crystal (about 0.5 mg) of sodium borohydride was stirred carefully into the solution in the cuvette and the reduced spectrum determined. The peak present in the oxidized solution at 275 nm shifts to 290 nm and decreases in intensity. From the decrease in extinction at 275 nm the concentration of ubiquinone can be calculated $(E_{ox.} - E_{red.}) = 12250$ mole^{-1} cm^{-1}.

E. Extraction and Determination of Flavin.

For the extraction of flavin, 30 mg of trypsin was added to 5 ml of the cell membrane fraction and mixed thoroughly. The mixture was incubated at 37° for 90 minutes, then heated in a boiling water bath for 10 minutes and cooled in ice. Protein was precipitated with 1.0 ml of cold 3M perchloric acid and centrifuged at 3,000 x g for 15 minutes. The precipitate was washed with 3 ml water and the washings combined with the first extract. The extract was neutralized with 2 N KOH and the $KClO_4$ removed by centrifuging. The extract was taken to dryness in a rotary evaporator and dissolved in 2 ml H_2O. The absorbance at 445 nm was read on a Unicam SP 800 recording spectrophotometer. An extinction coefficient of 12 x 10^{-3}

mole cm^{-1} was assumed for flavin.

F. Total Cytochrome and Flavoprotein Concentration.

The amounts of cytochrome and flavoprotein in the particulate fraction (P-144) were determined spectrophotometrically from dithionite reduced minus oxidised difference spectra using the wavelength pairs and extinction coefficients as given by Asano and Brodie (1964). These values are as follows:

	Wavelength Pair (nm)		E mM
Cytochrome a	598,	623	16
Cytochrome b	562,	574	20
Cytochrome c	551,	540	19
Flavoprotein	445,	510	11

G. Protein.

Protein was determined by the Folin method as modified by Lowry et al. (1951) using bovine serum albumin as the standard. Protein fractions that contained ammonium sulfate were dialysed against large volumes of distilled water to remove ammonium ions before analysis.

H. Total Protein Nitrogen.

Total protein nitrogen was determined by the micro-

Kjeldahl nitrogen method (Ballentine, 1957).

VI. MASS SPECTROMETRY.

Qualitative determination of gases formed by actively proliferating cultures was made by growing the cells for 24 hours in the standard medium to which had been added ^{15}N-labelled KNO_3 as the sole electron acceptor. The incubation was carried out in Rittenberg tubes which were rigorously evacuated to 10^{-3} mm Hg and then placed in an anaerobic jar which was also evacuated to minimize the risk of atmospheric contamination during the long incubation period. Resting cell suspensions or cell-free extracts were incubated as described above for 2 hours before testing for gas production in the mass spectrometer. About 2 ml of 20 per cent (w/v) KOH was included in a side-arm of all the tubes to absorb any CO_2 formed. The gaseous products formed during the incubation period were transferred from the Rittenberg tubes under high vacuum into an A.E.I. MS-2 mass spectrometer for analysis.

VII. DIFFERENCE SPECTRA.

Absorption spectra were recorded on a Unicam SP 800 spectrophotometer fitted with a scale expansion attachment and external recorder. Difference spectra were obtained by comparing cell suspensions or extracts

reduced with $Na_2S_2O_4$, $NaBH_4$, or formate with an identical sample that had been treated with oxygen or with nitrate or nitrite.

VIII. MICHAELIS CONSTANTS.

Initial reaction rates were measured as a function of the concentration of one substrate while the other substrate was held at a constant, saturating level. Michaelis constants were calculated from double reciprocal plots of the reaction velocity versus substrate concentration as described by Lineweaver and Burk (1934).

IX. REACTION OF 2,4-DINITROPHENOL WITH REDUCED FMN.

FMN was reduced with hydrogen gas using palladised asbestos as the catalyst. The 2,4-dinitrophenol solution was thoroughly deoxygenated in a bottle by bubbling with oxygen-free nitrogen through hypodermic needles inserted through a serum cap. The reduced FMN was removed from the hydrogenation vessel and injected anaerobically into the 2,4-DNP solution using a Hamilton gas-tight syringe. The mixture was allowed to react for 16 hours at 37°. The products of the reaction were separated by thin-layer chromatography on silica gel plates using a 10 per cent ethanol-90 per cent benzene (v/v) mixture, and authentic samples of 2-amino-4-nitrophenol and 2-nitro-4-aminoni-

trophenol as markers. The separated products were scraped off and eluted from the silica gel in 0.1 M phosphate buffer (pH 7.5) and the ultraviolet absorption spectra compared with those of the standards on a Unicam SP 800 spectrophotometer.

X. ULTRAVIOLET IRRADIATION OF EXTRACTS.

Ultraviolet irradiation of the cell-free extracts was carried out with an Oliphant probe-type ultraviolet lamp (maximum emission 253.7 nm). The lamp was placed about 3 cm above a layer of extract 2-3 mm deep that was stirred throughout the irradiation period with a magnetic stirrer, and kept cold in an ice bath. The extracts were irradiated for 30 minutes and then assayed for nitrate reductase activity with formate of $NADH_2$ as an electron donor and also for any change in ubiquinone content.

XI. CYANIDE DIALYSIS.

The enzyme preparation was dialysed against 5×10^{-3} M KCN on 0.05 M Tris-HCl buffer (pH 7.0) at 2° for 24 hours. A sample was removed for assay of activity, and the remainder was dialysed against several changes of 0.05 M Tris-HCl (pH 7.0) for 36 hours. Samples were removed at 12 and 24 hours to test activity.

The method of Kubowitz (1938) was also employed. The procedure was carried out in a double side-armed Warburg flask. The main compartment contained the purified enzyme (9 mg protein), and 1 ml 0.5 M phosphate (pH 7.5) in a total volume of 3 ml; one side arm contained 5 umole $NaBH_2$ and the other contained 0.03 ml 0.05 M KOH. The flasks were evacuated and flushed with oxygen-free nitrogen. The $NaBH_2$ was tipped into the main compartment and allowed to reduce the enzyme. The KCN or KOH was added and allowed to stand at room temperature for 30 minutes. The protein was then precipitated with ammonium sulfate, centrifuged and washed with saturated ammonium sulfate solution containing 0.05 M Tris-HCl (pH 7.0) and dialysed against several changes of the same buffer for 24 hours at 2°. The treated enzyme was then assayed for nitrite reductase activity.

XII. ASSIMILATION OF INORGANIC NITROGEN.

Cells were grown anaerobically in the basal medium, collected by centrifuging and washed aseptically and suspended in the aerobic medium supplemented with either 2 µmole/ml of $K^{15}NO_3$, $Na^{15}NO_2$, or $^{15}NH_4Cl$ (32, 25, 31.3 and 32.2 atom per cent excess enrichment, respectively). After incubating aerobically for 2 hours at 37°, the cells

were centrifuged, washed 3 times with cold 0.85 per cent (w/v) saline to remove residual labelled nitrogen compounds. The cells were suspended in 10 per cent (w/v) trichloroacetic acid for 12 hours, and the precipitated protein centrifuged at 10,000 x g for 20 minutes. It was then washed twice with trichloroacetic acid. Total nitrogen was determined by the micro-Kjeldahl method and the atom per cent excess ^{15}N in the protein determined in an A.E.I. MS-2 mass spectrometer.

XIII. CO-FACTORS AND CHEMICALS.

Sigma Chemical Corp., St. Louis, (U.S.A.) supplied NAD^+, $NADPH_2$, FMN, FAD, riboflavin, bovine serum albumin, co-enzyme Q_{10}, Dicoumerol, Rotenone, Amytal, Trypsin, and 2-heptyl-4-hydroxyquinoline-N-oxide.

^{15}N-labelled potassium nitrate, sodium nitrite, and ammonium chloride (32, 25, 31.3, and 32.3 atom per cent excess enrichment, respectively) were purchased from the Office National Industriel de l'Azote, France. The Australian Atomic Energy Commission, Lucas Heights, N.S.W. supplied the ^{14}C-labelled sodium formate.

The 2,4-dinitrophenol derivatives were gifts from Dr. D. Woodcock, Long Ashton Research Station, University of Bristol. The 2-amino-4-nitrophenol and 2-nitro-4-aminonitrophenol were a gift from Fluka AG, Buchs, Switzer-

land. Piericidin A was a gift from Professor S. Tamura, Department of Agricultural Chemistry, University of Tokyo.

Benzyl viologen, methyl viologen, dichlorophenol indophenol, and tetramethylphenylenediamine were purchased from British Drug Houses, Poole, England.

All other chemical reagents were of analytical grade and were obtained either from British Drug Houses, Poole, England, or from May and Baker Ltd., Dagenham, England.

RESULTS

I. STUDIES ON GROWTH AND ON THE ELECTRON TRANSPORT CHAIN IN WHOLE CELLS.

The effects of cultural conditions on cell growth and the composition of the electron transfer chain are reported in this section.

A. Effect of Cultural Conditions on Growth.

Long-term stock cultures maintained on anhydrous silica gel remained viable for at least 2 years. Use of this technique provided a convenient means of maintaining strain viability and stability over long periods of time without the necessity of frequent subculturing.

Studies on the growth of *Pseudomonas denitrificans* (A.T.C.C. 13876) in a variety of culture solutions showed that adequate growth and good production of the nitrate dissimilating system occurred under anaerobic conditions at 37° in the basal medium described in Methods. A mean generation time of about 40 minutes was recorded under these conditions. No differences were observed between cultures bubbled with oxygen-free nitrogen and those left as deep, still, 10 liter cultures with no bubbling. The bacterium also grew well when aerated in the basal medium with or without nitrate. Aerobic growth produced

approximately 25 per cent higher cell yield than anaerobic growth with nitrate.

No growth occurred in the nitrate-free medium under anaerobic conditions, or with sodium nitrite (1 gm/l) as the sole electron acceptor, even with prolonged incubation periods. Aerobic growth without nitrate was not affected by the addition of sodium nitrite at concentrations up to 2 gm/l.

Good growth occurred aerobically or anaerobically in the complete sodium glutamate medium used in place of the standard peptone medium for trace metal deficiency studies. Growth of cells in the iron-deficient medium resulted in a reduction in yield of 40 per cent compared with the complete cultures. Attempts to produce copper-deficient cultures were not successful. Equally good growth occurred in both copper-deficient and complete media. This was probably a reflection of the extremely low amount of copper required for normal growth and metabolism.

B. Breakage of Cells.

Three methods of cell-disruption were compared on the basis of the proportion of the total protein found in the 10,000 x g supernatant fraction after centrifuging for 20 minutes and by microscopic examination. Repeated

freezing and thawing proved to be the least satisfactory method, since only about 20 per cent of the total protein was located in the supernatant fraction. Microscopic examination of the homogenate showed that most of the cells were undamaged.

Breakage in the French pressure cell and by ultrasonication (5 minutes at 20 kcycles/sec.) both gave a more efficient disruption of cells than did repeated freezing and thawing. Both preparative methods resulted in about 80-90 per cent breakage of the cells. There was a similar distribution of nitrate and nitrite reducing activities in extracts prepared by the two methods. The French pressure cell technique was adopted as the standard method for preparing cell homogenates because of its simplicity and the less drastic disruption of cell membranes compared with the ultrasonic technique.

C. Gas Production During Denitrification.

Experiments were carried out to determine the products of nitrate and nitrite reduction by whole cells using the mass spectrometric technique previously described.

1. Gas Production from Nitrate.

It was observed that copious quantities of gases were formed during anaerobic growth with nitrate in still cultures. To identify these gases, the cells were grown

in evacuated, sealed tubes on the basal medium with ^{15}N-labelled nitrate (32.25 atom per cent excess) as the sole electron acceptor. The relative amounts of gaseous products after 24 hours incubation are shown in Figure 2. Large amounts of gases with masses 28, 29 and 30 were formed (corresponding to $^{14}N_2$, $^{14}N^{15}N$, and $^{15}N_2$, respectively). Very little product of mass 31 (^{15}NO) was formed, and only small quantities of gases of mass 44, 45, and 46 (corresponding to $^{14}N_2O$, $^{14}N^{15}NO$, and $^{15}N_2O$, respectively) were detected. No significant amounts of gas were formed in the uninoculated control vessels.

2. Gas Production from Nitrite.

Although P. denitrificans will not grow with nitrite as the sole electron acceptor, gas production from ^{15}N-labelled nitrite (31.3 atom per cent excess) was detected. Figure 3. presents data from a mass spectrometric assay of the gases formed by cultures after 24 and 96 hours incubation. After 24 hours incubation, the major products have masses of 30 and 31 (corresponding to ^{14}NO and ^{15}NO respectively). After 96 hours, more ^{14}NO and ^{15}NO were present, and in addition significant amounts of products

FIGURE 2.

PRODUCTS OF NITRATE REDUCTION BY WHOLE CELLS

Mass spectrometer assay of gaseous products formed by actively growing cultures of P. denitrificans after 24 hours incubation with ^{15}N-labelled KNO_3 (32.25 atom % enriched) as sole electron acceptor. See text for details of growth conditions.

Open bars: Actively growing cultures

Solid bars: Uninoculated controls

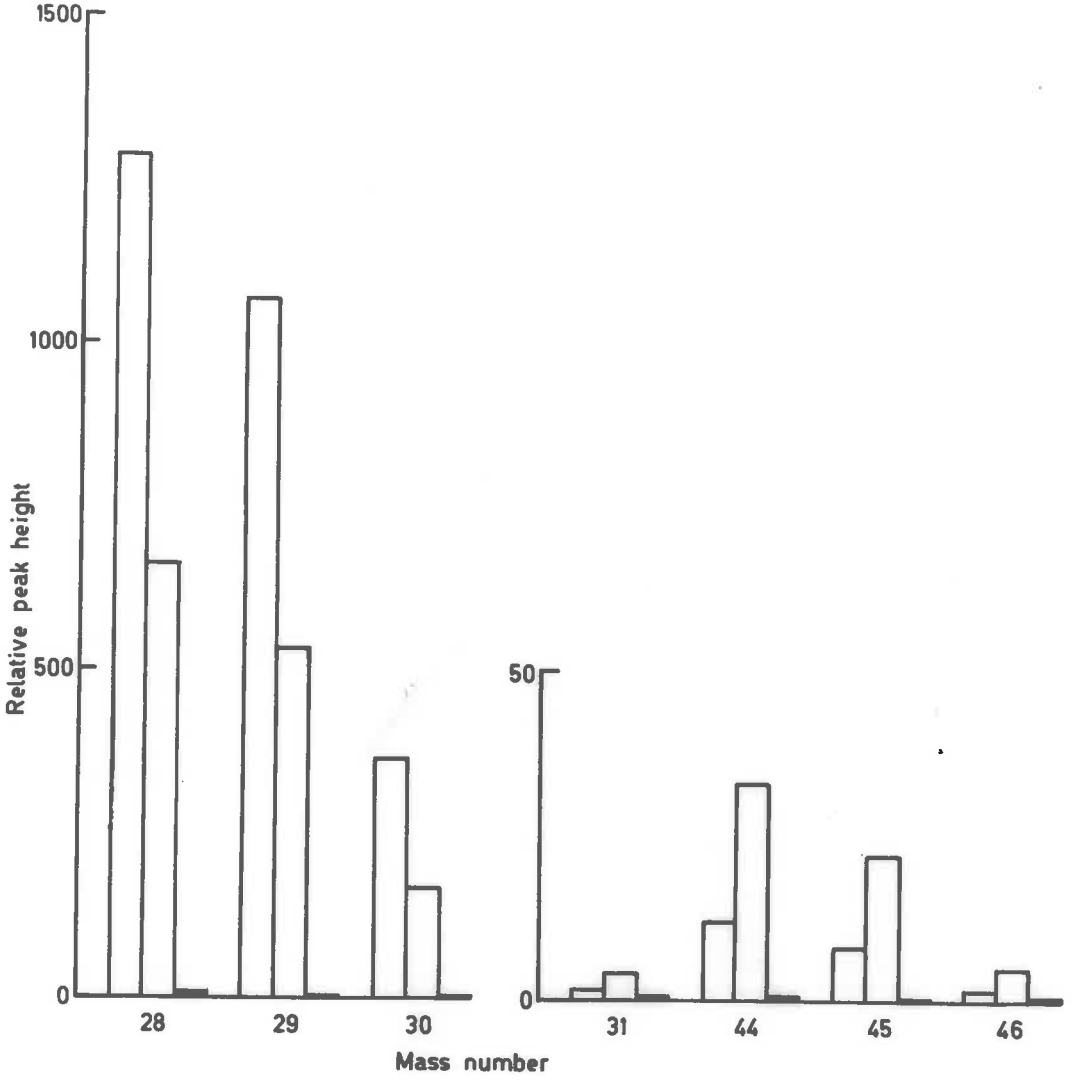

1500
1000
500
0
Relative peak height
50
0
28
29
30
31
44
45
46
Mass number

FIGURE 3.

PRODUCTS OF NITRATE REDUCTION BY WHOLE CELLS

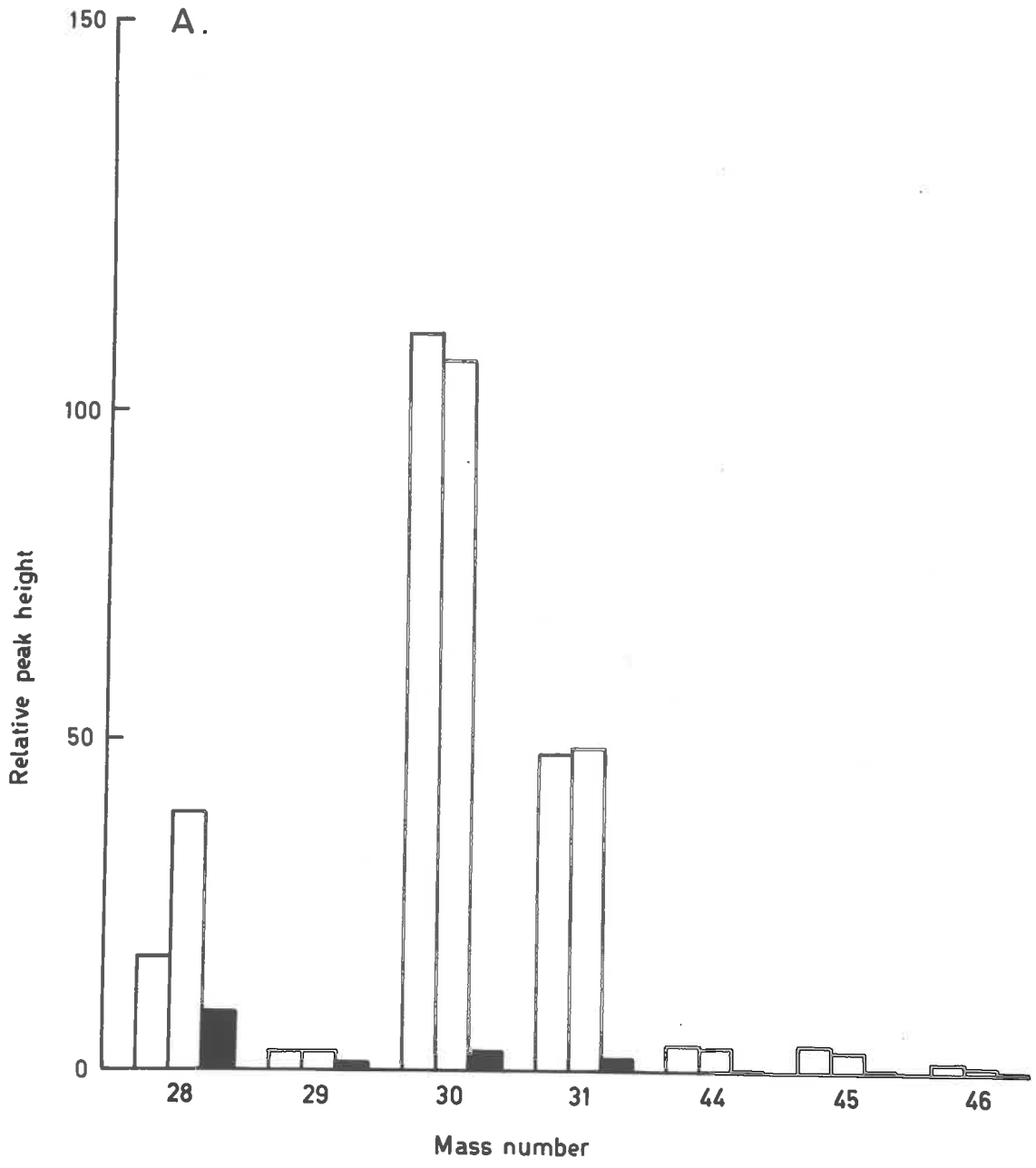
Mass spectrometer assay of gaseous products of actively growing cultures of *P. denitrificans* after 24 hours and 96 hours incubation with ^{15}N-labelled $NaNO_3$ (31.3 atom % enrichment). See text for details of growth conditions.

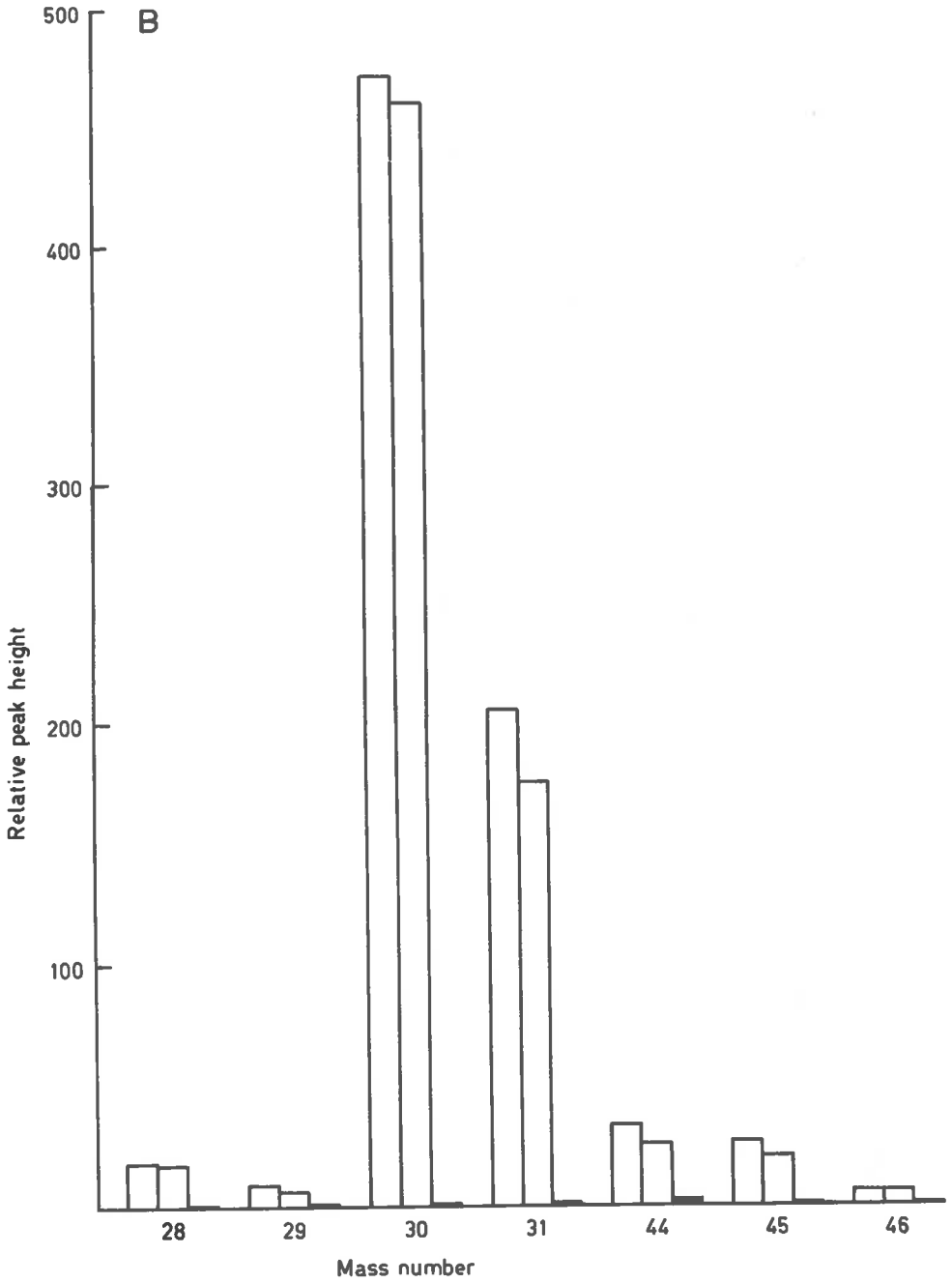
A. 24 hour incubation

B. 96 hour incubation

Open bars: Actively growing cultures

Solid bars: Uninoculated controls


A.
150
100
50
0
Relative peak height
28
29
30
31
44
45
46
Mass number


B
500
400
300
200
100
Relative peak height
28
29
30
31
44
45
46
Mass number

with masses of 44, 45, and 46 (corresponding to $^{14}N_2O$, $^{14}N^{15}NO$, and $^{15}N_2O$, respectively) were detected.

D. Effect of Terminal Electron Acceptor on Cells.

1. Cell Yield.

Cultures grown aerobically gave a 25 per cent higher yield than cultures grown anaerobically with nitrate: 4.1 gm/10 l when grown on oxygen versus 3.0 gm/10 l when grown anaerobically with nitrate.

2. Electron Transport System

Both qualitative and quantitative differences were observed in the composition of the electron transfer chains of aerobically and anaerobically grown cells. In Figure 4a. and b. difference spectra (dithionite reduced versus oxidized) for particles derived from cells grown in air and for those from cells grown anaerobically with nitrate are shown. In particles from oxygen grown cells, an a-type cytochrome (α-peak, 500 nm), a b-type cytochrome (α-peak, 560 nm), and a c-type cytochrome (α-peak, 551 nm) were observed. In particles derived from nitrate grown cells, only the b- and c-type cytochromes could be detected; there was no absorption band in the 600 nm region. A difference spectrum (dithionite reduced versus dithionite reduced plus carbon monoxide) showed a

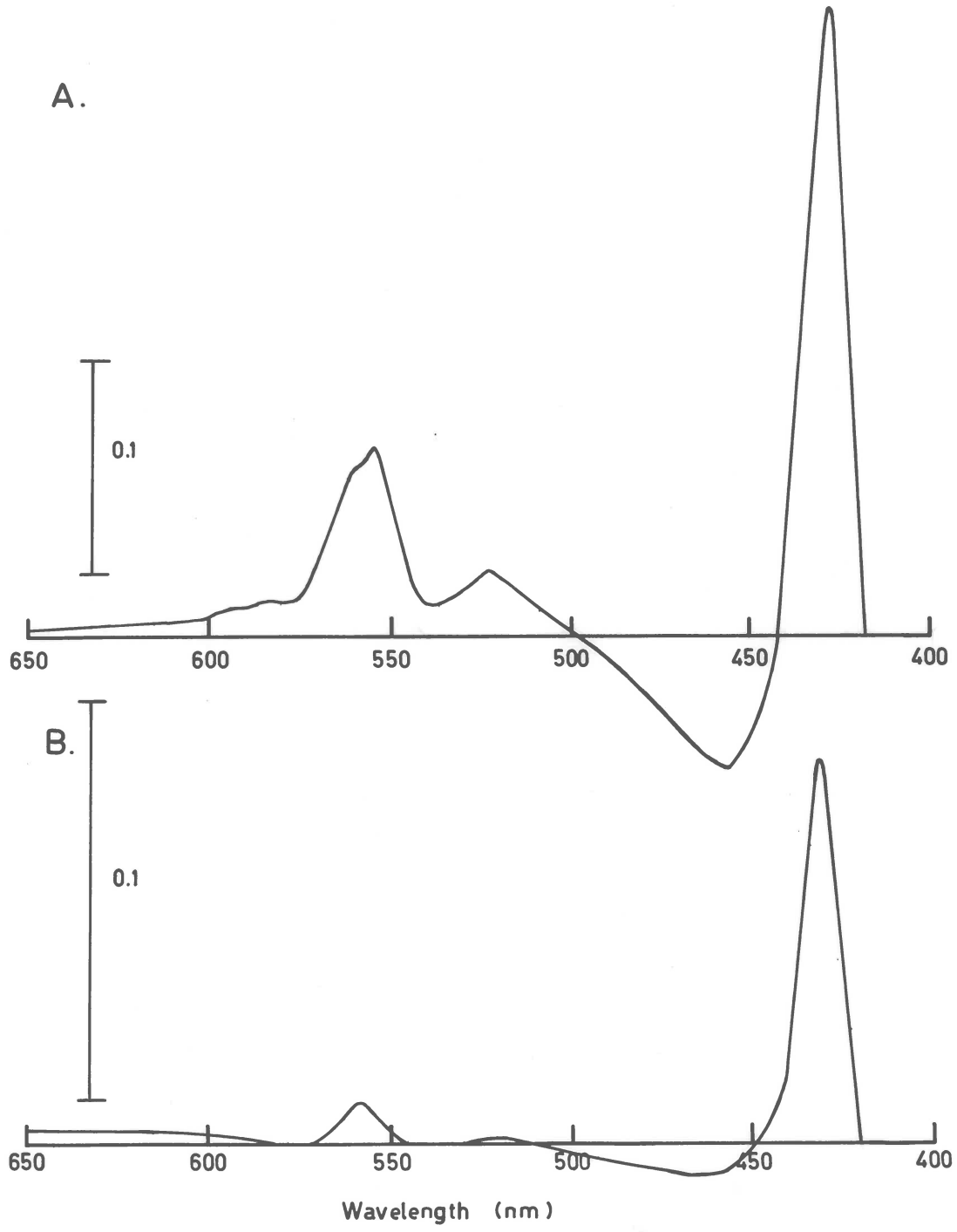
FIGURE 4.

DIFFERENCE SPECTRA OF PARTICLES FROM
CELLS GROWN AEROBICALLY OR ANAEROBICALLY

Dithionite reduced versus oxidised difference spectra of particles from aerobically and anaerobically grown cells.

A. Particles from oxygen-grown cells
(16 mg protein/ml)

B. Particles from nitrate-grown cells
(11 mg protein/ml)


A.
0.1
650
600
550
500
450
400
B.
0.1
650
600
550
500
450
400
Wavelength (nm)

cytochrome g type component in the nitrate-grown cells with absorption maxima at 419, 540, and 571 nm and minima at 437 and 558 nm.

Table II. sets out the quantitative differences in some of the components of the electron transfer chain. When calculated on an equivalent protein basis, electron transport particles from nitrate-grown cells contained 70 per cent more cytochrome b than did those from oxygen-grown cells. Cytochrome g content in the preparations of nitrate-grown cells was only slightly higher (20 per cent) than that of those grown in air. Cells grown with nitrate, however, had more than twice the flavoprotein content of those grown aerobically. Quinone concentration varied little in the two preparations.

3. Other Enzyme Activities.

Table III. compares the activities of a number of enzymes or enzyme systems in the particulate fractions from aerobically and anaerobically (nitrate) grown cells. Formate dehydrogenase activity was increased 8-fold by growing the cells with nitrate. $NADH_2$ oxidase activity was only slightly higher in nitrate grown cells than in those grown in air. An active succino-oxidase system was present in aerobically grown cells, but no activity was detected in particles from nitrate grown cells. Growing

TABLE II.

EFFECT OF TERMINAL ELECTRON ACCEPTOR ON COMPOSITION OF ELECTRON TRANSFER CHAIN

Components of the electron transfer system were estimated in the membrane fraction (144P). Cytochromes and flavoproteins were estimated from difference spectra (dithionite-reduced minus oxidised) by the method of Asano and Brodie (1964). Flavin and ubiquinone were extracted and determined as described in Methods (p. 63-65).

Electron transfer chain component	Concentration (n μmole/mg protein)	
	Terminal electron acceptor	
	O_2	NO_3^-
Cytochrome *a*	0.06	not detectable
Cytochrome *b*	0.44	0.74
Cytochrome *c* + c_1	0.68	0.81
Flavoprotein	0.68	1.61
Flavin (tryptic digest)	0.35	0.65
Ubiquinone	1.4*	1.2*

* μg/mg protein in crude extract

TABLE III.

ENZYMIC ACTIVITIES IN PARTICLES FROM CELLS GROWN WITH OXYGEN OR NITRATE

Activities of various enzyme systems in the particulate fraction (P-144) were assayed as described in Methods. Particles were prepared from cells grown with either oxygen or nitrate as the terminal electron acceptor.

Enzyme system	Activity (μmole substrate consumed/ mg protein/10 min.)	
	Type of Particles	
	O_2	NO_3^-
$NADH_2$ oxidase	4.6	6.3
Formate dehydrogenase	1.6*	13.0*
$NADH_2$-Nitrate reductase	0.25	1.52
Formate-nitrate reductase	0.96	8.45
Succinate oxidase	14.5	Not detectable

* μl CO_2 evolved/mg protein/10 min

the cultures anaerobically with nitrate resulted in a 6-fold increase in $NADH_2$-nitrate reductase activity and a 9-fold increase in the formate-nitrate reductase system.

E. Effect of Inhibitors on Electron Transport Chain.

The effects of a number of electron transport chain inhibitors on oxygen uptake are shown in Table IV. These inhibitors restricted oxygen uptake to a similar degree in particles from both types of cells. Amytal (0.2 mM) inhibited oxygen uptake 67 and 57 per cent in nitrate and oxygen particles, respectively. Rotenone (0.2 mM) restricted oxygen uptake 35 per cent (nitrate particles) and 31 per cent (oxygen particles). Piericidin A (0.04mM) decreased oxygen uptake by 47 and 51 per cent in nitrate and oxygen particles respectively. When 0.4 mM ubiquinone Q_{10} was added to the reaction mixtures, a 40 per cent reversal of this inhibition was observed. Antimycin A (20 µg/ml) and HOQNO (0.3 mM) each inhibited oxygen uptake by particles from either oxygen or nitrate grown cells by about 50 per cent.

F. Effect of Nitrate and Nitrite on $NADH_2$ Oxidation and Oxygen Uptake.

The effects of adding nitrate or nitrite to particles oxidising $NADH_2$ in air are shown in Table V. The spectrophotometrically measured rate of oxidation of $NADH_2$ by

TABLE IV.

EFFECT OF INHIBITORS ON $NADH_2$ OXIDATION BY RESPIRATORY PARTICLES FROM CELLS GROWN ON NITRATE OR OXYGEN

The uptake of oxygen with $NADH_2$ as electron donor was assayed using the oxygen electrode as described on p.58. The particulate fraction (144P) was prepared from cells grown with either nitrate or oxygen as the terminal electron acceptor; 0.5 mg protein was used for each assay.

Inhibitor	Concentration (mM)	Cells grown on:	Inhibition of O_2 uptake (%)
Amytal	2	NO_3^-	67
		O_2	57
Rotenone	0.2	NO_3^-	35
		O_2	31
Piericidin A*	0.04	NO_3^-	47
		O_2	51
HOQNO	0.3	NO_3^-	47
		O_2	50
Antimycin A	20 μg/ml	NO_3^-	51
		O_2	50

* A 40% reversal of inhibition occurred upon addition of ubiquinone Q_{10} (0.4 mM final concentration)

TABLE V.

EFFECT OF NITRATE AND NITRITE ON $NADH_2$ OXIDATION

The rate of $NADH_2$ oxidation was assayed spectrophotometrically, and the rate of oxygen uptake was assayed using the oxygen electrode as described on p. 57-58. The particulate fractions used were prepared from cells grown with either nitrate or oxygen as the terminal electron acceptor.

Cells grown on:	Addition	$NADH_2$ reduced (μmole/mg protein/10 min)	O_2 consumed (μmole/mg protein/10 min)
NO_3^-	None	6.3	4.7
	0.5 μmole KNO_3	6.3	-
	1 μmole $NaNO_2$	6.3	4.7
	10 μmole KNO_3	-	2.5
	10 μmole $KClO_3$	-	2.5
O_2	None	4.6	2.4
	0.5 μmole KNO_3	4.6	-
	1 μmole $NaNO_2$	4.5	2.4
	10 μmole KNO_3	-	2.4

particles from either oxygen or nitrate grown cells was unaffected by the addition of 0.5 µmole of potassium nitrate or 1 µmole of sodium nitrite. The rate of oxygen consumption by particles from nitrate grown cells was however decreased by adding 10 µmoles of KNO_3 or $KClO_3$. Addition of 10 µmoles of KNO_3 had no effect on oxygen uptake by particles from oxygen-grown cells. Oxygen uptake by either preparation was unaffected in including 1 µmole of sodium nitrite in the reaction mixture.

Inhibition of oxygen uptake in particles from nitrate-grown cells was accompanied by the reduction of nitrate or nitrite as shown in Table VI. The inhibition of oxygen uptake and the reduction of nitrate were stoichiometric.

G. Uptake of ^{15}N-labelled Nitrate, Nitrite, or Ammonia.

Anaerobically grown cells were tested to ascertain if they were able to assimilate inorganic nitrogen compounds. Table VII. shows that no significant increase in the atom per cent excess of ^{15}N of cell-nitrogen occurred over the controls when the cells were supplied with either $^{15}NO_3^-$, $^{15}NO_2^-$, or $^{15}NH_4^+$.

TABLE VI.

RELATIONSHIP BETWEEN INHIBITION OF OXYGEN UPTAKE BY NITRATE AND AMOUNT OF NITRATE REDUCED

The rate of oxygen uptake during $NaHN_2$ oxidation in the absence or presence of potassium nitrate was assayed by the oxygen electrode technique described on p. 58. The particulate fraction from nitrate-grown cells was used. The amount of nitrite formed was determined as described in Methods.

Addition	Inhibition in O_2 uptake (μmole)	NO_3^- reduced (μmole)
10 μmole NO_3^-	0.49	0.43
10 μmole NO_3^-	0.50	0.47

TABLE VII.

INCORPORATION OF ^{15}N-LABELLED NITRATE, NITRITE, AND AMMONIA BY WHOLE CELLS

The cells were grown anaerobically on the basal medium, then collected and washed aseptically and resuspended in aerobic medium without peptone. ^{15}N-labelled NH_4Cl, KNO_3, or $NaNO_2$ (2 μmole/ml) was added to each tube. The cells were incubated aerobically for 2 h at 30°, collected by centrifuging, and washed twice with 0.85% (W/V) saline to remove unincorporated ^{15}N. The cells were treated with 10% trichloroacetic acid for 12 h in the cold; the protein was centrifuged down and washed twice with 10% trichloroacetic acid. Total nitrogen and per cent enrichment with ^{15}N was determined for each sample.

^{15}N-labelled compound	Total N (mg)	Enrichment (atom % excess ^{15}N)
NH_4Cl	8.70	0.005
NH_4Cl	8.60	0.004
KNO_3	8.45	0.004
KNO_3	8.75	0.004
$NaNO_2$	8.30	0.003
$NaNO_2$	8.62	0.003
None	8.50	0.004
None	8.55	0.003

II. NITRATE REDUCTASE.

A. Particulate Nitrate Reductase.

Nitrate reductase in P. denitrificans is found associated with the cell-membrane fraction, and some of its properties will be described first. All cell-free extracts were prepared by the standard method using the French pressure cell as previously described.

1. Distribution.

Table VIII. shows the distribution of formate-nitrate reductase activity in cell-free homogenates of the bacterium. After centrifuging these homogenates at 144,000 x g for 2 hours, all the activity originally present in the crude extract was found in the pellet (P-144). No activity was detected in the supernatant fraction. Breaking the cells by ultrasonication or by freezing and thawing resulted in the same distribution as when the cells were broken in the French pressure cell. Thus the nitrate reducing system appears to be tightly bound to the cell-membrane fraction that also contains the electron transfer components of the cell.

2. Gas Production During Nitrate Reduction.

Figure 5. shows mass spectrometer data for gases produced by resting whole cells and by a particulate

TABLE VIII.

DISTRIBUTION OF FORMATE-NITRATE REDUCTASE IN CELL-FREE EXTRACTS

The enzyme activity in cell-free extracts was assayed anaerobically in Thunberg tubes using sodium formate as the electron donor. Details of the standard assay are described on p. 52.

Fraction	Total enzyme units (μmoles NO_3^- reduced per 10 min)	Total protein (mg)
1. Crude extract left after centrifuging homogenate at 10,000 x g for 20 min.	160	504
2. Pellet left after centrifuging Fraction 1 for 2 h at 144,000 x g	160	296
3. 144,000 x g supernatant fraction from 2	0	234

FIGURE 5

PRODUCTS OF NITRATE REDUCTION BY WHOLE CELLS AND CELL EXTRACTS

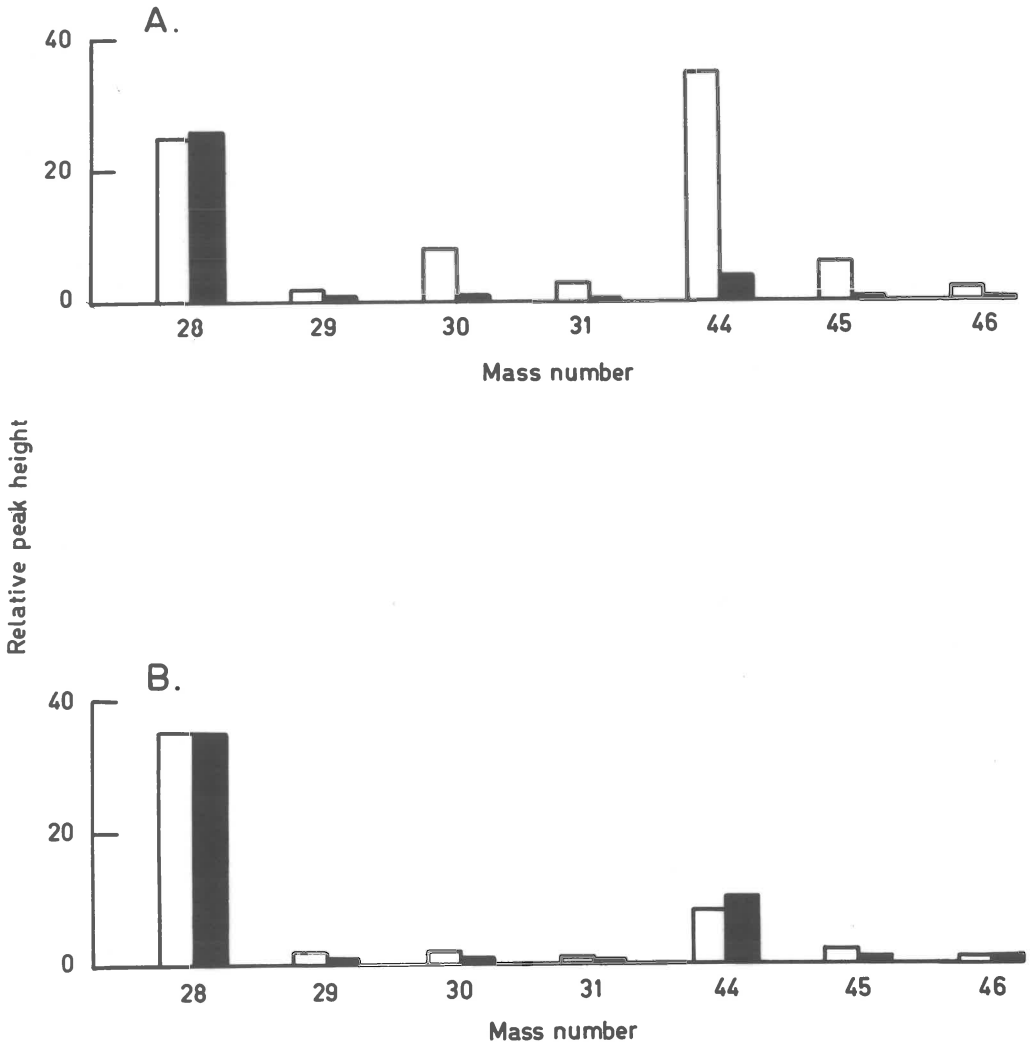
Mass spectrometer assay of gaseous products formed by resting cells or cell extracts from ^{15}N-labelled KNO_3 (32.25 atom % enriched) under anaerobic conditions after 2 hours incubation.

A. Resting cells

B. Particulate fraction (P-144)

Open bars: test material

Solid bars: controls


A.
40
20
0
28
29
30
31
44
45
46
Mass number
Relative peak height
B.
40
20
0
28
29
30
31
44
45
46
Mass number

preparation (P-144) after two hours incubation. Figure 5a. shows that after the short incubation period (2 hours) with whole cells, no nitrogen gas (mass 28 or 29) was formed, but that small quantities of intermediate products with mass 30 and 31 (^{14}NO and ^{15}NO) and with masses 44, 45, and 46 ($^{14}N_2O$, $^{14}N^{15}NO$, and $^{15}N_2O$) were formed. Figure 5b. shows that no gaseous products were formed by the particulate fraction (P-144) alone. This indicates that with only the cell-membrane fraction present, nitrate reduction proceeds only as far as a non-volatile intermediate, probably nitrite.

3. Electron Donors.

The relative efficiency of various electron donors with the particulate nitrate reductase (P-144) is shown in Table IX. Activity with $NADH_2$ was taken as 100. Very little or no nitrate reduction occurred with either $NADPH_2$, sodium succinate, or sodium lactate as electron donor. Sodium formate was as active with this preparation as was $NADH_2$. Activity with $NADH_2$ as donor was not stimulated by adding 10^{-3} M FMN. Only very slight (8 per cent) nitrate reduction occurred without the addition of an exogenous electron donor. The two most active electron donors, $NADH_2$ and sodium formate, have E'_0 of -0.32 and

TABLE IX.

RELATIVE EFFICIENCY OF VARIOUS ELECTRON DONORS FOR PARTICULATE NITRATE REDUCTASE

See text (p. 51-52) for details of standard assay; 1.9 mg protein (144P fraction) was used for each assay. Activity with $NADH_2$ taken as 100.

Electron donor	Relative efficiency	E_0^1 (pH 7.0)
$NADH_2$	100	-0.32
$NADH_2$ + 0.1 μmole FMN	89	
$NADPH_2$	13	-0.32
Sodium succinate	16	-0.03
Sodium formate	96	-0.42
Sodium lactate	0	-0.19
None	8	-

-0.42 volts, respectively, while sodium lactate and sodium succinate, which were least active, are more electropositive, and have E'_0 of -0.19 and -0.03 respectively.

4. Effect of Time of Incubation.

The effect of time of incubation on the production of nitrite by nitrate reductase is shown in Figure 6. Nitrite production by the particulate nitrate reducing system was linear with time up to 20 minutes incubation, when 60 per cent of the substrate had been consumed.

5. Effect of Temperature.

The effect of temperature on nitrate reduction is shown in Figure 7. The enzyme was completely inactivated at 70° when $NADH_2$ was the donor and at 80° when formate was the donor. All enzyme activity was lost with either donor upon heating to 100° for 5 minutes.

6. Effect of pH.

Figure 8. shows the effect of pH on the activity of nitrate reductase. Optimal activity occurred at pH 6.5 when sodium formate was the electron donor. A particularly sharp drop in activity occurred below pH 6.0 with a more gradual diminution of activity above pH 7.0. When $NADH_2$ was the electron donor, maximal activity occurred at pH 7.5. Variation in activity over the pH range 4.8 to 10.0

FIGURE 6.

THE EFFECT OF INCUBATION TIMES ON
NITRITE PRODUCTION BY NITRATE REDUCTASE

Nitrate reductase (1 mg of particulate fraction) was incubated for times varying from 0 to 20 minutes with sodium formate as electron donor as described in the text.

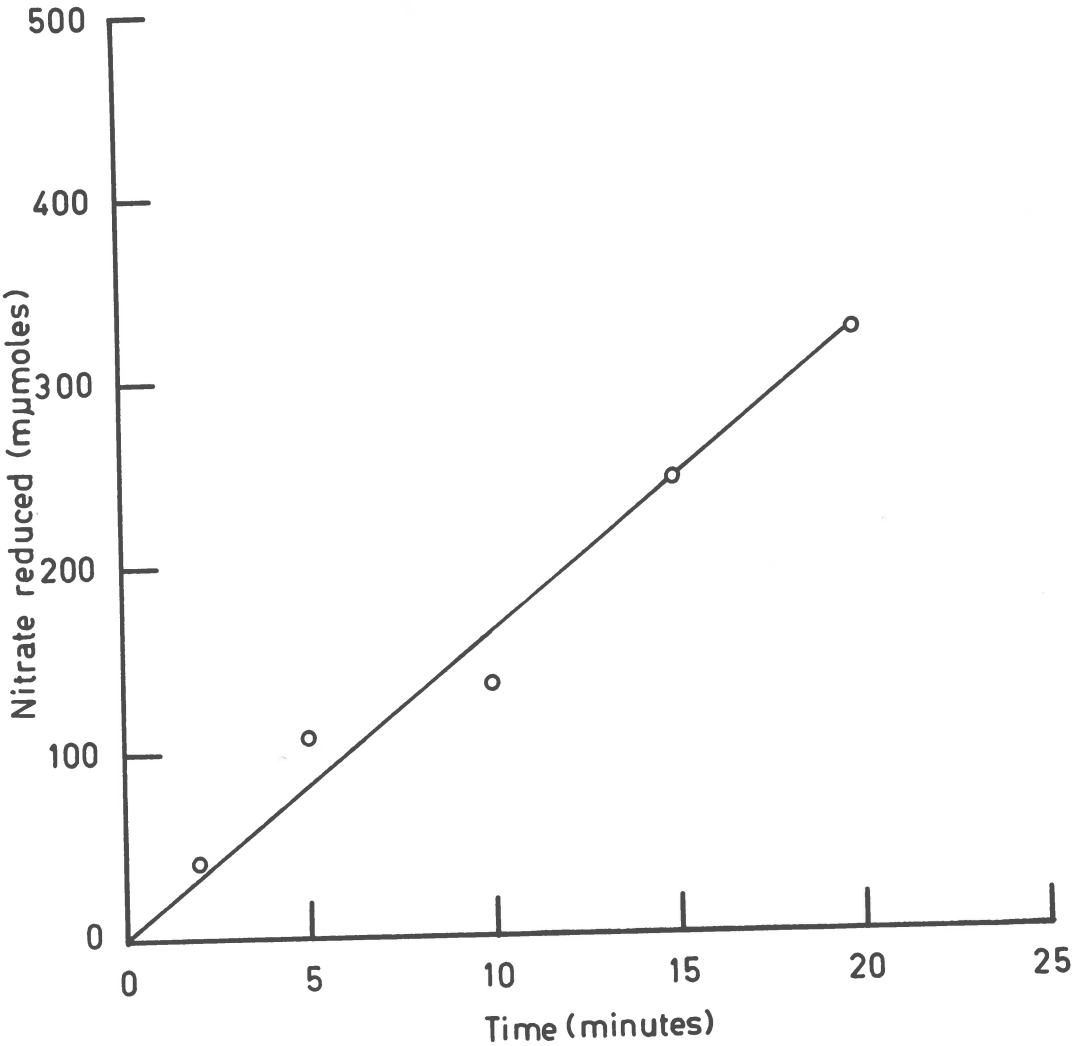

500
400
300
200
100
0
Nitrate reduced (mμmoles)
0
5
10
15
20
25
Time (minutes)

FIGURE 7.

THE EFFECT OF TEMPERATURE ON THE RATE OF NITRATE REDUCTION

Nitrate reductase activity at various temperatures was determined using the crude extract with sodium formate or $NADH_2$ as electron donors. Details of the assay are as given in the text.

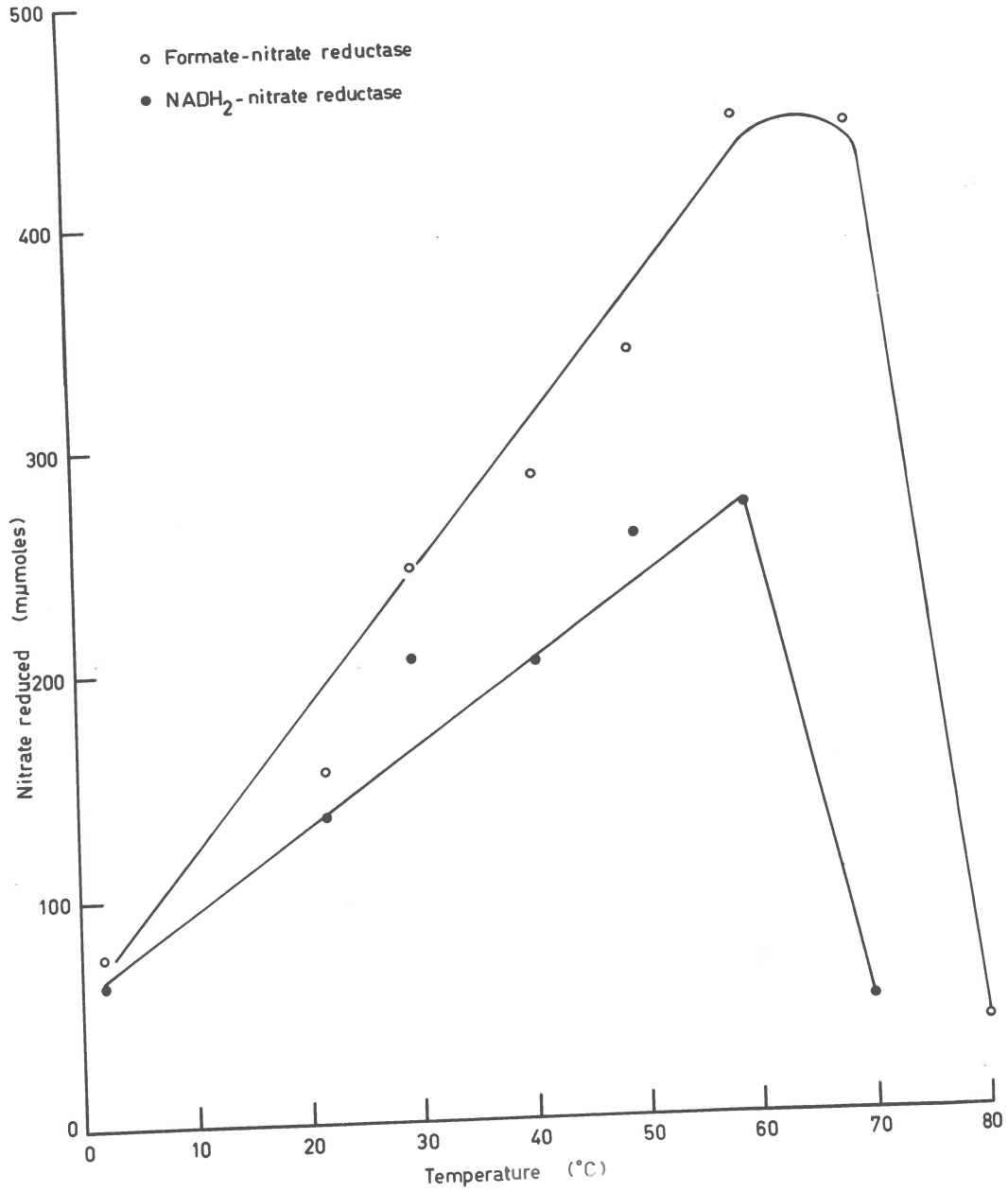
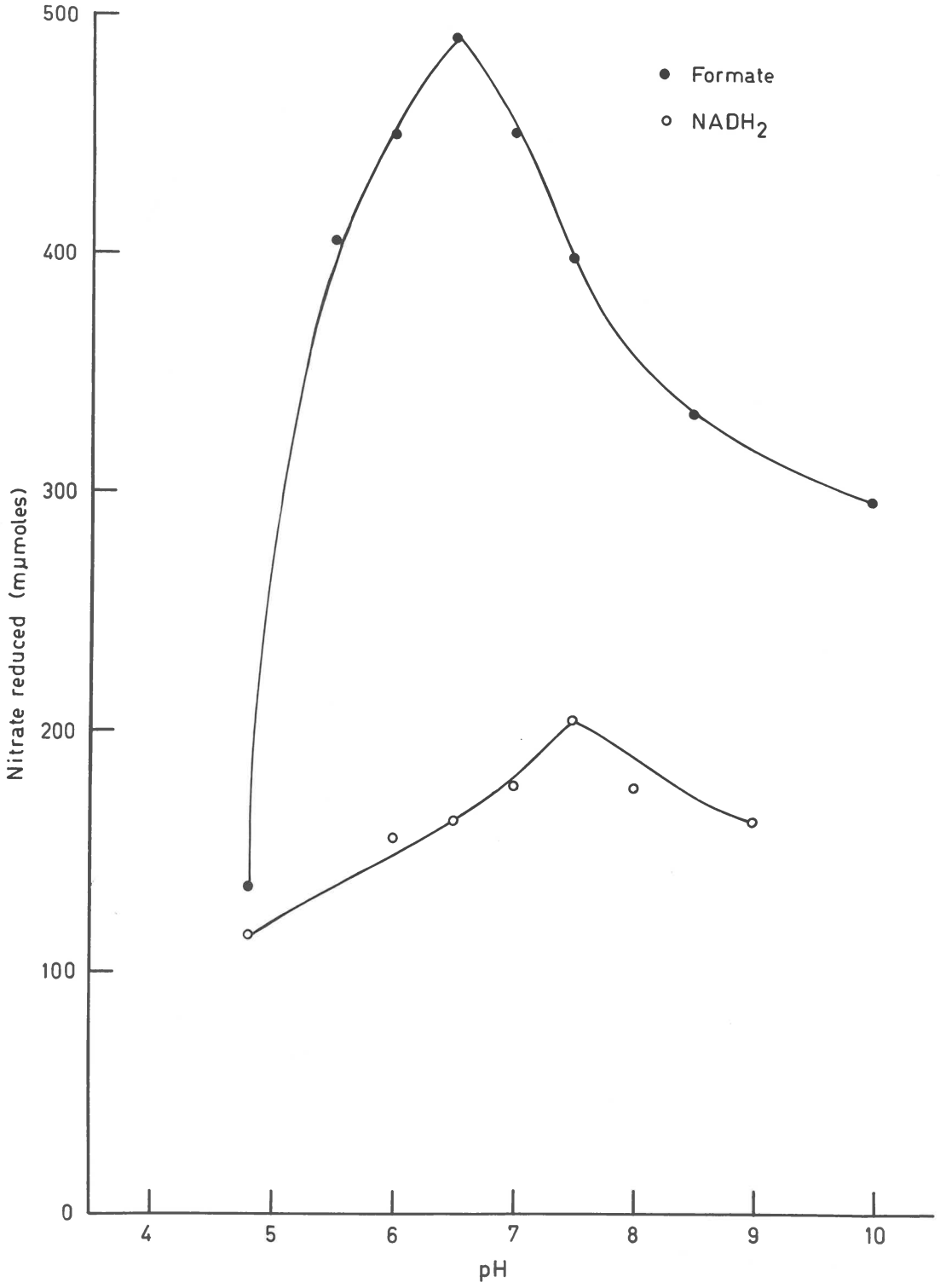

500
400
300
200
100
0
Nitrate reduced (mμmoles)
Formate-nitrate reductase
NADH2-nitrate reductase
0
10
20
30
40
50
60
70
80
Temperature (°C)

FIGURE 8.

THE EFFECT OF pH ON THE ACTIVITY OF NITRATE REDUCTASE

Nitrate reductase activity at various pH values was determined with the crude extract using sodium formate or $NADH_2$ as electron donor. Details of assay are given in the text.


500
400
300
200
100
0
Nitrate reduced (mμmoles)
Formate
NADH2
4
5
6
7
8
9
10
pH

was much less than with formate as the hydrogen donor.

7. Stoichiometry of Nitrate Reduction.

In Table X. the relationship between the oxidation of $NADH_2$ or sodium formate and nitrate reduced is presented. One mole of $NADH_2$ was utilized per mole of nitrate reduced. Likewise with formate as hydrogen donor, one mole of formate was oxidized to carbon dioxide per mole of nitrate reduced.

8. Michaelis Constants.

Figures 9, 10, and 11 are double reciprocal plots (Lineweaver and Burk, 1934) of the velocity of nitrate reduction when the concentration of one of the substrates was varied while the other substrate was held at a constant, saturating concentration. In Figure 9. the electron donor $NADH_2$ was the substrate varied. The apparent K_m value was found to be 1.9×10^{-4} M. At high concentrations of $NADH_2$, the rate of nitrate reduction fell off sharply. This may be interpreted as indicating the occurrence of substrate inhibition (Dixon and Webb, 1964).

In figure 10. the effect of varying the concentration of sodium formate is shown. At high concentrations of formate a falling off of the reaction rate was observed but not to the extent of that occurring with $NADH_2$. By

TABLE X.

STOICHIOMETRY OF NITRATE REDUCTION BY THE PARTICULATE PREPARATION

Formate- and $NADH_2$-nitrate reductase assays were performed as described in Methods with one substrate present in limiting amount and the other in excess as shown below. The reactions were allowed to go to completion and nitrite formed determined in the usual way.

Limiting substrate	Amount of substrate (μmole)	NO_3^- reduced (μmole)	$\frac{NO_3^- \text{ reduced}}{\text{substrate}}$
$NADH_2$	0.225	0.295	1.31
	0.450	0.467	1.04
	0.675	0.630	0.93
	0.900	0.750	0.83
Formate	2.0	2.2	1.10
	1.25	1.25	1.00
	0.50	0.48	0.96
NO_3^-	5.5	5.7	1.04
	2.5	2.45	0.98
	2.0	2.0	1.0

FIGURE 9.

MICHAELIS CONSTANT OF NITRATE REDUCTASE FOR $NADH_2$

Double reciprocal plot (Lineweaver and Burk, 1934) of the velocity of nitrate reduction at varying concentrations of $NADH_2$ using the particulate fraction (0.6 mg protein). Details of assay are given in text.

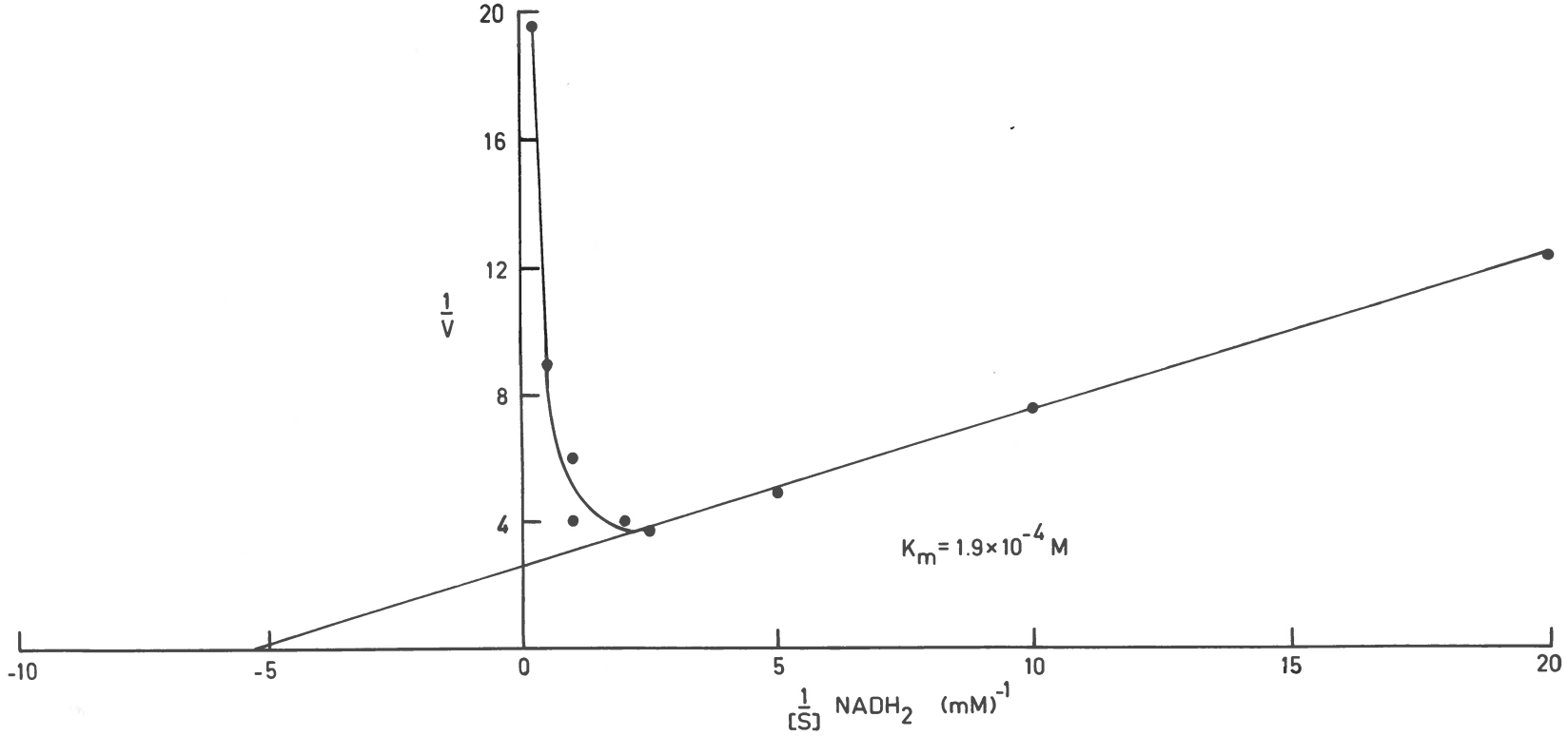

20
16
12
8
4
1/V
$K_m = 1.9 \times 10^{-4}$ M
-10
-5
0
5
10
15
20
1/[S] $NADH_2$ $(mM)^{-1}$

FIGURE 10.

MICHAELIS CONSTANT OF NITRATE REDUCTASE FOR FORMATE

Double reciprocal plot (Lineweaver and Burk, 1934) of velocity of nitrate reduction versus concentration of sodium formate. Assays were carried out with the particulate fraction (1 mg protein). Details of assay are given in the text.

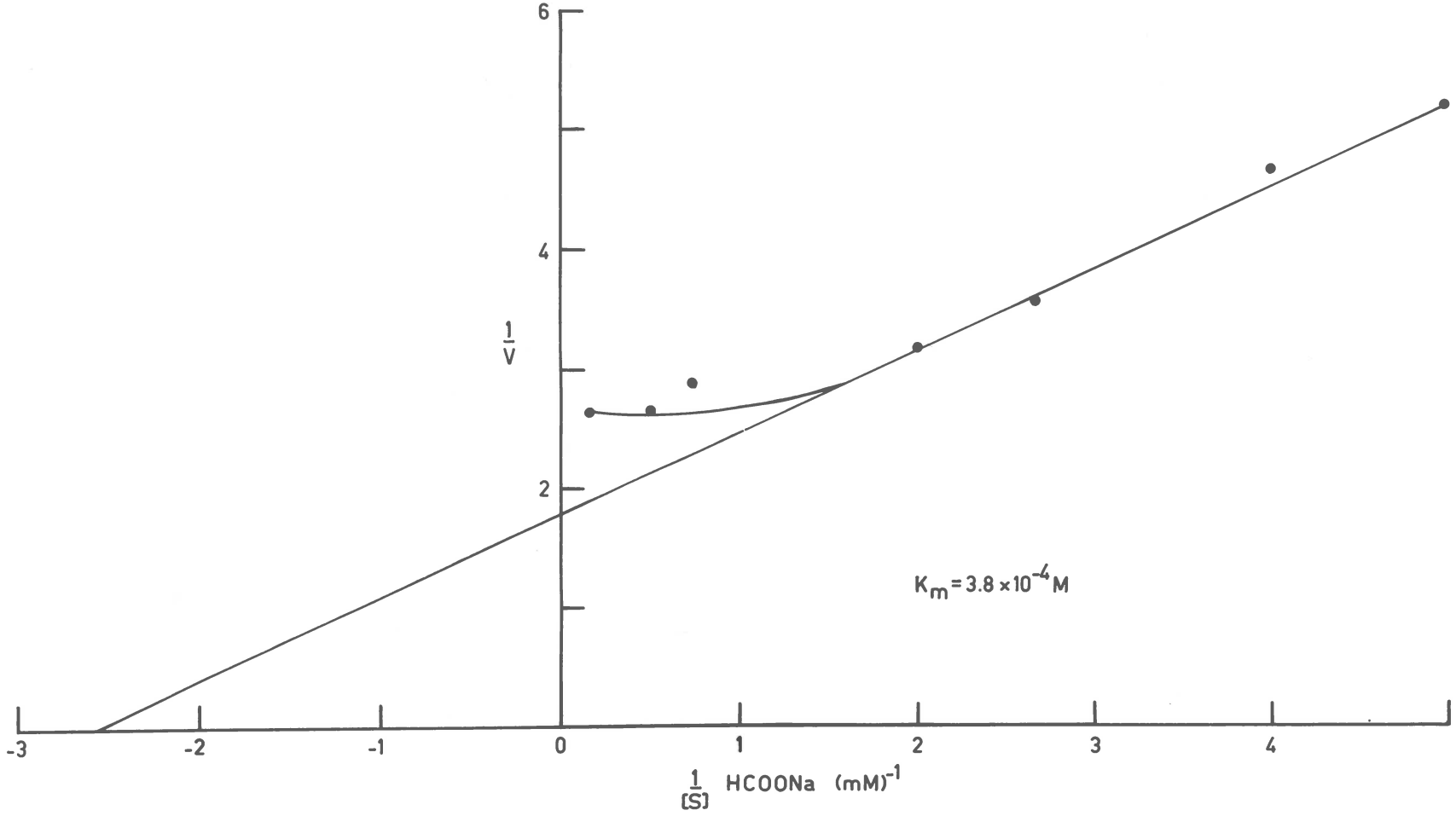
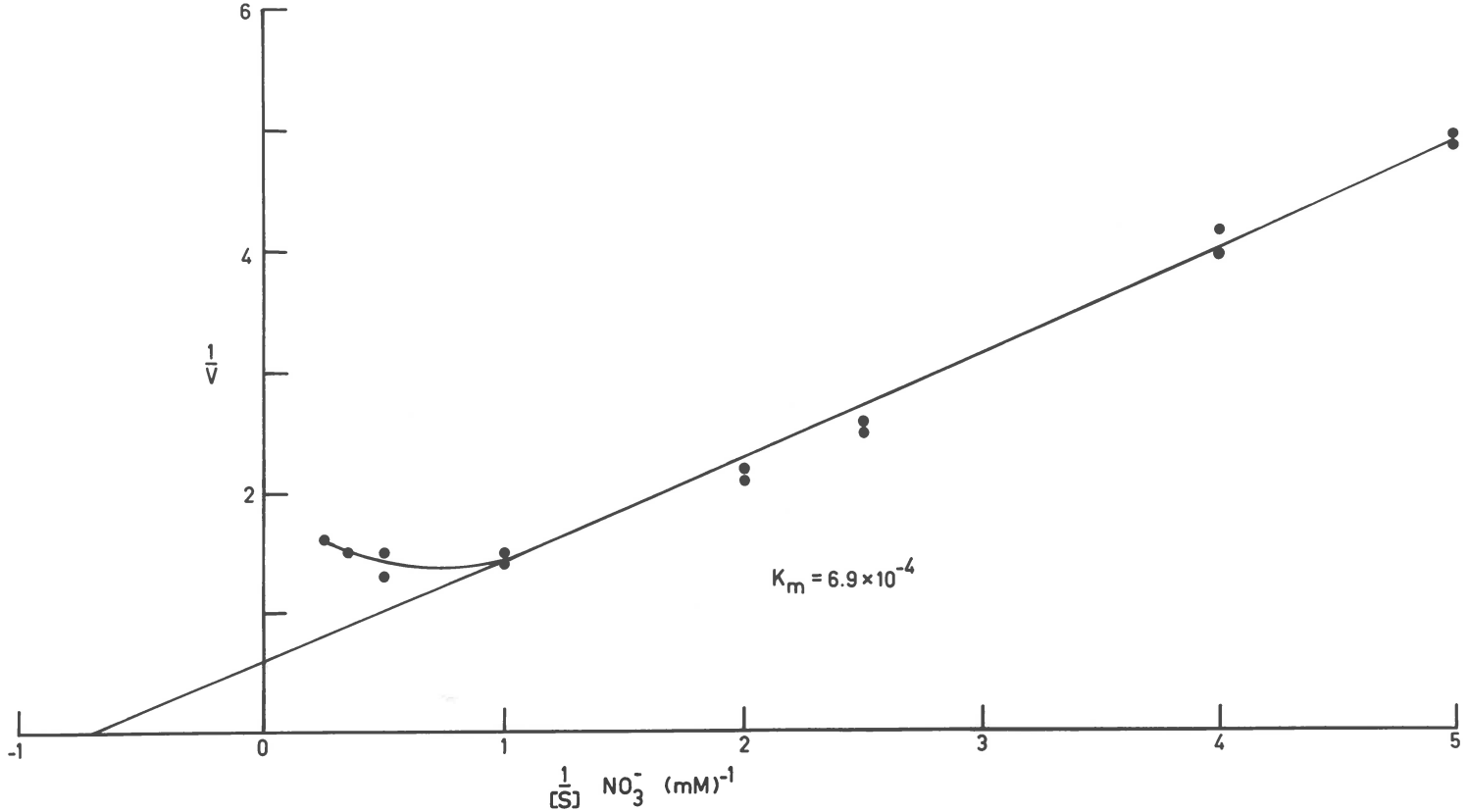

6
4
2
1/V
-3
-2
-1
0
1
2
3
4
1/[S] HCOONa (mM)⁻¹
Km = 3.8 × 10⁻⁴ M

FIGURE 11.

MICHAELIS CONSTANT OF NITRATE REDUCTASE FOR NITRATE

Double reciprocal plot (Lineweaver and Burk, 1934) of velocity of nitrate reduction versus concentration of KNO_3. Assays were carried out on particulate fraction (1 mg protein). Details of assay are given in text.


6
4
2
1/V
$K_m = 6.9 \times 10^{-4}$
-1
0
1
2
3
4
5
1/[S] NO_3^- $(mM)^{-1}$

extrapolating the straight line formed at lower formate concentrations, the apparent K_m for formate was calculated to be 3.8×10^{-4} M.

The effect of varying the concentration of the electron acceptor, potassium nitrate is shown in Figure 11. when $NADH_2$ was the donor. At nitrate concentrations above 1 mM, a slight degree of substrate inhibition was again observed; in this case it was more marked than with formate but less than with $NADH_2$. By extrapolating the linear portion of the graph, the apparent K_m value was found to be 6.9×10^{-4} M KNO_3.

9. Inhibitor Studies.

In Table XI. the results of studies with a number of inhibitors are presented. When using the particulate fraction (P-144) with sodium formate as the electron donor, nitrate reduction was strongly inhibited by KCN (92 per cent at 5 mM and 80 per cent at 0.5 mM). Nitrate reductase was also inhibited to a lesser extent by the metal chelating agents o-phenanthroline, potassium thiocyanate, thiourea, and dithiol (43, 25, 20, and 52 per cent, respectively, the first three at 5 mM and dithiol at 0.5 mM final concentration). Mepacrine inhibited nitrate reduction by 50 per cent at 1 mM suggesting the participation of a flavin component in the system. Nitrate

TABLE XI.

EFFECT OF INHIBITORS ON PARTICULATE NITRATE REDUCTASE

Enzyme activity assayed by standard method as described on p. 51-52. Particulate fraction (144P) was used with 1 mg protein per assay. Inhibitors incubated with enzyme for 15 min before initiating reaction.

Inhibitor	Final concn. (mM)	Donor: Formate	$NADH_2$
KCN	5	92	90
	0.5	80	80
KSCN	5	25	30
2,2'-Bipyridyl	10	0	0
o-Phenanthroline	5	43	45
	1	39	40
Thiourea	5	20	15
Dithiol	0.25	35	35
	0.50	52	50
p-Chloromercuribenzoate	2	70	71
	1	30	65
Amytal	1	0	30
	2	20	75
Hepacrine	1	50	50
HO·NO	0.3	0	0
Antimycin A	50 µg/ml	0	0
Piericidin A*	0.2	35	70
	0.1	23	60
	0.04	-	45
Rotenone	0.2	-	30
	1	0	50
Dicoumarol	0.2	0	0
CO	extract bubbled for 90 sec	0	0
$KClO_3$	1	85	80
$NaBrO_3$	1	40	40
$KClO_4$	1	15	18
KIO_3	1	10	10
Na_2SO_4	1	0	0

* A 50% reversal of inhibition occurred upon addition of a 10-fold excess of ubiquinone Q10.

reduction was inhibited 70 per cent by PCMB at 2 mM thus implicating a requirement for sulfhydryl groups in the system. Piericidin A (0.2 mM) and amytal (5.5 mM) inhibited 35 and 20 per cent respectively. Rotenone had no effect.

With $NADH_2$ as the electron donor, KCN and the other metal chelating agents and mepacrine inhibited to a similar extent as when the formate system was used. At low concentrations (1 mM) PCMB inhibited the $NADH_2$-nitrate reductase system more strongly than the formate system. Amytal inhibited 75 per cent at 5.5 mM. Piericidin A inhibited 75 per cent at 0.2 mM and this effect was reversed by about 50 per cent upon adding a 10-fold excess of ubiquinone Q_{10}. Rotenone inhibited 30 per cent at 1 mM.

Both systems were strongly inhibited by potassium chlorate, and to a lesser extent by bromate, perchlorate and iodate. Antimycin A, HOQNO, and CO had no effect on either system.

Irradiation of the particles with ultraviolet light (360 nm) had no effect on either nitrate reductase activity or on the ubiquinone level in the particles.

10. Effect of Chlorate and Bromate

The effect of potassium chlorate and sodium bromate on the formate-nitrate reductase are shown in Figures 12. and 13. In each experiment the concentration of nitrate

was varied in the presence or absence of 1 mM $KClO_3$ or 3 mM $NaBrO_3$. The data are present by means of double reciprocal plots (Lineweaver and Burk, 1934). In Figure 12. extrapolation of the linear portion of the graph that occurs at low substrate concentration converges on the vertical axis of the graph thus indicating a competetive type of inhibition (Dixon and Webb, 1964). The K_i value for chlorate as calculated from the graph was 1.7×10^{-3} M. The experiment represented in Figure 13. for bromate was carried out at lower nitrate concentrations than was the previous example so that no substrate inhibition was evident. As in the case of chlorate, the two lines meet on the vertical axis of the graph indicating that bromate also acts as a competetive inhibitor of nitrate reduction. The K_i value calculated for bromate was 1.25×10^{-2} M.

11. Effect of Nitrite.

Nitrite, the initial product of nitrate reduction, had no effect on the activity of the $NADH_2$ or formate nitrate reducing systems up to a concentration of 1 mM (twice the initial concentration of nitrate in the reaction mixture).

12. Stability of Nitrate Reducing System.

One storage at -17° the particulate preparation

FIGURE 12.

EFFECT OF CHLORATE ON NITRATE REDUCTASE ACTIVITY

Double reciprocal plot (Lineweaver and Burk, 1934) of velocity of nitrate reduction at varying concentrations of KNO_3. Potassium chlorate (10^{-3} M) was added to each assay of one series. Assays were carried out using the particulate fraction (1 mg protein) and formate as electron donor. Details of assay are given in the text.

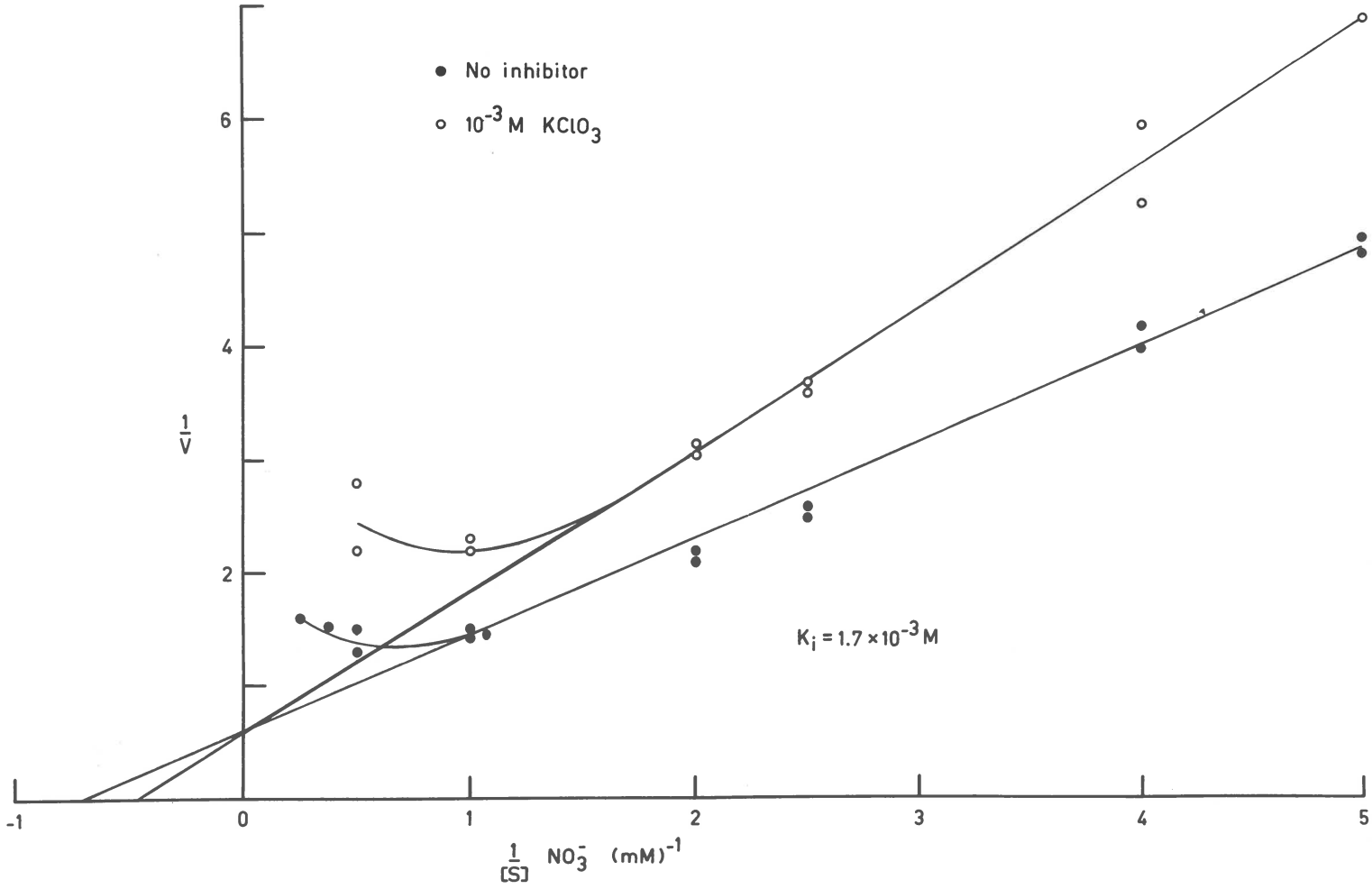
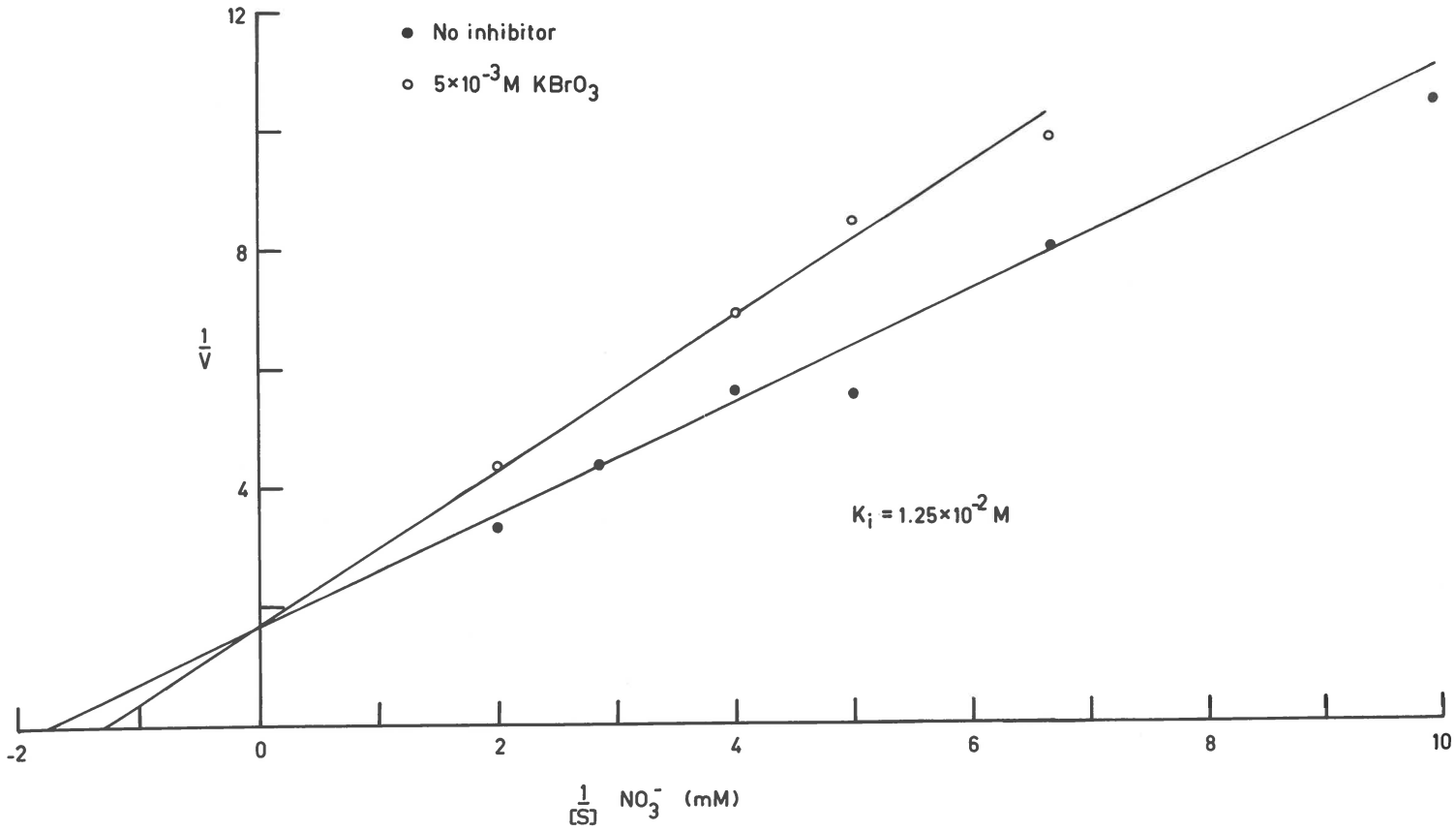

● No inhibitor
○ 10^{-3} M $KClO_3$
$K_i = 1.7 \times 10^{-3}$ M
$\frac{1}{V}$
$\frac{1}{[S]}$ NO_3^- $(mM)^{-1}$
-1
0
1
2
3
4
5
2
4
6

FIGURE 13.

EFFECT OF BROMATE ON NITRATE REDUCTASE

Double reciprocal plot (Lineweaver and Burk, 1934) of nitrate reductase activity at varying concentrations of KNO_3. Potassium bromate (5×10^{-3} M) was added to each assay of one series. Details of assay are given in the text. Sodium formate was used as electron donor.


12
8
4
1/V
● No inhibitor
○ 5×10⁻³M KBrO₃
K_i = 1.25×10⁻² M
-2
0
2
4
6
8
10
1/[S] NO₃⁻ (mM)

(P-144) exhibited a rapid loss of activity with $NADH_2$ as the electron donor. The formate-nitrate reductase activity was much more stable upon storage than was $NADH_2$ system. Particulate preparations lost none of their formate-nitrate reductase activity upon storage for 1 week at -17^{o}, while in the same period 90 per cent of the $NADH_2$-nitrate reductase activity was inactivated. The supernatant fraction (S-144), although devoid of nitrate reductase activity itself, exerted a stimulatory and stabilizing effect on the $NADH_2$-nitrate reductase as shown in Table XII. and Figure 14.

It is seen from the data presented in Table XII. that addition of the supernatant fraction resulted in an activation of the order of 100 per cent when $NADH_2$ was used as the hydrogen donor. Boiled supernatant fraction also stimulated to the extent of 30 per cent. FAD and FMN did not substitute for the supernatant fraction. When formate was used as hydrogen donor a similar but much smaller stimulation was observed, being in the order of 25 per cent.

Figure 14. shows the effect of the supernatant fraction on the stability of $NADH_2$-nitrate reductase. The crude extract was separated into particulate and supernatant fractions by centrifuging at 144,000 x g for 2 hours.

TABLE XII.

EFFECT OF ADDING SUPERNATANT FRACTION TO PARTICULATE NITRATE REDUCTASE

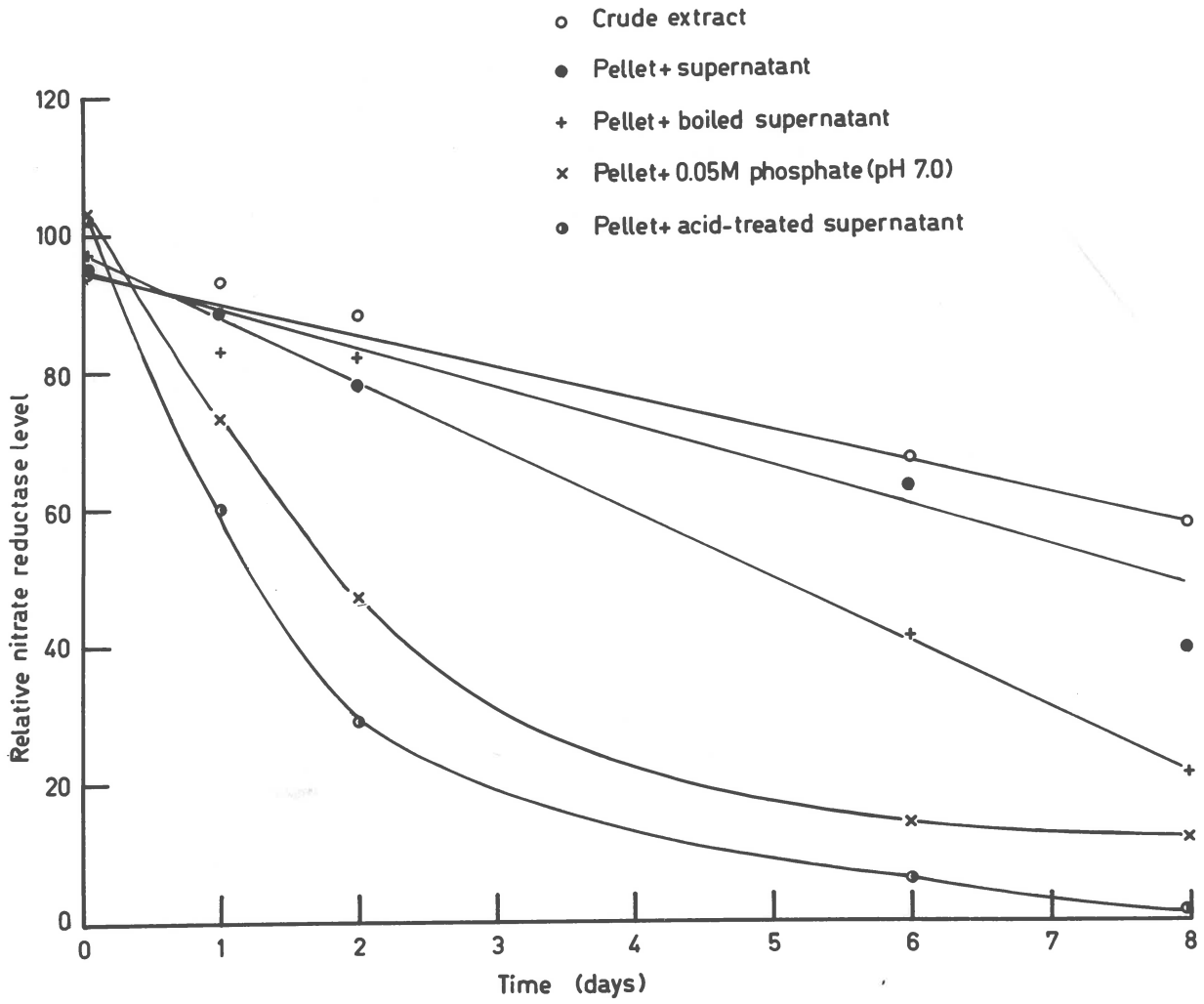
$NADH_2$- and formate-nitrate reductase activity assayed by the standard technique as described in Methods.

Fraction	Protein	Donor	Nitrate reduced (mμmole/10 minutes)
Particles	1.7 mg	NADH	266
Supernatant	2 mg	NADH	40
Particles + supernatant	3.7 mg	NADH	552
Boiled supernatant	2 mg	NADH	0
Particles + boiled supernatant	3.7 mg	NADH	387
Particles + 0.1 μ-mole FMN	2 mg	NADH	316
Particles + 0.1 μmole FAD	2 mg	NADH	286
Particles	1.7 mg	Formate	480
Supernatant	2 mg	Formate	0
Particles + supernatant	3.7 mg	Formate	622

FIGURE 14.

STABILITY OF $NADH_2$-NITRATE REDUCTASE

Crude extract was centrifuged for 2 hours at 144,000 x g to given a pellet and a supernatant fraction. The pellet was then treated in one of the following ways: (1) pellet and supernatant recombined immediately; (2) pellet suspended in 0.05 M phosphate buffer (pH 7.0); (3) supernatant boiled for 5 minutes, then recombined with pellet; (4) supernatant adjusted to pH 5.0 with HCl, then neutralised with NaOH and recombined with pellet. Activity tested after storage at -17^{0} for varying periods by the standard $NADH_2$-nitrate reductase assay.


Crude extract
Pellet+ supernatant
Pellet+ boiled supernatant
Pellet+ 0.05M phosphate (pH 7.0)
Pellet+ acid-treated supernatant
Relative nitrate reductase level
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Time (days)

The particles were then resuspended in 0.1 M phosphate buffer (pH 7.0), or in the supernatant fraction that had been either untreated, boiled for 5 minutes, or acid-treated by adjusting to pH 5.0 for 10 minutes and then neutralizing with NaOH. The recombined preparations were stored frozen at -17° and thawed out at intervals when the nitrate reducing activity was tested. The particulate fraction that was resuspended in the untreated supernatant lost activity only slowly and at about the same rate as the crude extract with about 70 per cent of the $NADH_2$-nitrate reductase activity remaining after six days storage. The sample that was recombined with the boiled supernatant lost activity about twice as fast, and only 44 per cent of the original activity remained after six days. The samples that were resuspended in the acid-treated supernatant or phosphate buffer were least stable with only 6 per cent and 14 per cent of their original activity left, respectively, after six days storage. In addition the initial rate of inactivation was much more rapid with these last two treatments.

13. Effect of Oxygen.

The effect of oxygen on nitrate reduction with either formate or $NADH_2$ as the electron donor is shown in Table XIII. Formate-nitrate reductase activity is

TABLE XIII.

EFFECT OF OXYGEN ON NITRATE REDUCTION

Standard $NADH_2$- and formate-nitrate reductase assayed as described in Methods (p. 51-52) except for the modifications given in the Table using crude extract (0.9 mg protein/assay).

Electron donor	Assay conditions	NO_3^- reduced (mμ-mole/10 minutes)
NADH	Thunberg tubes; anaerobic	382
NADH	Open tubes; air	341
NADH	Thunberg tubes; under pure O_2	240
Formate	Thunberg tubes; anaerobic	388
Formate	Open tubes; air	224
Formate	Thunberg tubes; under pure O_2	87

much more sensitive to oxygen than is the $NADH_2$-dependent system. Nitrate reduction with $NADH_2$ as the hydrogen donor was inhibited only 13 per cent by air and 37 per cent when the assay was carried out in an atmosphere of pure oxygen. Formate-nitrate reductase, on the other hand, was inhibited 43 per cent by air and 78 per cent when incubated with pure oxygen. These data suggest that the formate dehydrogenase portion of the electron transfer system is more sensitive to oxygen than is the $NADH_2$ dehydrogenase moiety.

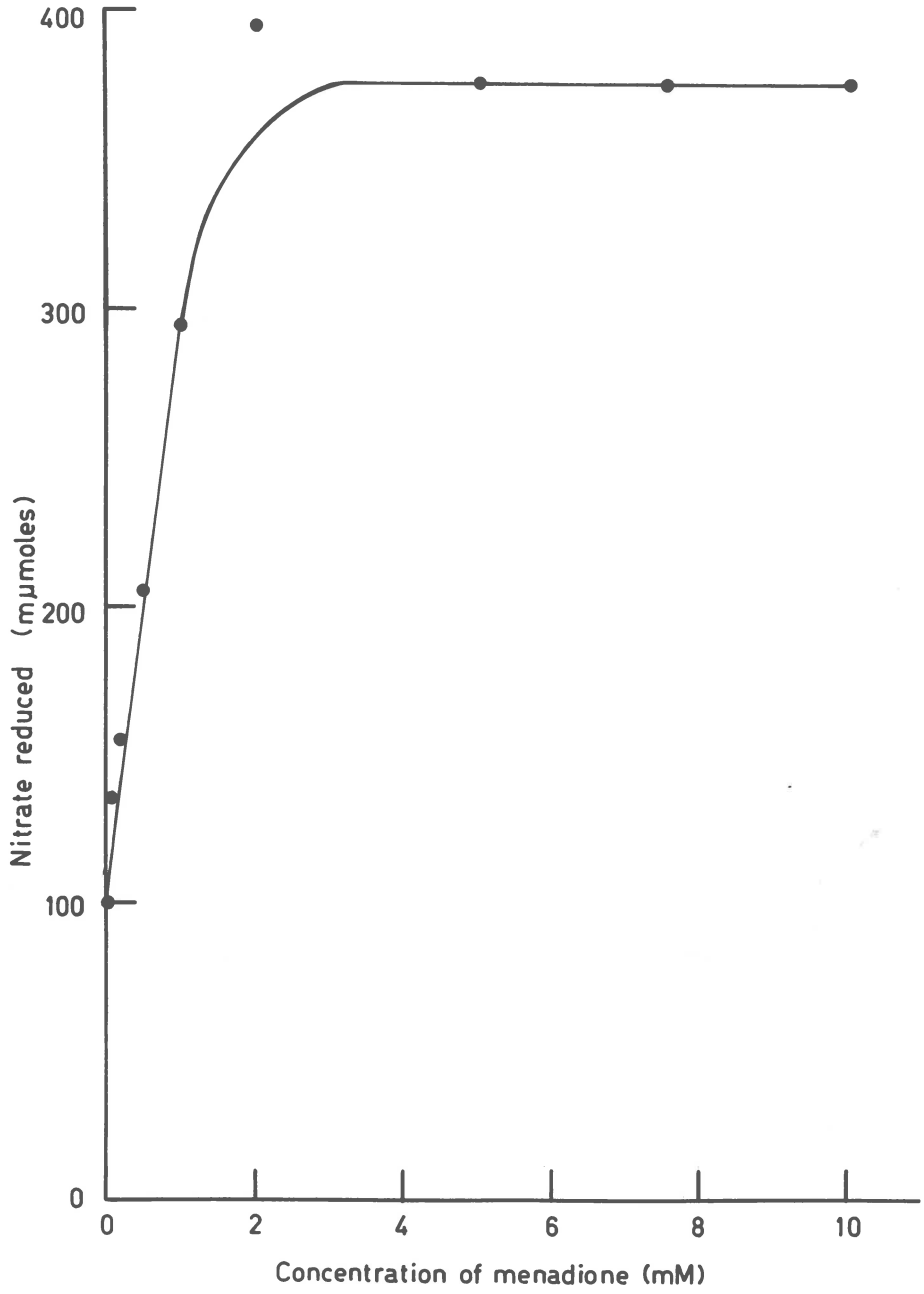
14. Effect of Menadione.

The addition of menadione to the reaction mixture stimulated $NADH_2$-nitrate reductase activity as shown in Figure 15. Maximum enzyme activation which occurred at 2 mM menadione was inhibited 60 per cent by 0.1 mM dicoumarol. Dicoumarol had no effect on nitrate reduction when menadione was not added to the enzyme mixture. Menadione provided an effective bypass of the rotenone sensitive portion of the electron transfer chain from $NADH_2$. Rotenone (1 mM) inhibited nitrate reduction by 50 per cent with $NADH_2$ alone, but had no effect with $NADH_2$ plus menadione.

FIGURE 15.

EFFECT OF MENADIONE ON RATE OF NITRATE REDUCTION

Menadione (sodium bisulfite salt) was added to standard $NAPH_2$-nitrate reductase assay mixture in varying concentrations up to 10 mM. Particulate fraction (0.6 mg protein) was used.


400
300
200
100
0
Nitrate reduced (mμmoles)
0
2
4
6
8
10
Concentration of menadione (mM)

15. Effect of Iron Deficiency.

Table XIV. shows the effect of iron deficiency on the level of nitrate reductase in the cells. The cells were subcultured sequentially 3 times on iron-deficient media prepared as described in Methods. Enzyme activities of crude extracts prepared from the iron-deficient cells were compared with those of normal cells. Formate-nitrate reductase activity in iron-deficient cells was one-fifth that in normal cells while $NADH_2$-nitrate reductase activity was reduced to one-tenth of the level of normal cells.

B. Formate Dehydrogenase.

Since formate is such an effective hydrogen donor for nitrate reductase, some properties of formate dehydrogenase in the extracts were studied.

1. Distribution.

Table XV. shows the distribution of formate dehydrogenase activity in cell-free homogenates from nitrate and oxygen grown cells. In both cases all the activity was found in the particulate fraction.

2. Electron Acceptors.

The activity of formate dehydrogenase with a variety of electron acceptors is shown in Table XVI. The highest

TABLE XIV.

INFLUENCE OF IRON DEFICIENCY ON NITRATE REDUCTASE ACTIVITY

The cells were subcultured 5 times in iron deficient media prepared as described in Methods. Cells from an 18 hr culture were collected, washed and the crude extrate prepared. Activity with NADH and formate assayed in crude extract.

Sample	Electron donor	NO_3^- Reduced nμmole/mg protein/10 min
Fe-deficient	NADH	64
Complete	NADH	317
Fe-deficient	Formate	32
Complete	Formate	310

TABLE XV.

DISTRIBUTION OF FORMATE DEHYDROGENASE ACTIVITY IN CELL-FREE EXTRACTS

Formate dehydrogenase activity in the various fractions from cell-free extracts of cells grown with nitrate or oxygen as terminal electron acceptor was assayed by the manometric method with methylene blue as acceptor as described on p. 53.

Fraction	Cells grown on:	CO_2 evolved (μl/hr)	Protein (mg)	μl CO_2 evolved per mg per hr
Crude extract	NO_3^-	111	2.2	50.4
Pellet (144P)	NO_3^-	81	1.1	73.8
Supernatant (144S)	NO_3^-	3	1.4	2.4
Crude extract	O_2	27	2.8	9.6
Pellet (144P)	O_2	15	1.6	11.4
Supernatant (144S)	O_2	0	1.8	0

TABLE XVI.

ACTIVITY OF FORMATE DEHYDROGENASE WITH VARIOUS ELECTRON ACCEPTORS

The rate of CO_2 evolution by formate dehydrogenase with a variety of electron acceptors was measured manometrically as described on p. 53. Crude extract (4 mg protein) was used.

Acceptor	Concn. (mM)	CO_2 evolved (μl/10 min)
Methylene blue	1.67	129
Dichlorophenolindophenol	1.67	23
Benzyl viologen	0.83	10
KNO_3	0.67	43
$KClO_3$	0.67	30
$KBrO_3$	0.67	30
$KClO_4$	0.67	14
KIO_3	0.67	7
None	-	0

rate of carbon dioxide evolution was with the dye methylene blue; little activity was found with dichlorophenol-indophenol or benzyl viologen as acceptors. Potassium nitrate was about one third as effective an acceptor as methylene blue. Chlorate and bromate exhibited about three-quarters the activity of nitrate. Perchlorate and iodate were relatively ineffective as acceptors.

3. Stoichiometry.

The relation between formate oxidation and nitrate reduction is illustrated in Figures 16, 17, and 18. In Figure 16. it is shown that when nitrate was the limiting substrate, nitrate reduced: nitrite formed: carbon dioxide evolved were approximately in the ratio 1:1:1. When formate was the limiting substrate as in the experiment represented in Figure 17, a similar result was obtained; formate supplied: nitrite formed: carbon dioxide evolved were in the ratio 1:1:1. When chlorate was the only acceptor (Figure 18.), one µmole of carbon dioxide was evolved per mole of chlorate supplied, thus indicating that chlorate acted as an acceptor for formate dehydrogenation.

Figure 19. shows that the Michaelis constant for chlorate was 2.8×10^{-3} M.

FIGURE 16

STOICHIOMETRY OF NITRATE LINKED FORMATE DEHYDROGENASE

Enzyme activity was assayed by manometric method described in text. Each flask contained excess sodium formate and varying amounts of potassium nitrate. CO_2 evolution and total nitrite formed were measured. Crude extract (8 mg protein) was used.

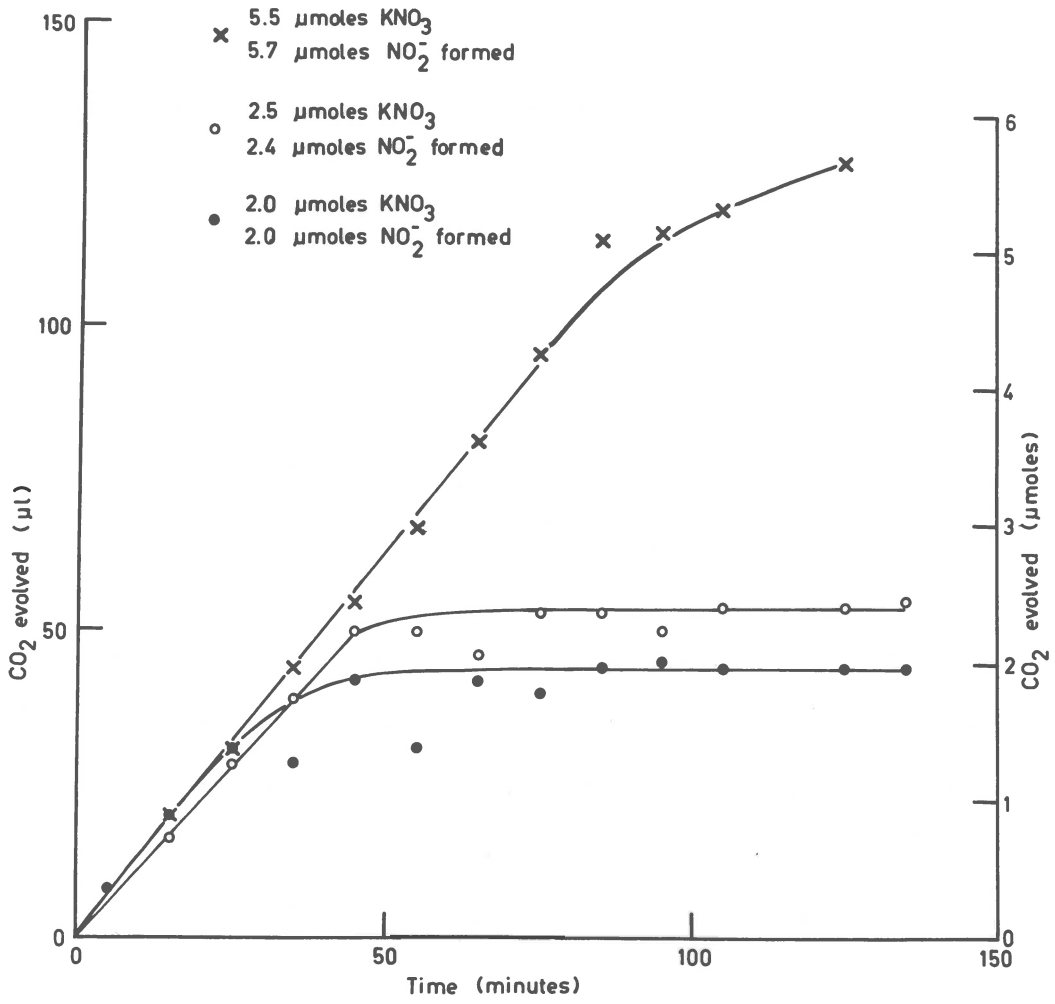

5.5 µmoles KNO3
5.7 µmoles NO2- formed
2.5 µmoles KNO3
2.4 µmoles NO2- formed
2.0 µmoles KNO3
2.0 µmoles NO2- formed
CO2 evolved (µl)
CO2 evolved (µmoles)
Time (minutes)
0
50
100
150
1
2
3
4
5
6

FIGURE 17.

STOICHIOMETRY OF NITRATE LINKED FORMATE DEHYDROGENASE

Enzyme activity was assayed by manometric method described in text. Each flask contained excess KNO_3 and limiting amounts of sodium formate. CO_2 evolution and total nitrite formed were measured. Crude extract (8 mg protein) was used.

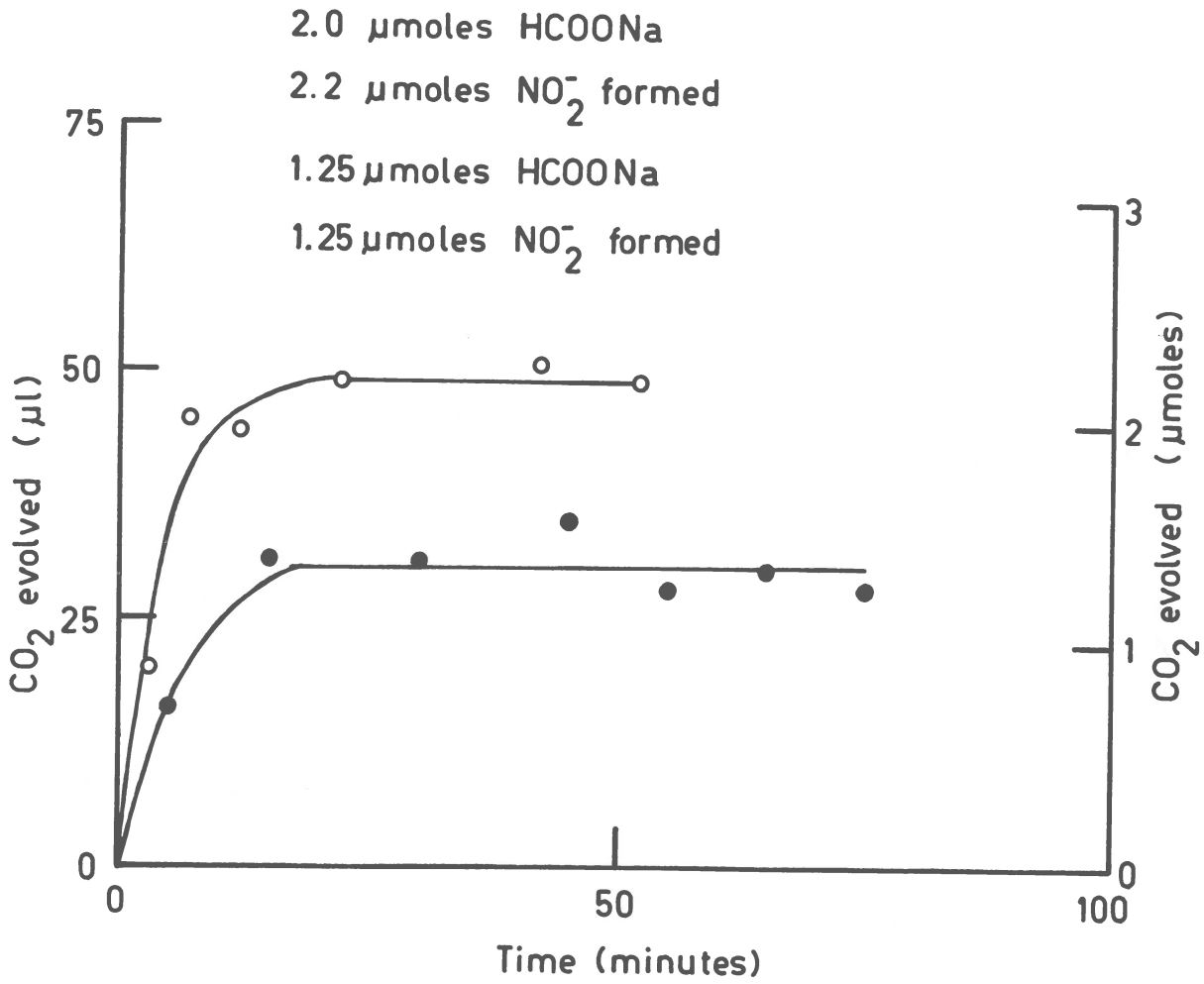

2.0 µmoles HCOONa
2.2 µmoles NO_2^- formed
1.25 µmoles HCOONa
1.25 µmoles NO_2^- formed
75
50
25
0
CO_2 evolved (µl)
3
2
1
0
CO_2 evolved (µmoles)
0
50
100
Time (minutes)

FIGURE 18.

STOICHIOMETRY OF FORMATE DEHYDROGENASE WITH $KClO_3$ AS ACCEPTOR

Enzyme activity assayed by manometric method described in text. Each flask contained excess formate and limiting amounts of $KClO_3$. CO_2 evolution was measured. Crude extract (8 mg protein) was used.

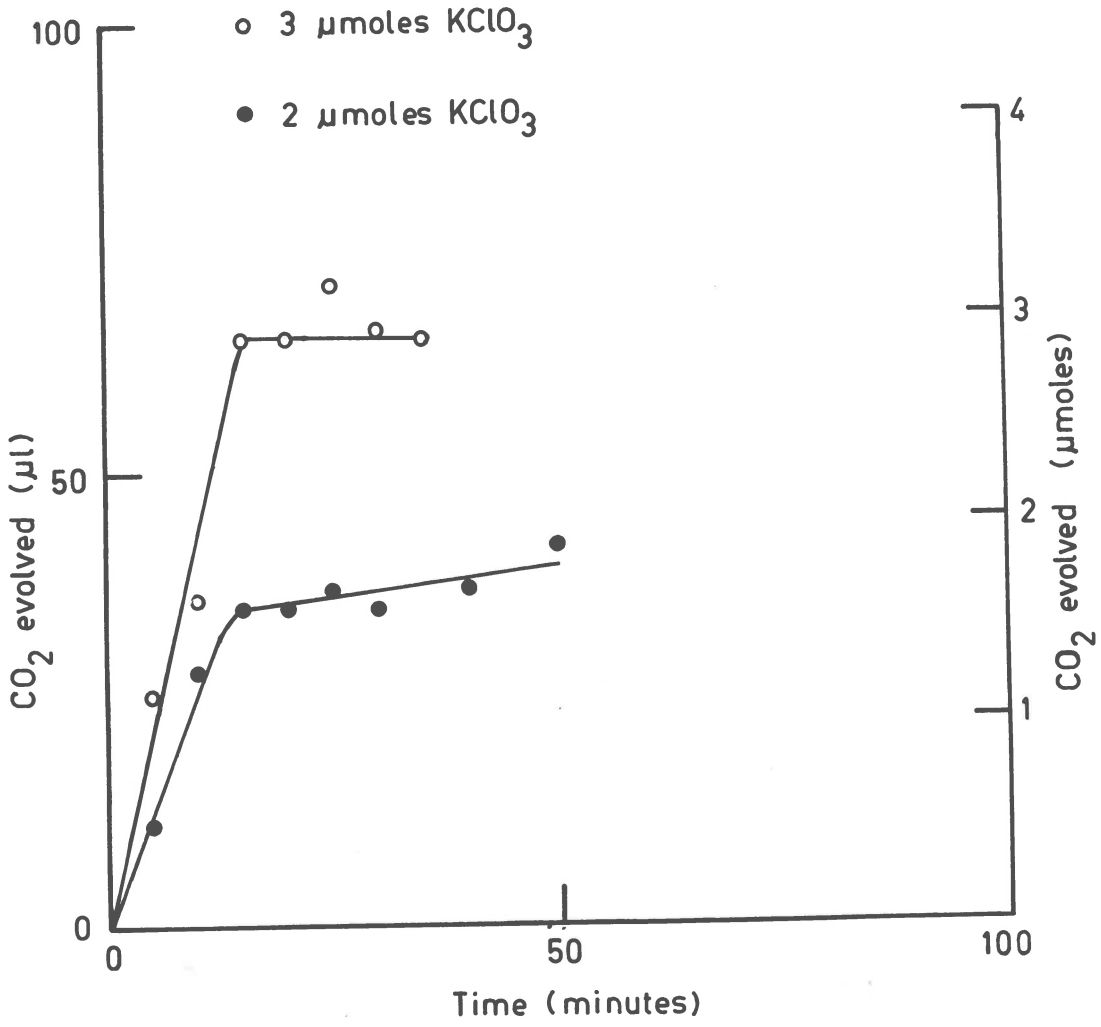
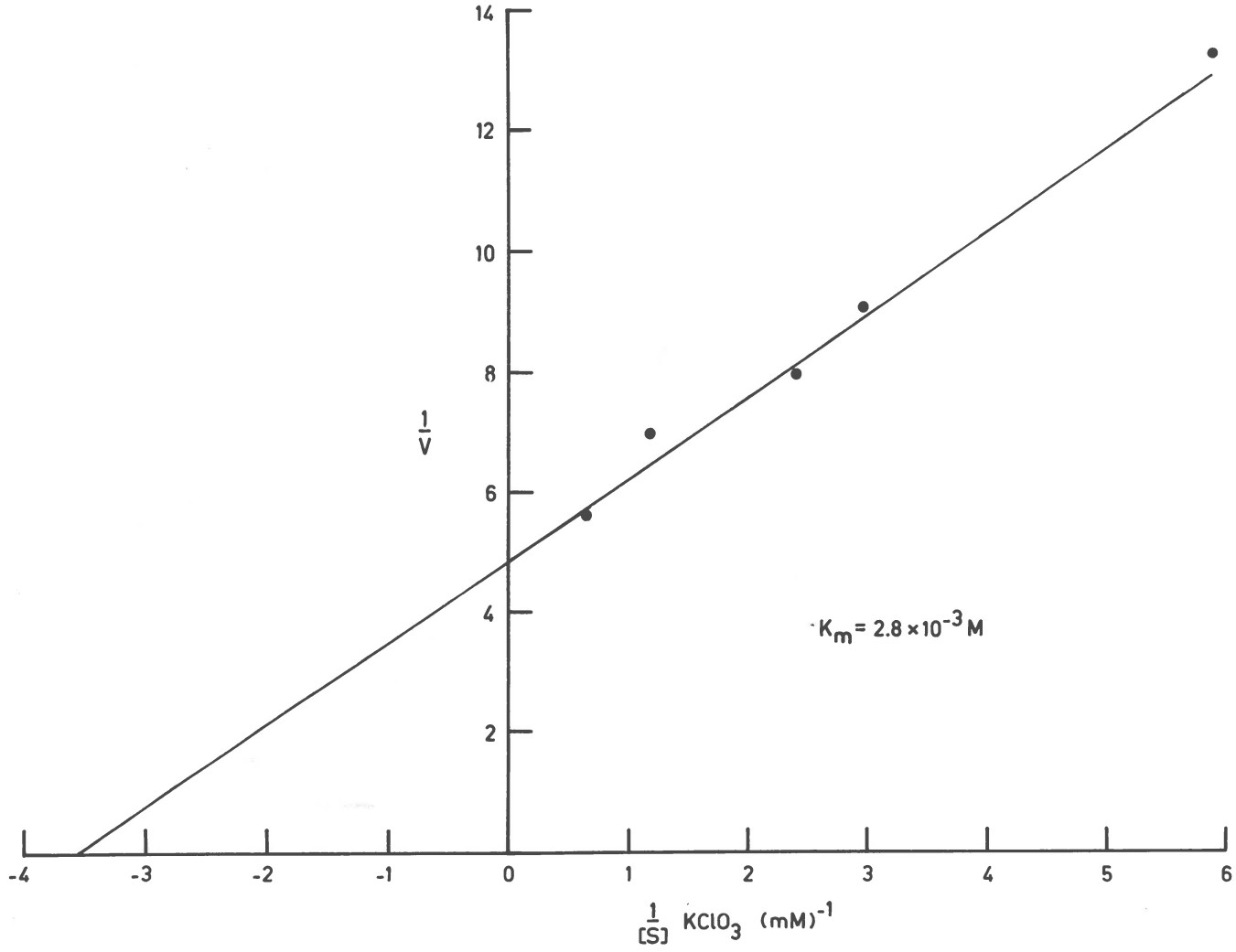

100
50
0
0
50
100
Time (minutes)
CO2 evolved (µl)
CO2 evolved (µmoles)
4
3
2
1
3 µmoles KClO3
2 µmoles KClO3

FIGURE 19

MICHAELIS CONSTANT OF NITRATE REDUCTASE FOR CHLORATE

Double reciprocal plot (Lineweaver and Burk, 1934) of the velocity of chlorate reduction determined by the manometric assay as described in the text at varying concentrations of $KClO_3$. Crude extract (1.6 mg protein) was used.


14
12
10
8
6
4
2
1/V
$K_m = 2.8 \times 10^{-3}$ M
-4
-3
-2
-1
0
1
2
3
4
5
6
1/[S] $KClO_3$ $(mM)^{-1}$

4. Inhibitor studies.

The effect of various inhibitors on formate dehydrogenase was studied. Figure 20. shows the effect of chlorate on formate dehydrogenase with nitrate as acceptor. Addition of chlorate at the same concentration as nitrate had no effect on the rate of carbon dioxide evolution, but the production of nitrite from nitrate was reduced 35 per cent from the level found with no chlorate present.

Table XVII. shows the effect of some other inhibitors on formate dehydrogenase activity with methylene blue as the acceptor. Formate dehydrogenase was inhibited by azide (57 per cent), mepacrine (25 per cent), and PCMB (52 per cent) each at 1 mM. Inhibition by PCMB was completely reversed by adding 1 mM reduced glutathione. Thiourea, potassium thiocyante, and o-phenanthroline had no effect on the rate of formate dehydrogenation.

Table XVIII. shows the effect of inhibitors on formate dehydrogenase activity when chlorate was the acceptor. Dithiol (0.17 mM) inhibited the enzyme completely. Potassium cyanide (5 mM) and o-phenanthroline (5 mM) inhibited 92 per cent and 84 per cent respectively. Mepacrine (1 mM), PCMB (2 mM), and potassium thiocyante (5 mM) inhibited 40, 70, and 30 per cent, respectively. Antimycin A, HOQNO, and rotenone had no effect.

TABLE XVII.

INHIBITORS OF FORMATE DEHYDROGENASE WITH METHYLENE BLUE AS ELECTRON ACCEPTOR

The influence of inhibitors on the activity of formate dehydrogenase with methylene blue as electron acceptor was assayed manometrically as described in Methods. Particulate fraction (144F) was used with 1 mg protein per assay.

Inhibitor	Concn. (mM)	Inhibition (%)
Sodium azide	1	57
Thiourea	1	0
o-Phenanthroline	1	0
Mepacrine	1	25
p-Chloromercuribenzoate	1	42
p-Chloromercuribenzoate + reduced glutathione	1	0
KSCN	1	0

TABLE XVIII.

INHIBITORS OF FORMATE DEHYDROGENASE WITH CHLORATE AS ACCEPTOR

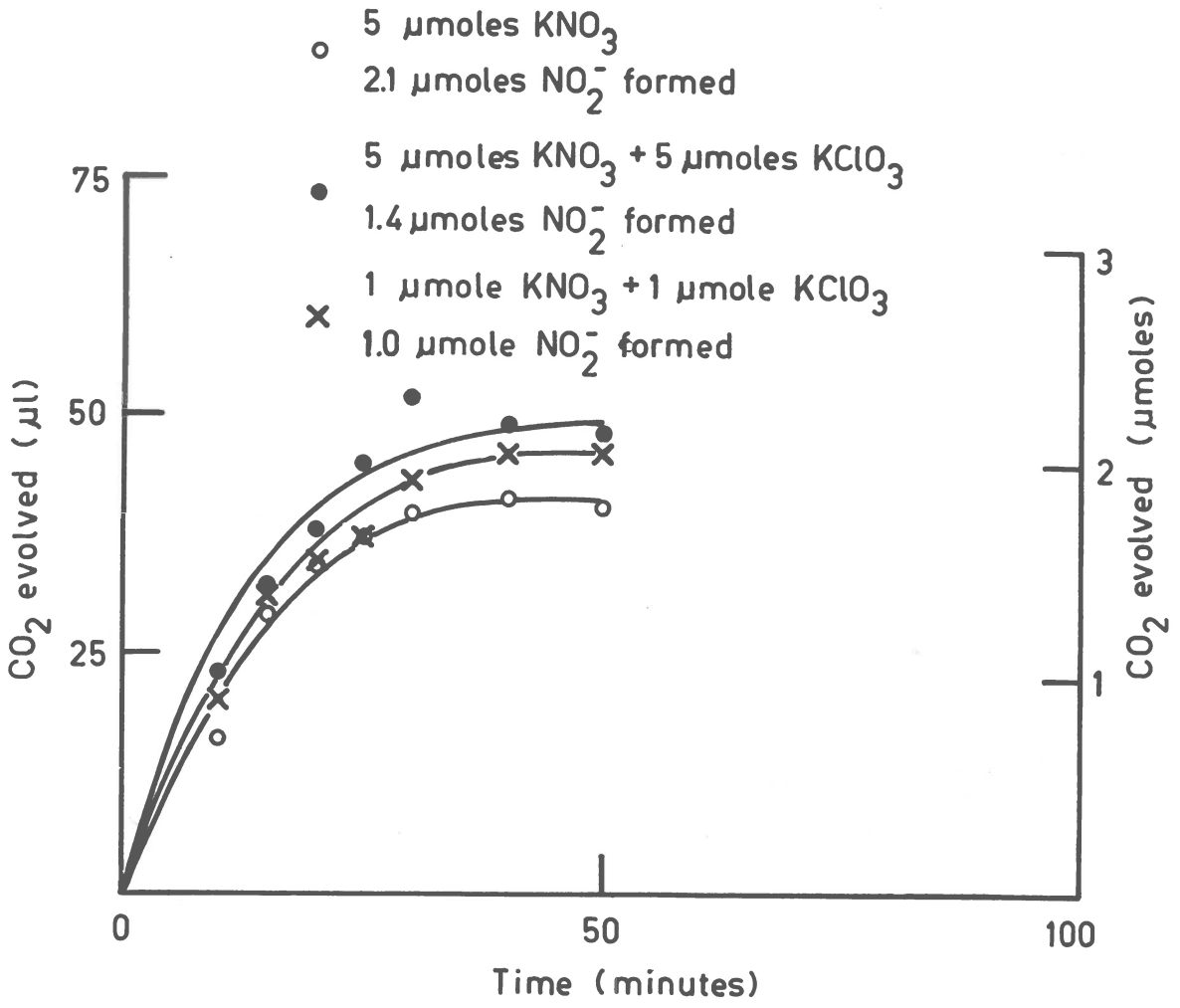
Effect of inhibitors on formate dehydrogenase activity in particulate fraction (1 mg protein) with $KClO_3$ as electron acceptor was determined using the manometric assay described in Methods.

Inhibitor	Concn. (mM)	Inhibition (%)
KCN	5	92
o-Phenanthroline	5	84
EDCH	5	30
Dithiol	0.17	100
p-Chloromercuribenzoate	2	70
Hepacrine	1	40
HO_2NO	0.3	0
Antimycin A	50 µg/ml	0
Rotenone	1	0

FIGURE 20.

EFFECT OF CHLORATE ON FORMATE DEHYDROGENASE

Each flask contained 2 μmole sodium formate and varying amounts of nitrate and chlorate as acceptor. CO_2 evolution and total nitrite formed were measured by the manometric assay described in the text. Crude extract (8 mg protein) was used.


5 µmoles KNO_3
2.1 µmoles NO_2^- formed
5 µmoles KNO_3 + 5 µmoles $KClO_3$
1.4 µmoles NO_2^- formed
1 µmole KNO_3 + 1 µmole $KClO_3$
1.0 µmole NO_2^- formed
75
50
25
CO_2 evolved (µl)
3
2
1
CO_2 evolved (µmoles)
0
50
100
Time (minutes)

C. Solubilization of Nitrate Reductase.

In order to purify the nitrate reductase enzyme itself, it was necessary to separate it from the rest of the cell-membrane bound respiratory chain. Of the three methods of solubilization attempted, two resulted in the complete inactivation of both nitrate reductase and formate dehydrogenase. These two methods were alkaline incubation after heat treatment and cold acetone treatment.

The third technique, incubation with sodium deoxycholate (1 mg/mg protein) at pH 8.0 for 30 minutes at 30°, resulted in the solubilization of around 70 per cent of the protein originally present in the particles and a good recovery of the nitrate reductase activity with reduced benzyl viologen as donor. Formate dehydrogenase was completely inactivated, however, by this treatment. Table XIX. sets out the details of the solubilization and purification scheme. This procedure resulted in a 36-fold purification of nitrate reductase.

1. Spectrum of solubilized material.

The solubilized material was a clear red solution. A difference spectrum (dithionite reduced minus oxidized) indicated that both c and b type cytochromes were present. The c type cytochrome appeared to be lost upon further purification by ammonium sulfate fractionation, but the b

TABLE XIX

PURIFICATION OF NITRATE REDUCTASE

Cell-free homogenates were prepared as described in Methods

Fraction	Volume (ml)	Protein (mg)	Total Activity (μ-moles NO_2^- reduced per 10 min	Specific Activity (μ-moles NO_2^- reduced per 10 min per mg protein	Purification
1. Crude extract - supernatant left after centrifuging cell-free homogenates at 10,000 x g for 20 min	20	1640	240	0.15	1
2. 144P - Pellet left after centrifuging at 144,000 x g for 2 hr resuspended in 0.1 M phosphate buffer (pH 7.0)	27	850	380	0.45	3
3. Fraction 2 adjusted to pH 8.0, sodium deoxycholate (1 mg/mg protein) added, and the mixture incubated at 30° for 30 min; the mixture then centrifuged at 225,000 x g for 2 hr. Activity in supernatant	50	510	1250	2.45	16.3
4. $(NH_4)_2SO_4$ added to Fraction 3 to give 30% saturation. Precipitate dissolved in 0.1 M phosphate buffer (pH 7.0) and dialysed for 12 hr against the same buffer	25	88	750	8.5	56

type cytochrome persisted. Difference spectra are shown in Figure 21.

2. Electron Donors.

The solubilized nitrate reductase was no longer active with the natural electron donors, formate and $NADH_2$. Reduced benzyl viologen and methyl viologen were however effective hydrogen donors. Ascorbate coupled to TMPD reduced cytochrome c, but not cytochrome b in the solubilized preparation (Fraction III). This system was ineffective as an electron donor. The activity of the solubilized nitrate reductase preparation with various electron donating systems is shown in Table XX.

3. Effect of pH.

The effect of pH on the activity of the solubilized nitrate reductase with reduced benzyl viologen as hydrogen donor is shown in Figure 22. Optimal activity occurred at pH 8.0 with a sharp decline in activity at the more acid and alkaline pH values.

4. Effect of Time of Reaction.

Figure 23. shows the effect of time of reaction on the BVH-nitrate reduction by the purified preparation. Nitrate reduction was linear with time for the first 10 minutes when about 75 per cent of the substrate had been

TABLE XX.

RELATIVE EFFICIENCY OF VARIOUS ELECTRON DONORS WITH SOLUBILIZED NITRATE REDUCTASE

Activity of solubilised nitrate reductase assayed as described in Methods using 50 μg protein per assay. Activity with reduced benzyl viologen taken as 100.

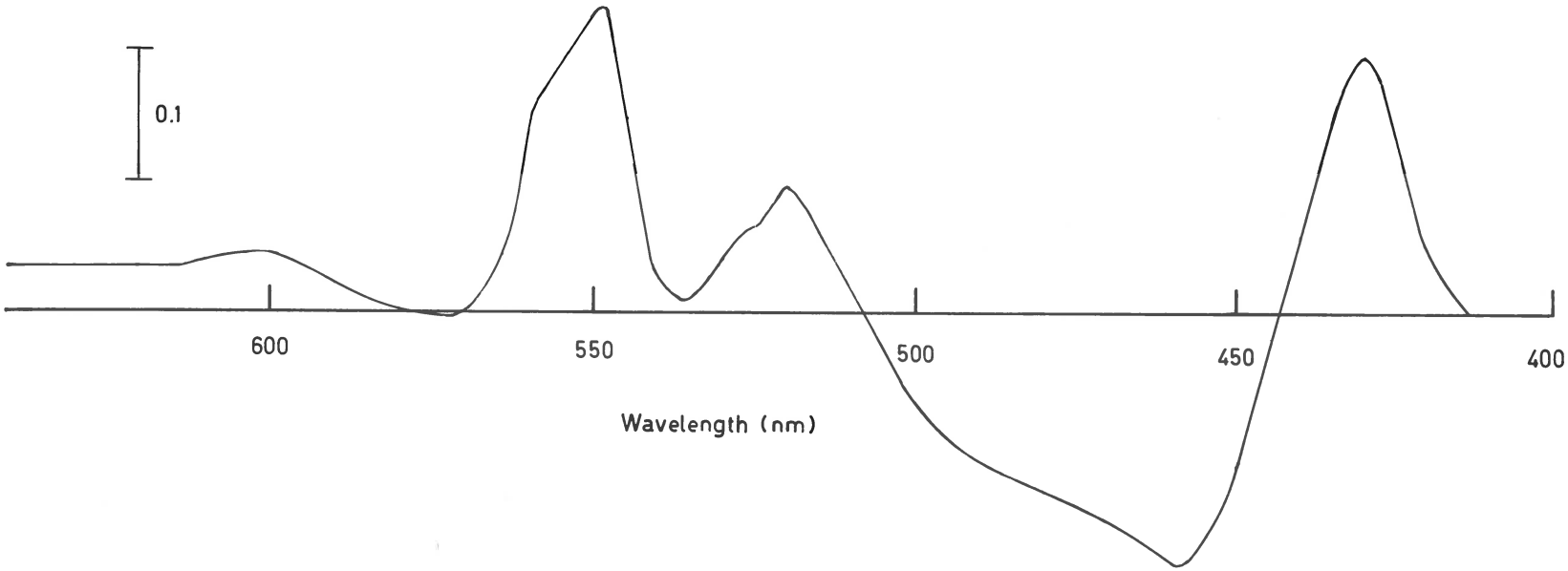
Electron Donor	Relative Activity
$NADH_2$	0
$NADH_2$ + 0.1 mM FMN	0
$NADPH_2$	0
Sodium formate	0
Ascorbate - TMPD	0
Reduced benzyl viologen	100
Reduced methyl viologen	100

FIGURE 21.

ABSORPTION SPECTRA OF SOLUBILIZED NITRATE REDUCTASE

Dithionite reduced minus oxidized difference spectra of solubilized nitrate reductase. (A) Deoxycholate solubilized preparation (Fraction III - 11 mg protein/ml). (B) 0-30% saturation ammonium sulfate precipitate (Fraction IV - 3.5 mg protein/ml).

A.



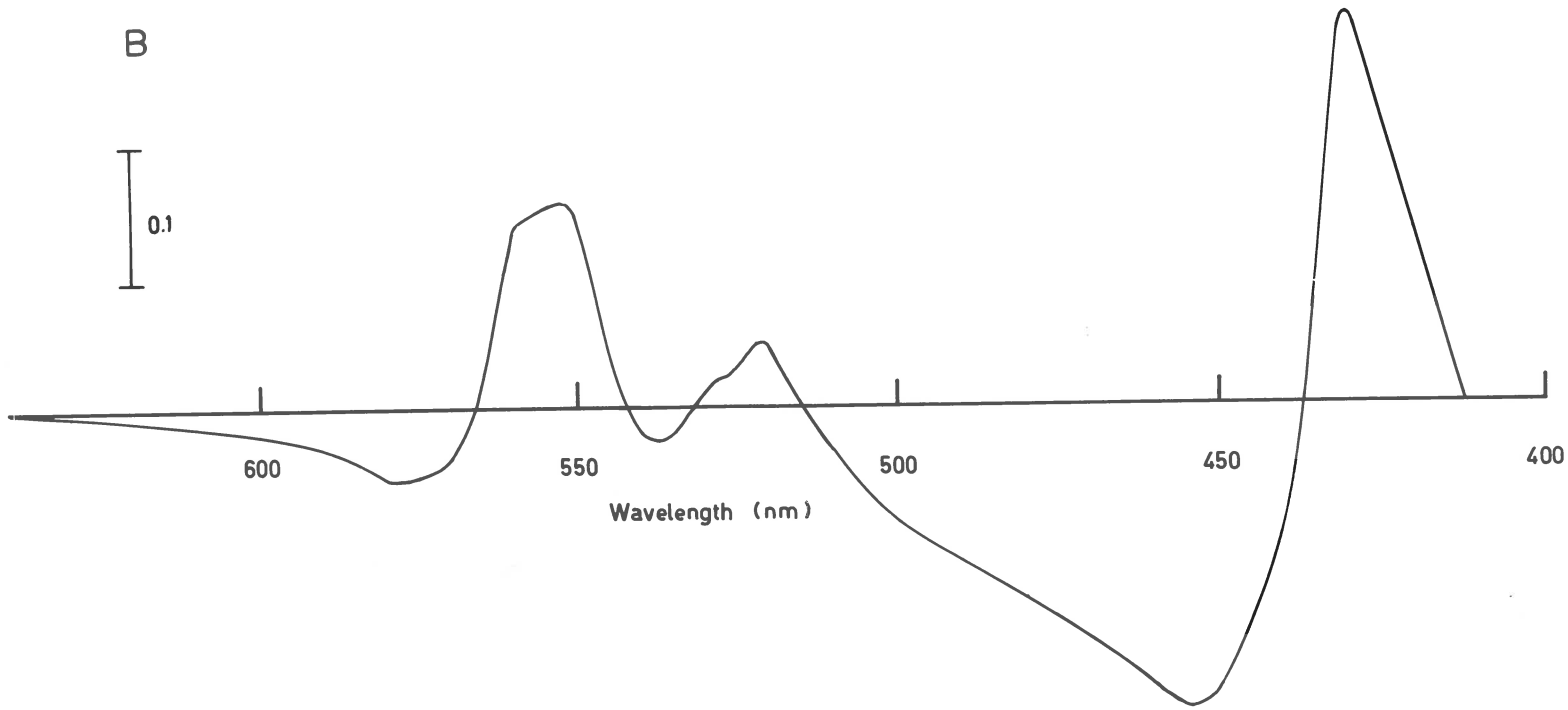

B
0.1
600
550
500
450
400
Wavelength (nm)

FIGURE 22.

INFLUENCE OF pH ON NITRATE REDUCTION BY SOLUBILIZED NITRATE REDUCTASE

Enzyme activity assayed using the purified nitrate reductase (Fraction IV - 0.14 mg protein) with reduced benzyl viologen as electron donor.

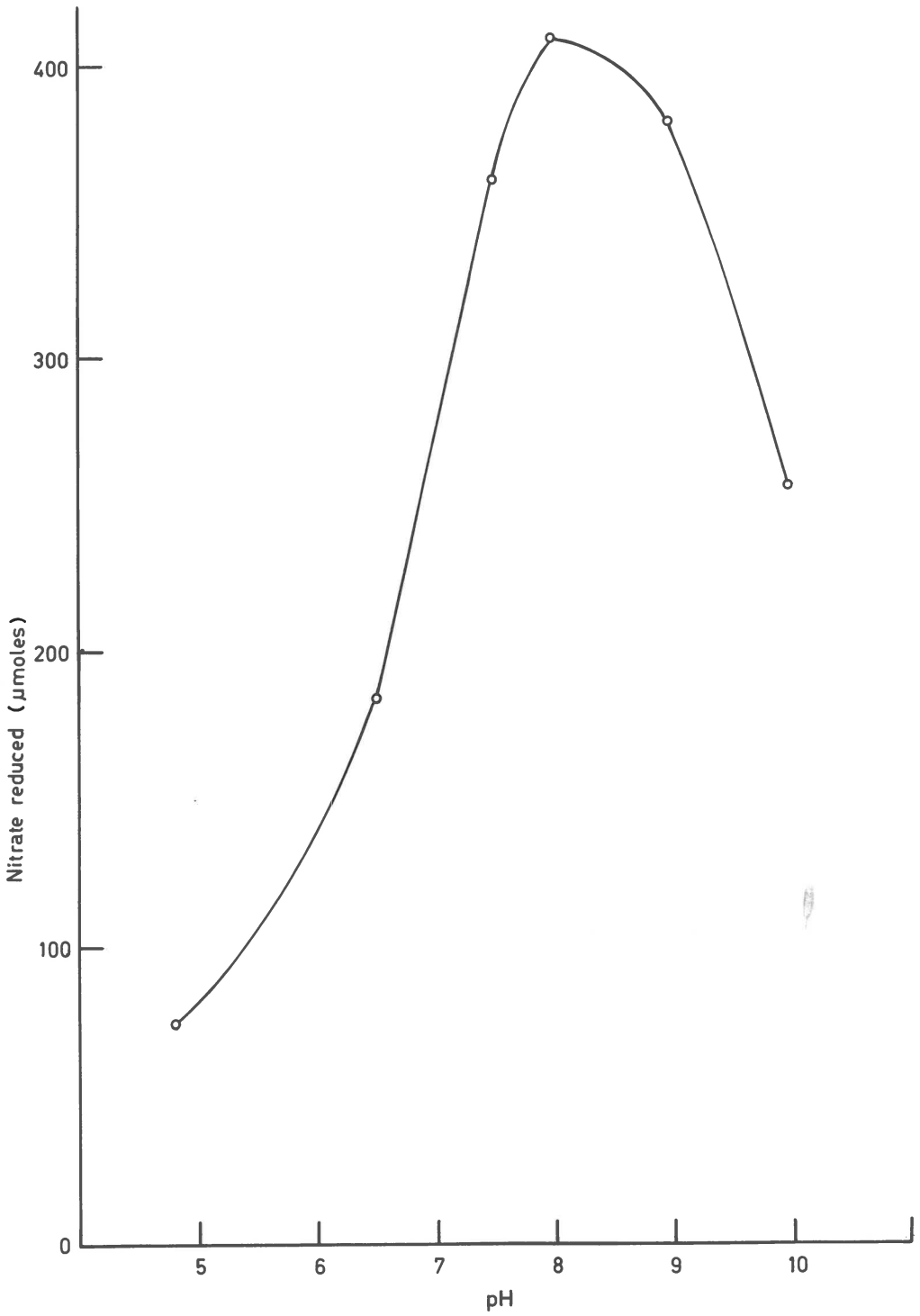
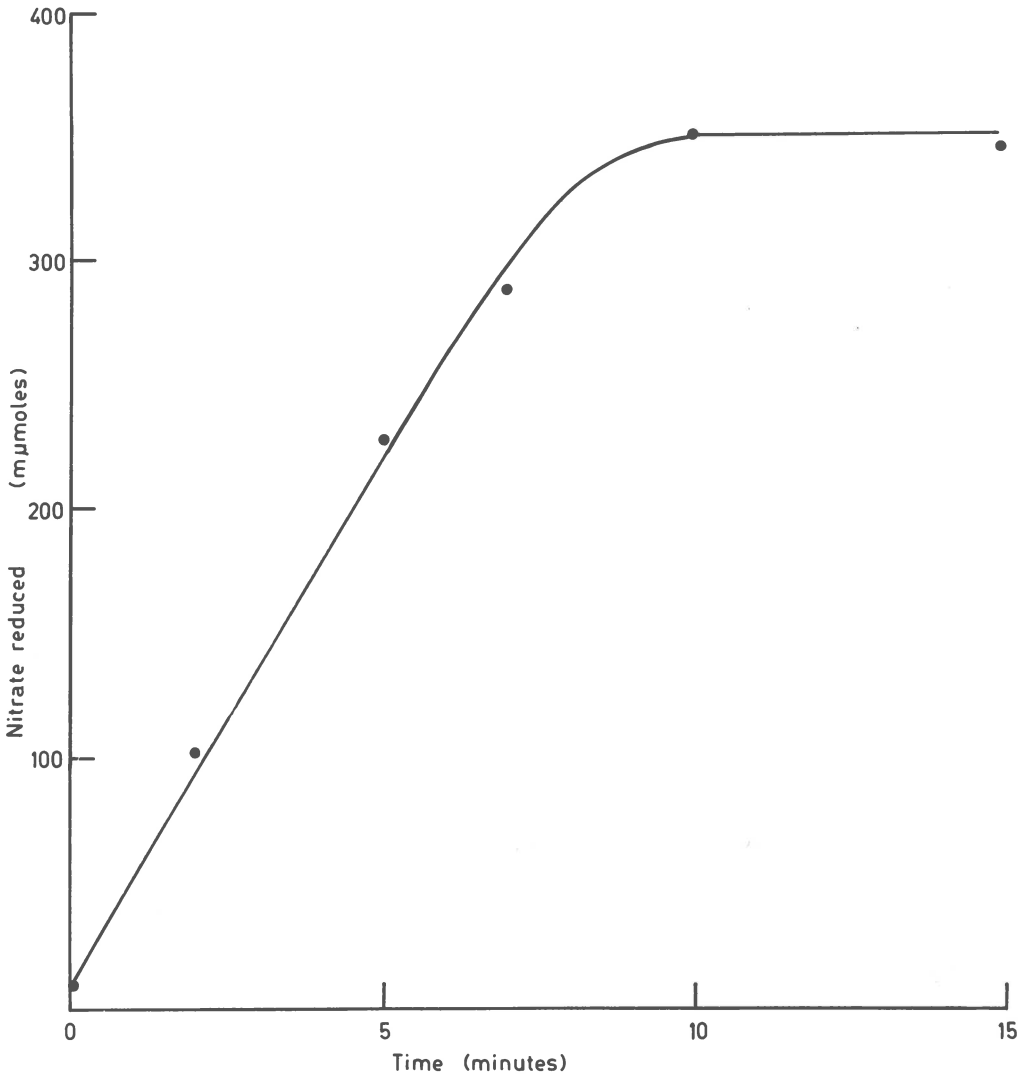

400
300
200
100
0
Nitrate reduced (µmoles)
5
6
7
8
9
10
pH

FIGURE 23.

EFFECT OF INCUBATION TIME ON PRODUCTION OF NITRITE BY SOLUBLE NITRATE REDUCTASE

Purified nitrate reductase (Fraction IV - 0.14 mg protein) incubated for times varying from 0 to 15 minutes with reduced benzyl viologen as donor as described in the text.


400
300
200
100
Nitrate reduced
(mμmoles)
0
5
10
15
Time (minutes)

exhausted.

5. Effect of Protein Concentration.

Figure 24. shows that the reaction rate increased linearly with increasing quantities of BVH-nitrate reductase.

6. Stoichiometry.

Table XXI. shows that nitrate was reduced quantitatively to nitrite by the purified preparation. The Michaelis constant for nitrate was 6.7×10^{-4} M as calculated from Figure 25.

7. Inhibitors.

The effect of a variety of inhibitors on the purified BVH-enzyme is shown in Table XXII. Dithiol (0.1 mM) and potassium thiocyanate (1.5 mM), agents that bind molybdenum, inhibited nitrate reduction by 95 and 75 per cent respectively. Amytal, Piericidin A, rotenone, carbon monoxide, mepacrine, 8-hydroxyquinoline, and 2,2'-dipyridyl were all without effect. Chlorate, perchlorate, bromate, and iodate all inhibited as they did in the case of the particulate nitrate reducing system.

8. Effect of Chlorate.

Figure 25. shows the effect on the rate of nitrate

TABLE XXI.

STOICHIOMETRY OF NITRATE REDUCTION BY SOLUBILIZED NITRATE REDUCTASE

The standard assay using reduced benzyl viologen as electron donor described on p. 52 was used with varying amounts of nitrate. The reaction was allowed to go to completion and nitrite formed determined as described in Methods.

NO_3^- Supplied (μmole)	NO_2^- Produced (μmole)	NO_3^-/NO_2^-
1.0	0.96	0.96
0.5	0.54	1.08
0.2	0.20	1.0

TABLE XXII.

EFFECT OF INHIBITORS ON SOLUBILIZED NITRATE REDUCTASE

The effect of a variety of inhibitors on solubilised nitrate reductase was determined using reduced benzyl viologen as electron donor as described on p. 52; 50 µg protein was used for each assay.

Inhibitor	Concn. (mM)	Inhibition (%)
KCN	1	81
KSCN	15	91
	1.5	75
Dithiol	0.1	95
o-Phenanthroline	3	25
p-Chloromercuribenzoate	2	60
Sodium diethyldithiocarbamate	5	20
Mepacrine	1	0
$KClO_3$	1	50
$KBrO_3$	1	50
$KClO_4$	1	33
KIO_3	1	40
Antimycin A	50 µg/ml	0
HO_2NO	0.3	0
Amytal	5.5	0
Piericidin A	0.2	0
Rotenone	0.2	0
CO	extract bubbled for 90 sec	0
8-hydroxyquinoline	5	0
2,2'-dipyridyl	10	0

FIGURE 24.

EFFECT OF QUANTITY OF SOLUBILIZED ENZYME ON RATE OF NITRATE REDUCTION

Different quantities of purified nitrate reductase (Fraction IV - 0.14 mg protein) were assayed using reduced benzyl viologen as electron donor as described in the text.

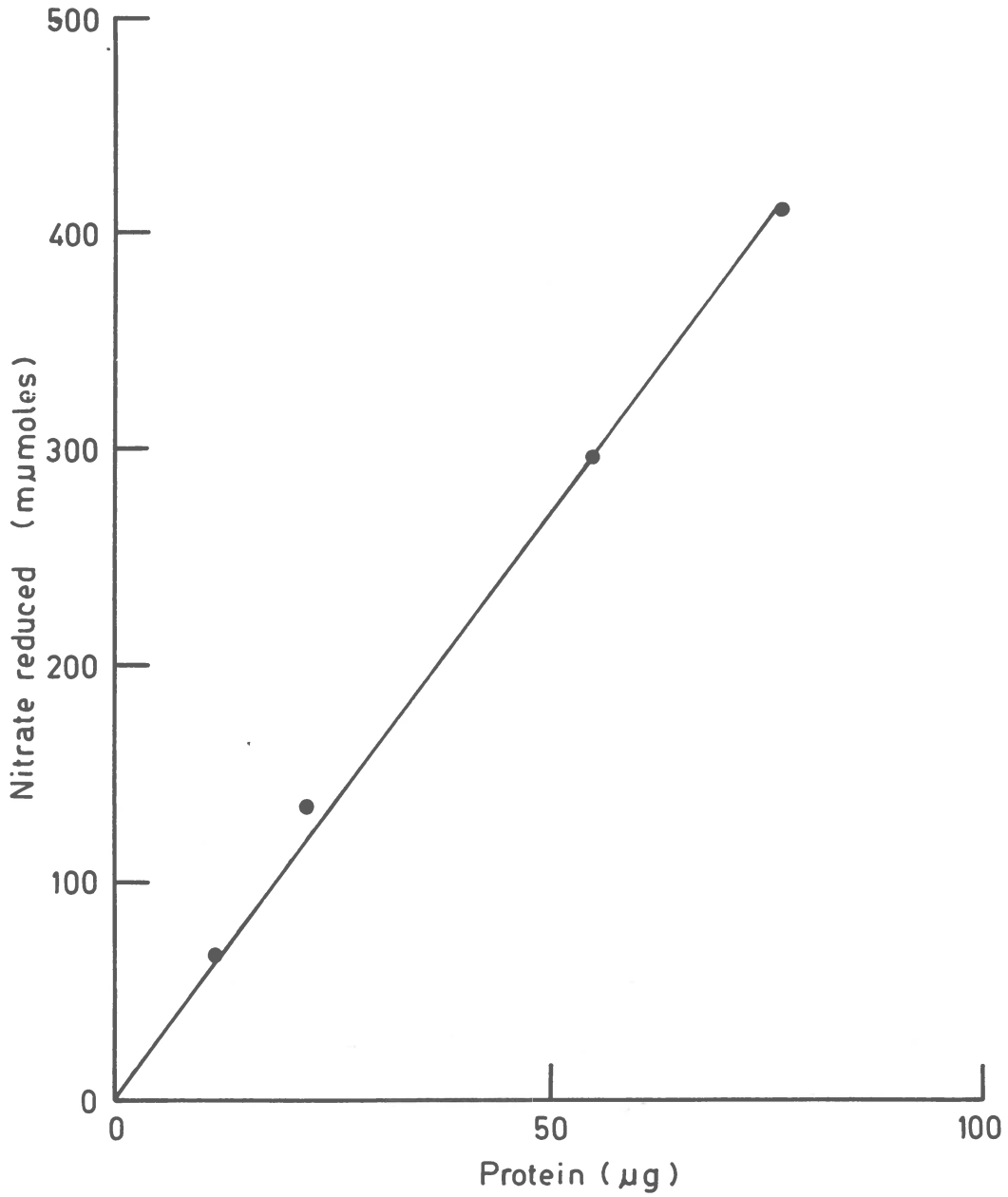
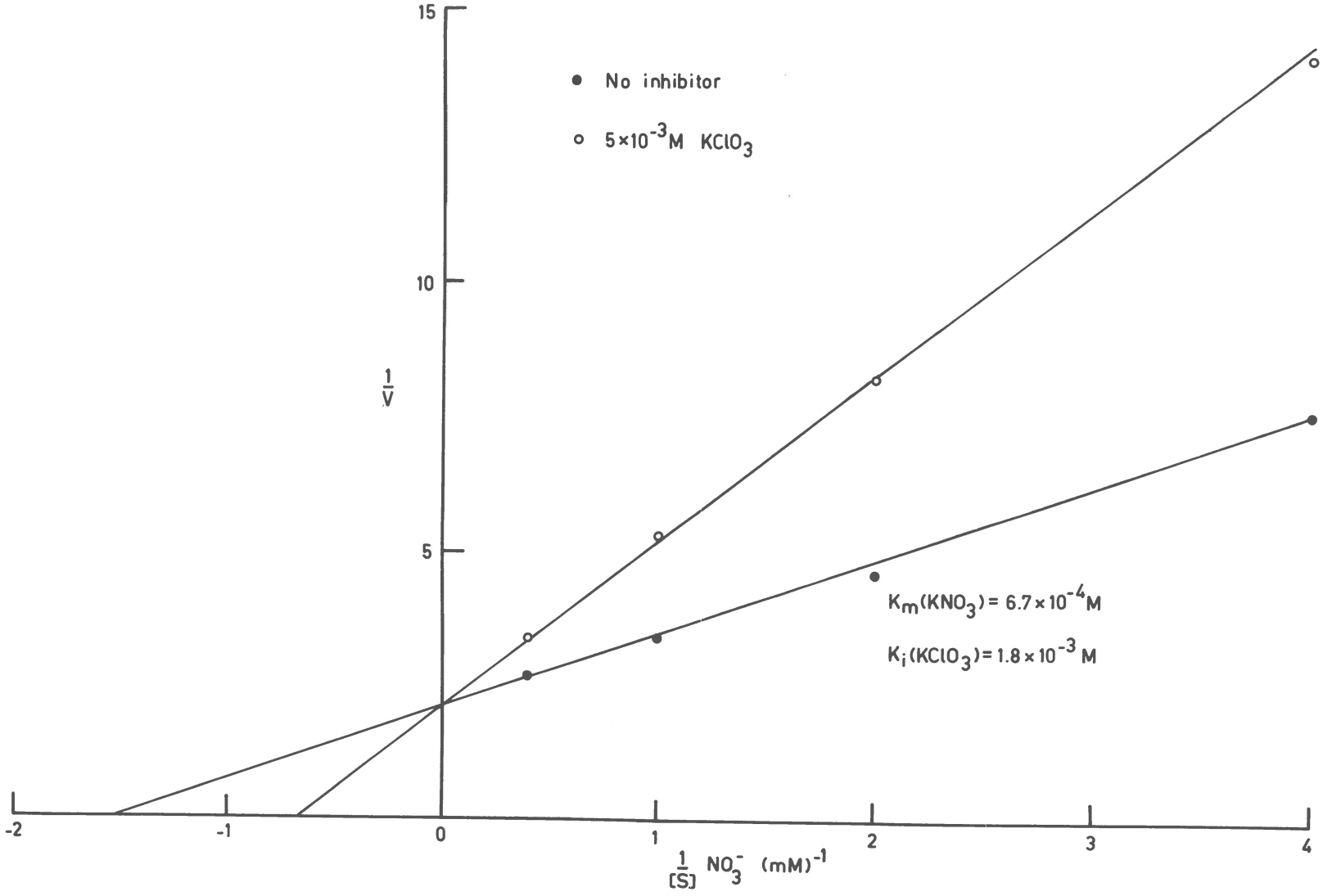

500
400
300
200
100
0
Nitrate reduced (mμmoles)
0
50
100
Protein (μg)

FIGURE 25.

EFFECT OF CHLORATE ON SOLUBILIZED NITRATE REDUCTASE

Double reciprocal plot (Lineweaver and Burk, 1934) of the rate of nitrate reduction occurring at different concentrations of nitrate. Potassium chlorate (5×10^{-3} M) was added to one series of assays. Reduced benzyl viologen was the electron donor, and purified nitrate reductase (Fraction IV - 0.14 mg protein) was used.


15
10
5
1/V
● No inhibitor
○ 5×10⁻³M KClO₃
Km(KNO₃) = 6.7×10⁻⁴M
Ki(KClO₃) = 1.8×10⁻³ M
-2
-1
0
1
2
3
4
1/[S] NO₃⁻ (mM)⁻¹

reduction by the purified enzyme of adding 10^{-3} M $KClO_3$ to the reaction mixture. This graph indicates that chlorate is a competetive inhibitor of the purified nitrate reductase. The K_i for chlorate was 1.8×10^{-3} M.

III. NITRITE REDUCTASE.

The distribution, purification, and properties of the nitrite reducing system from P. denitrificans are set out in detail below.

A. Distribution.

The results in Table XXIII. show the distribution of nitrite reducing activity in cell-homogenates. The homogenates were prepared by passing the cell suspensions (1 g cells/2 ml 0.1 M Tris-HCl, pH 7.4) through a French pressure cell at 20,000 lb·inch^{-2} as described in Methods. In contrast with other denitrifying bacteria such as Micrococcus denitrificans (Naik and Nicholas, 1966a), most of the $NADH_2$ and reduced benzyl viologen nitrite reductase activity (more than 95 per cent) was found in the 144,000 x g supernatant fraction. Little activity was associated with the particulate fraction when using either $NADH_2$ or reduced benzyl viologen as the electron donor. This same distribution pattern was found regardless of whether the cells were broken by ultrasonication, by the French

TABLE XXIII.

DISTRIBUTION OF NITRITE REDUCTASE IN CELL HOMOGENATES

The enzyme activity present in the cell homogenates was assayed anaerobically in Thunberg tubes using $NADH_2$ or reduced benzyl viologen as electron donor. Details of the standard assay are described on p. 55-56.

Fraction	Total enzyme units (μmoles NO_2^- reduced per 5 min)	
Hydrogen donor:	$NADH_2$	Reduced benzyl viologen
1. Crude extract left after centrifuging homogenate at 10,000 x g for 20 min.	428	2000
2. 144,000 x g supernatant left after centrifuging Fraction 1. for 2 h.	418	1500
3. 144,000 x g pellet from Fraction 2.	44	300

pressure cell, or by repeated freezing and thawing.

B. Purification.

The purification procedure for nitrite reductase is outlined in Table XXIV. The cells were broken in the French pressure cell, and cell debris and unbroken cells were removed by centrifuging at 20,000 x g for 20 minutes. The resulting crude extract was then centrifuged for 2 hours at 144,000 x g to give supernatant and particulate fractions. The supernatant fraction which contained more than 95 per cent of the nitrate reductase activity present in the crude extract was diluted with an equal volume of 0.1 M potassium phosphate buffer (pH 6.0) and ammonium sulfate added to give a 75 per cent saturation. The precipitate was removed by centrifuging for 10 minutes at 10,000 x g and discarded. Further amounts of ammonium sulfate were added to the residual solution until 80 per cent saturation was reached. The precipitate was collected by centrifuging for 10 minutes at 10,000 x g, dissolved in 0.1 M phosphate buffer (pH 6.0), and dialysed for 12 hours against large volumes of the same buffer. All procedures were carried out at 2^{0}. This purification procedure resulted in a 160-fold purification over the crude extract. Any further attempts at purification such as Sephadex gel filtration, alumina C-γ gel absorption,

TABLE XXIV.

PURIFICATION OF NITRITE REDUCTASE

Washed cells were suspended in 2 vol. 0.05 M Tris-HCl buffer (pH 7.0) and disrupted in the French pressure cell. The enzyme was assayed using reduced benzyl viologen as electron donor and protein determined by the Folin method.

Fraction	Vol. (ml)	Enzyme activity (moles NO_2^- reduced/5 min)	Recovery enzyme (%)	Protein (mg)	Specific Activity (moles NO_2^- reduced 5 min/mg protein)	Purification
1. Homogenate	185	-	-	-	-	-
2. Supernatant fraction after centrifuging homogenate at 10,000 x g for 20 min.	150	1500	100	4650	0.32	1
3. Supernatant fraction after centrifuging Fraction 2 at 144,000 x g for 2 h.	110	5400	360	1210	2.46	7.7
4. Fraction 3 diluted with an equal volume of 0.1 M phosphate buffer (pH 6.0) and precipitated between 75-80% satn with $(NH_4)_2SO_4$, dissolved in 0.1 M phosphate buffer (pH 6.0) and dialysed for 12 hr against the same buffer	35	2870	190	56	51.2	160

or starch gel electrophoresis resulted in rapid denaturation of the enzyme. All further data reported are for the purified preparation unless otherwise noted.

C. Electron Donors.

The suitability of a number of electron donors with a range of oxidation-reduction potentials was investigated and the results are given in Table XXV. Appropriate controls were included in each case to check the possibility of a non-enzymic reduction of nitrite. Reduced FMN and reduced benzyl viologen were found to be the most effective donors. When either $NADH_2$ or $NADPH_2$ was used there was a requirement for a flavin cofactor for enzyme activity which could be met equally well by either FAD, FMN, or riboflavin. Reduced methylene blue was also effective as an electron donor. Hydroxylamine hydrochloride, adjusted to pH 7.0 immediately before use, was inactive, while reduced methyl viologen and reduced phenazine methosulfate caused a large non-enzymic loss of nitrite from the reaction mixture.

D. Effect of Time of Incubation.

The effect of varying the incubation times on nitrite reduction is shown in Figure 26. Purified nitrite reductase (0.2 mg protein) was incubated for varying times up to 30 minutes with $NADH_2$ + FMN as the electron donating system.

TABLE XXV.

ELECTRON DONORS FOR NITRITE REDUCTASE

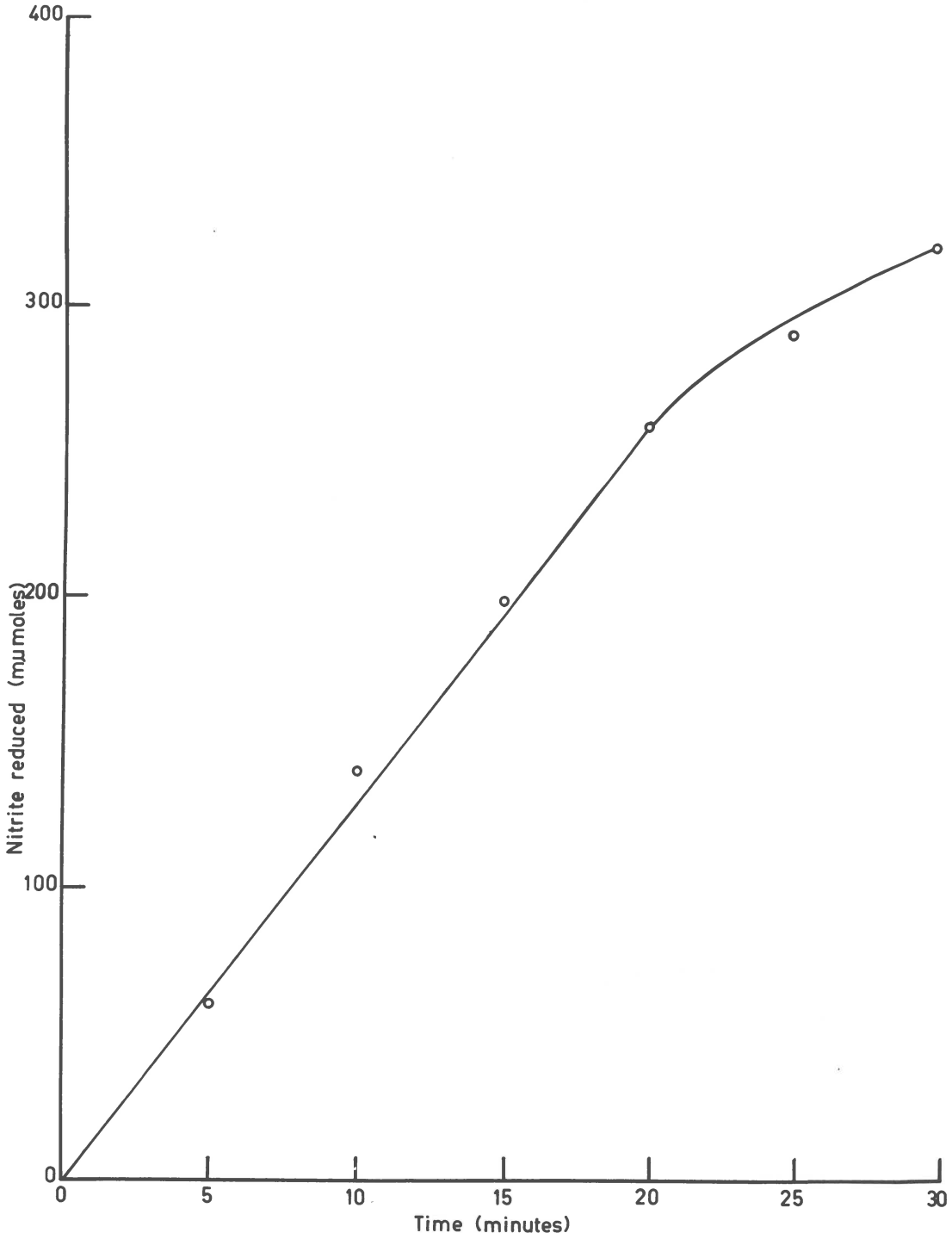
See text for details of assay (p. 55-56).

Electron Donor	Final conc. (M)	Relative activity for reduction of nitrite (NADH + FMN = 100)	Redox Potential at pH 7.0
NADH	10^{-3}	0	-0.320
NADH + FMN (0.1 mM)	10^{-3}	100	-0.320
NADPH	10^{-3}	0	-0.320
NADPH + FMN (0.1 mM)	10^{-3}	100	-0.320
Reduced methylene blue	2×10^{-4}	133	+0.011
Reduced benzyl viologen	3×10^{-3}	2500	-0.359
$FMNH_2$	10^{-3}	2500	-0.185
Reduced methyl viologen	10^{-3}	Non-enzymic reduction	-0.446
Reduced phenazine methosulfate	10^{-3}	Non-enzymic reduction	-0.080
Hydroxylamine-HCl (pH 7.0)	10^{-3}	0	+0.450

FIGURE 26.

EFFECT OF INCUBATION TIMES ON NITRITE REDUCTION

Nitrite reductase (0.2 mg protein) was incubated for times varying from 0 to 30 minutes with $NADH_2$ as donor as described in the text.


400
300
200
100
0
Nitrite reduced (mμmoles)
0
5
10
15
20
25
30
Time (minutes)

Nitrite reduction was linear with time for the first 20 minutes with only a slight falling off over the 20 to 30 minutes period.

E. Effect of pH.

In Figure 27, the effect of pH on enzyme activity is presented. When $NADH_2$ was the electron donor, optimal nitrite reduction occurred at pH 6.0. A particularly marked loss of activity was observed at lower pH values. The data show that the optimal activity with reduced benzyl viologen as electron donor occurred between pH 6.0 and 7.0.

F. Stability of the Enzyme.

Nitrite reductase, when stored at -17°, retained its activity for at least a year. Heat treatment at 80° for 10 minutes resulted in a 75 per cent loss of activity. The enzyme was completely inactivated by boiling for 5 minutes.

G. Gas Production by Nitrite Reductase.

1. Manometric experiments.

The results of a manometric assay of gas production by the purified enzyme are depicted in Figure 28. Warburg manometer vessels fitted with two side-arms were used so that gas absorbents could be included. All treatments

FIGURE 27.

THE EFFECT OF pH ON THE ACTIVITY OF NITRITE REDUCTASE

Nitrite reductase activity at various pH value was determined with (A) NADH and (B) reduced benzyl viologen as the electron donor. Details of assay are described in the text.

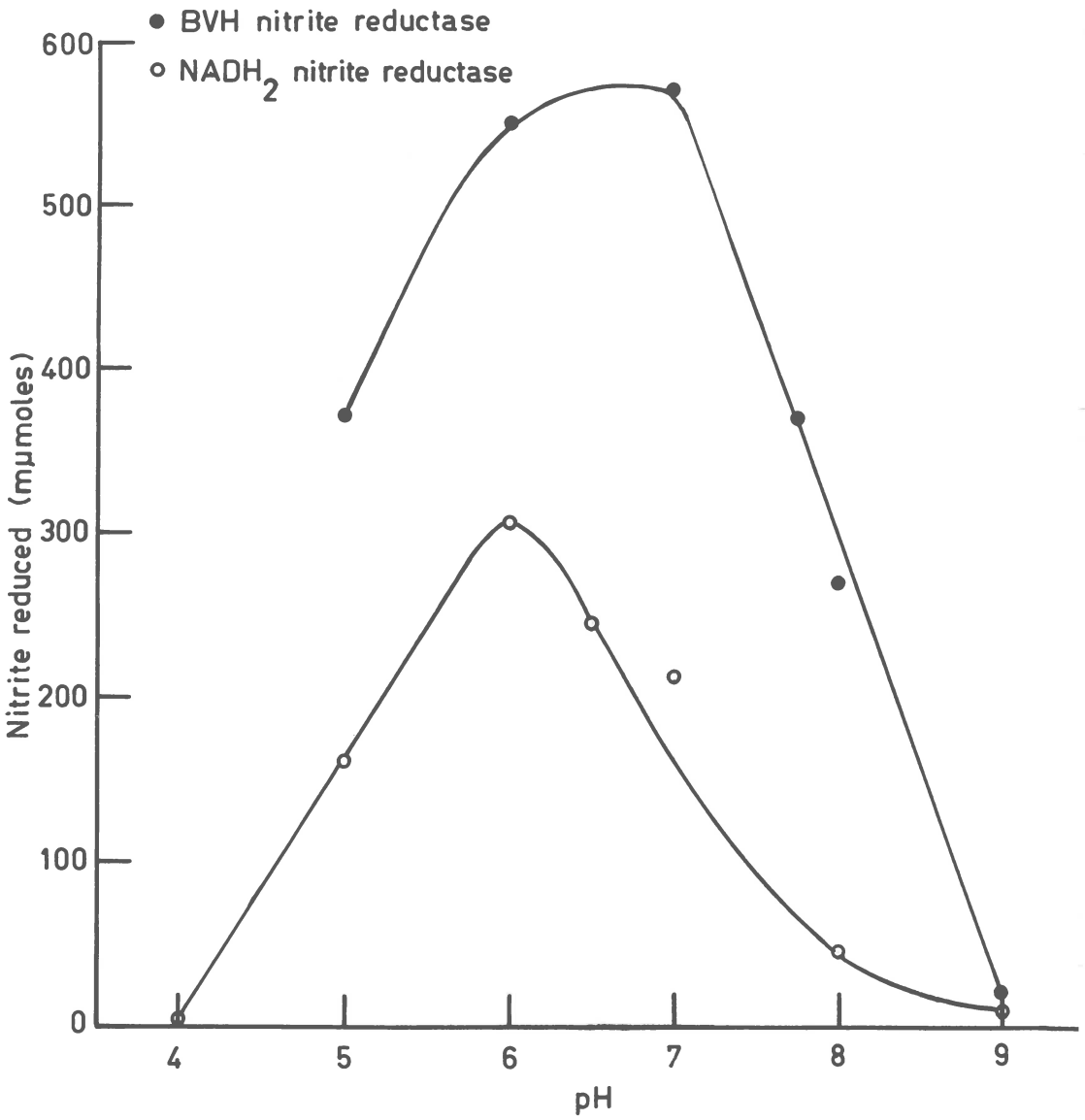
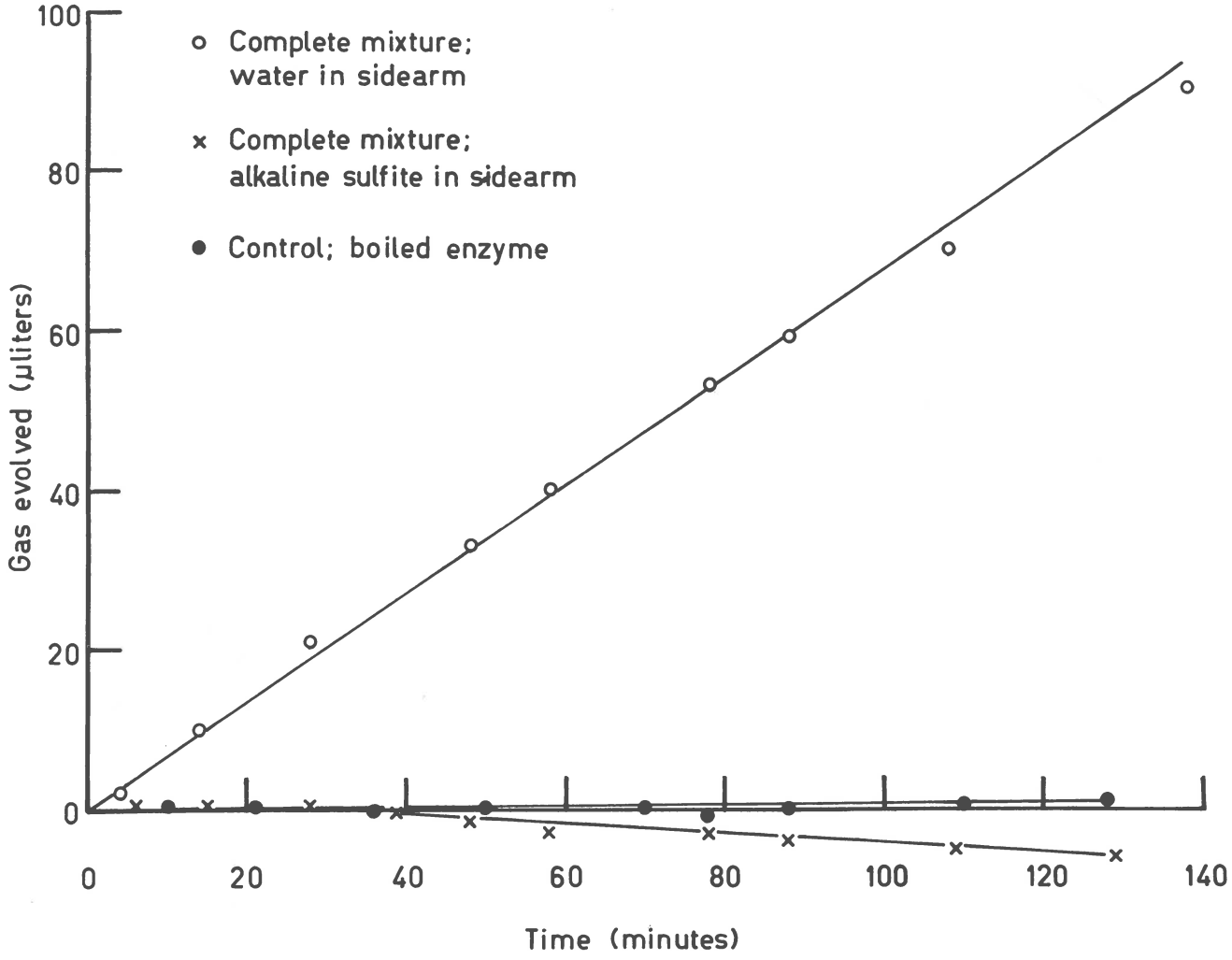

● BVH nitrite reductase
○ NADH2 nitrite reductase
Nitrite reduced (mμmoles)
600
500
400
300
200
100
0
4
5
6
7
8
9
pH

FIGURE 28.

GAS PRODUCTION BY NITRITE REDUCTASE

Manometric assay of nitrite reduction by soluble nitrite reductase. Reaction mixture consisted of: 5 moles Na NO_2; 5 moles NaN$_3$; 0.2 mole KCN; nitrite reductase (0.2 mg protein); 0.1 M phosphate buffer pH 6.0 to 3.0 ml. 0.2 ml 20% (V/V) KOH was placed in the center well and 0.2 ml alkaline sulfite solution or 0.2 ml H_2O in a sidearm.


o Complete mixture; water in sidearm
× Complete mixture; alkaline sulfite in sidearm
● Control; boiled enzyme
Gas evolved (µliters)
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Time (minutes)

included 0.2 ml of 20 per cent (w/v) KOH in the center well to absorb any CO_2 that might be formed. Linear production of gas with time, equivalent to about 4 umoles, occurred in the flask in which water was the only absorbent. No gas accumulation occurred in the flask which contained 0.4 ml of alkaline sulfite solution (20 per cent (w/v) $NaSO_3$ in 2 per cent (w/v) KOH) to absorb nitric oxide (Treadwell and Hall, 1942). This observation suggests that nitric oxide was the only gaseous product formed.

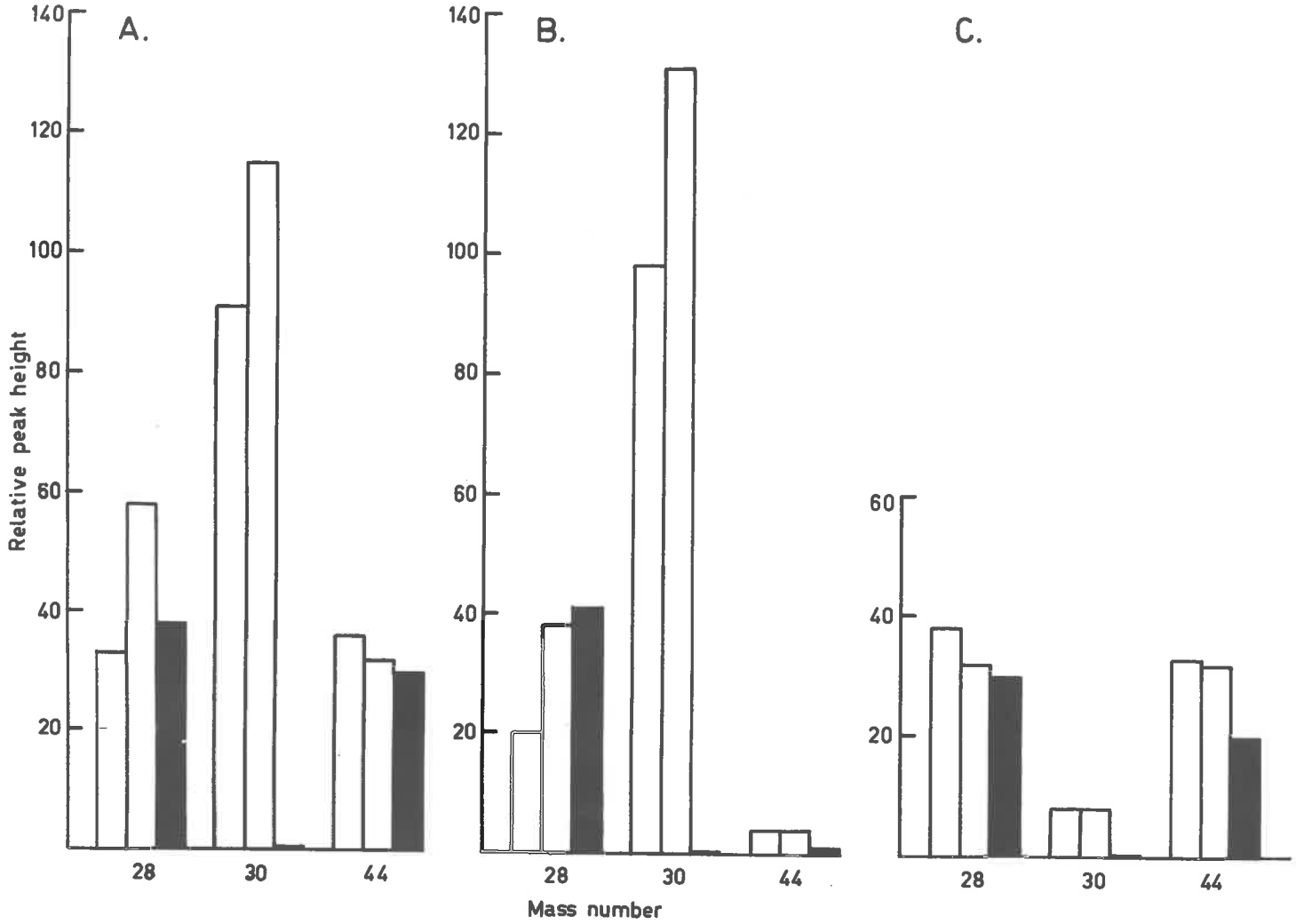
2. Mass Spectrometric Experiments.

In Figure 29. the results of a similar experiment using purified enzyme as shown, but in this case the mass spectrometer was used to analyze the gas produced. The mass spectrum of the gases present when water was included in a side-arm of the reaction vessel is shown in Figure 29a. The major gas produced was of mass 30 with some gas of mass 44. Figure 29b. shows the mass spectrum of the gases present when the side-arm contained 0.4 ml of alkaline permanganate solution (0.125 M $KMnO_4$ in 1 M KOH) which is an absorbent for nitric oxide (Anderson, 1965). In this case most of the gas with a mass of 30 had been absorbed. In Figure 29c. the mass spectrum of gases found in the reaction vessel when 0.4 ml of 20 per cent (w/v) KOH was included in the side-arm is shown.

FIGURE 29.

PRODUCTS OF NITRITE REDUCTION

Mass spectrometer assay of gaseous products formed by nitrite reductase under anaerobic conditions after 2 hours incubation. Reaction mixture contained 2 μmole $NaNO_2$, 2 μmole $NaBH_2$, 0.1 μmole FMN, purified nitrite reductase (0.16 mg protein), and 0.1 M phosphate buffer (pH 7.0) to 2.0 ml. Gas absorbents included in a side arm were (A) 0.4 ml water; (B) 0.4 ml 20% (W/V) KOH; (C) 0.4 ml alkaline permanganate solution.


A.
B.
C.
140
120
100
80
60
40
20
Relative peak height
28
30
44
Mass number

Nearly all of the gas with a mass of 44 was absorbed, suggesting that it was CO_2. The product with mass 30 was not absorbed by KOH. Little nitrogen gas (mass 28) was found above the level of the controls in any of the three treatments.

In Figure 30. the products of the reduction of ^{15}N-labelled nitrite by resting whole cells and by the purified enzyme are compared. The resting whole cells produced gases with masses of 44, 45, and 46 corresponding to $^{14}N_2O$, $^{14}N^{15}NO$, and $^{15}N_2O$, respectively in addition to gases with masses 30 and 31 (^{14}NO and ^{15}NO). The purified system produced large amounts of gas with mass number of 30 and 31, but very little at masses 44, 45, and 46.

These data indicate that while whole cells reduce nitrite to nitric and nitrous oxides, the purified nitrite reductase produces only nitric oxide.

H. Requirement for Flavin Co-factor.

The stimulatory effect of flavin co-factors is shown in Figure 31. Nitrite reductase was assayed with $NADH_2$ as the electron donor with varying amounts of FMN, FAD, or riboflavin. No activity was detected in the absence of a flavin compound. Maximal activity with any of the three flavins occurred at about 10^{-4} M, with no further

FIGURE 20.

PRODUCTS OF NITRITE REDUCTION

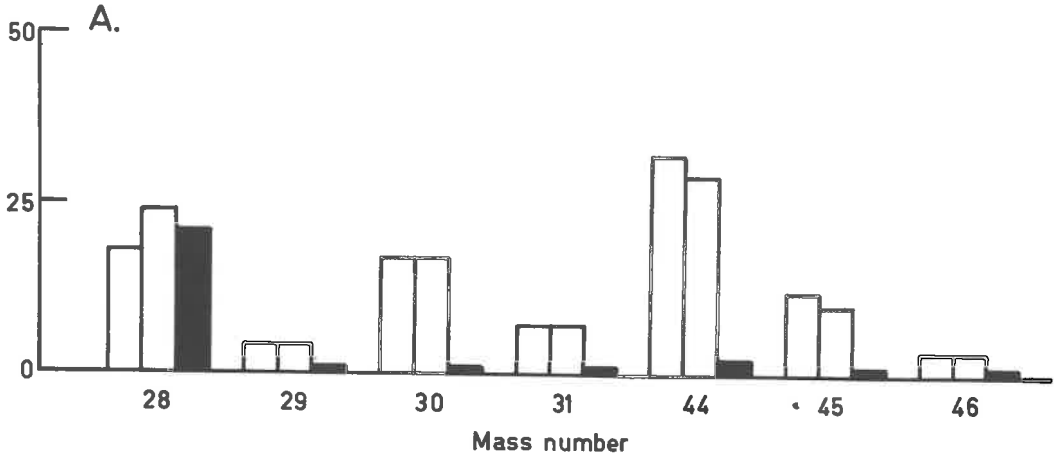
Mass spectrometer assay of gaseous products formed by resting whole cells or cell extracts from ^{15}N-labelled Na NO_2 (31.3 atom % enriched) under anaerobic conditions after 2 hours incubation.

A. Whole cells

B. Purified nitrite reductase

Open bars: test material

Solid bars: controls


A.
50
25
0
28
29
30
31
44
45
46
Mass number

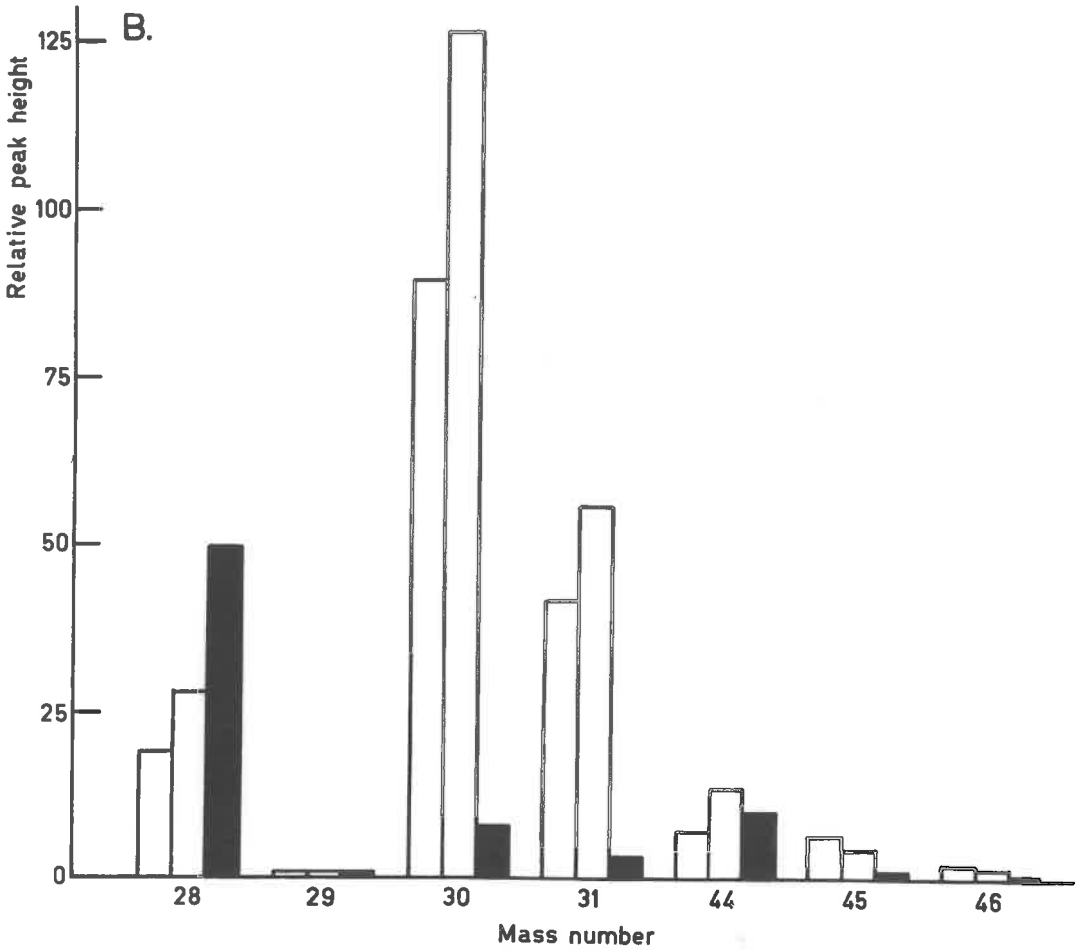

B.
Relative peak height
125
100
75
50
25
0
28
29
30
31
44
45
46
Mass number

FIGURE 31.

EFFECT OF ADDED FLAVIN COMPOUNDS ON NITRITE REDUCTASE ACTIVITY

Nitrite reductase activity assayed with varying amounts of exogenous flavin compounds from 0 to 0.2 mM. Standard assay carried out as described in the text.

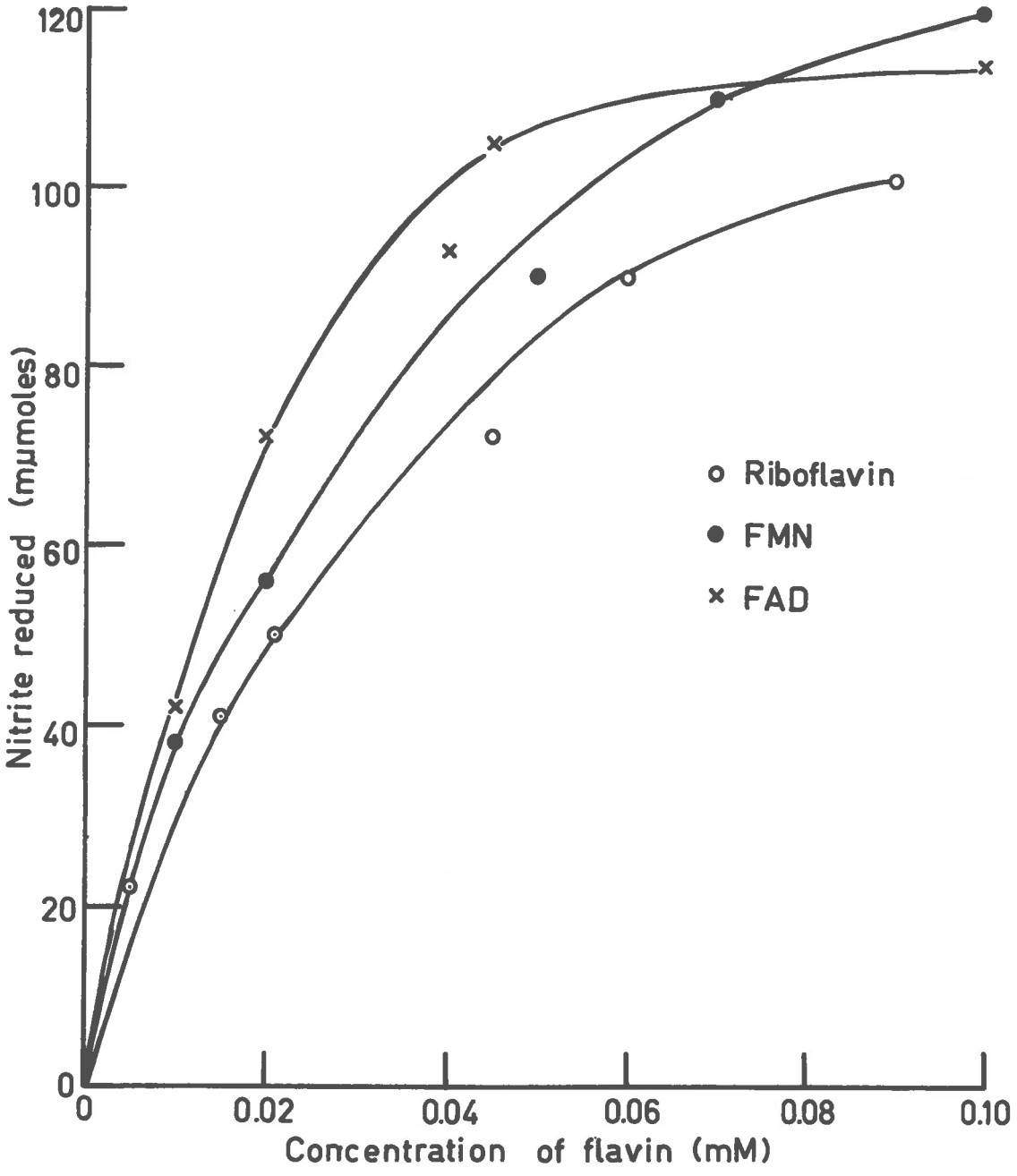

120
100
80
60
40
20
0
Nitrite reduced (mμmoles)
0
0.02
0.04
0.06
0.08
0.10
Concentration of flavin (mM)
o Riboflavin
● FMN
× FAD

FIGURE 32.

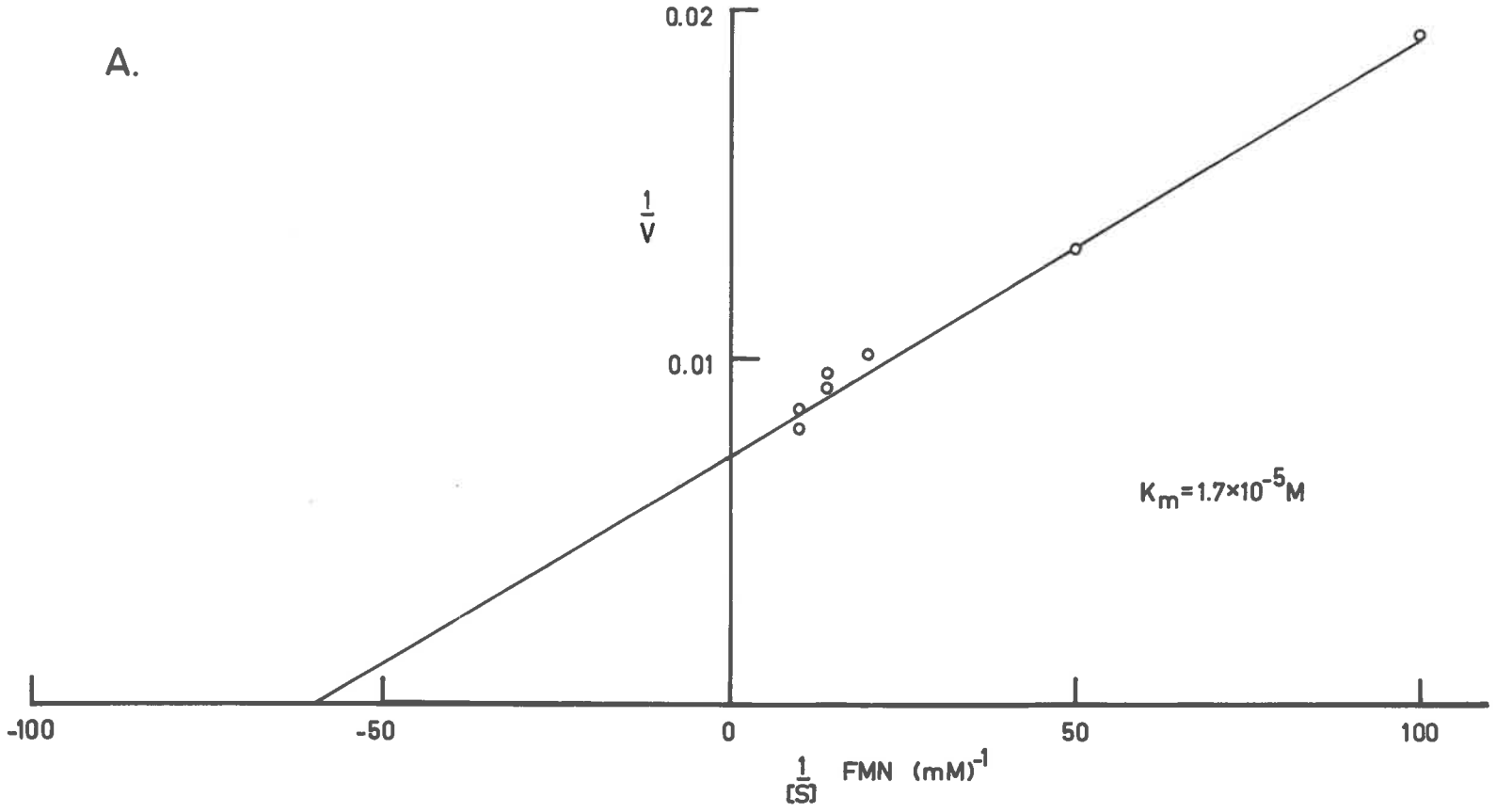
MICHAELIS CONSTANT OF NITRITE REDUCTASE FOR FLAVIN CO-FACTORS

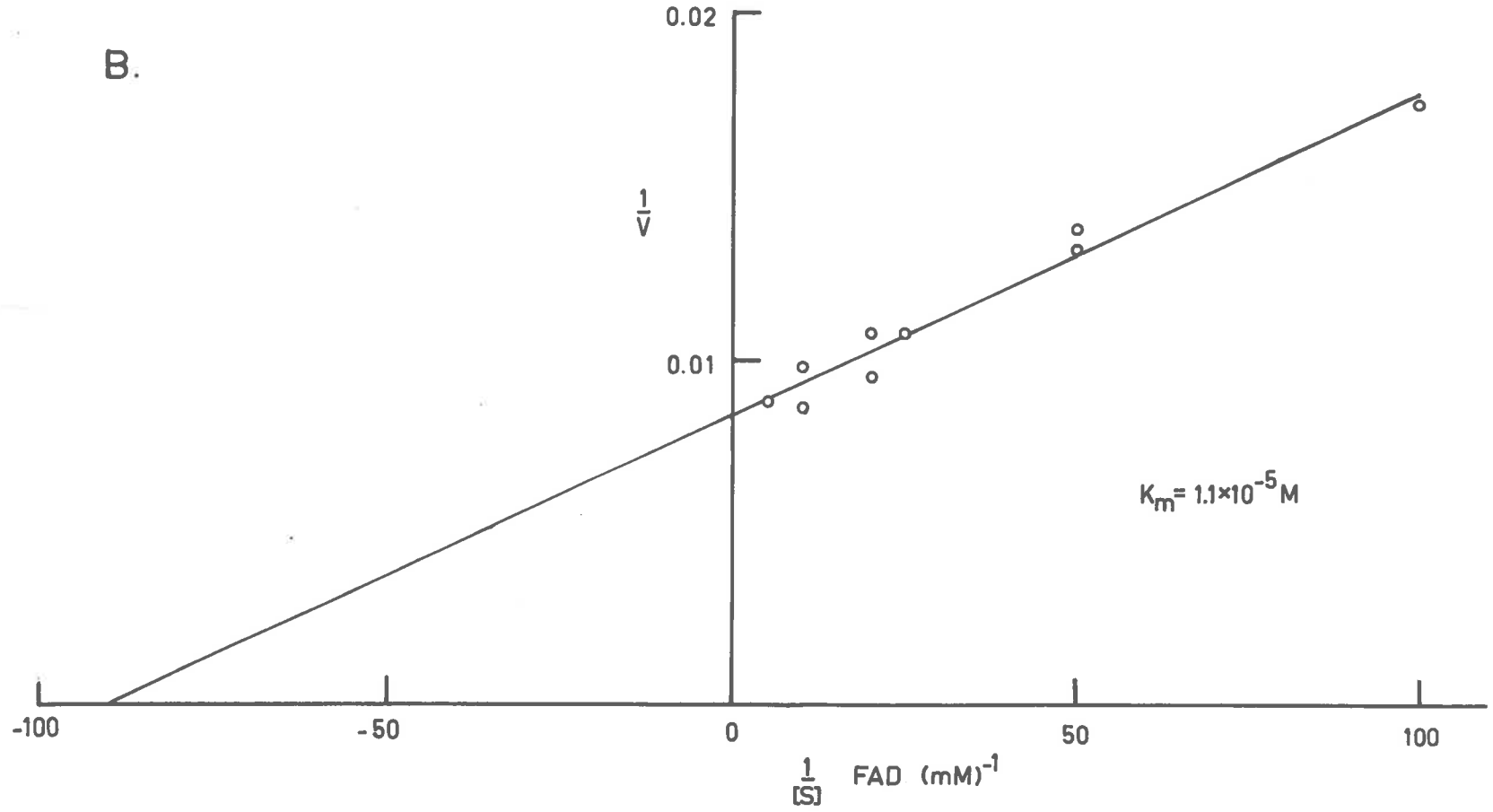
Double reciprocal plots (Lineweaver and Burk, 1934) of the rate of nitrite reduction versus the concentration of flavin co-factor. Details of assay are given in the text.

A. FMN

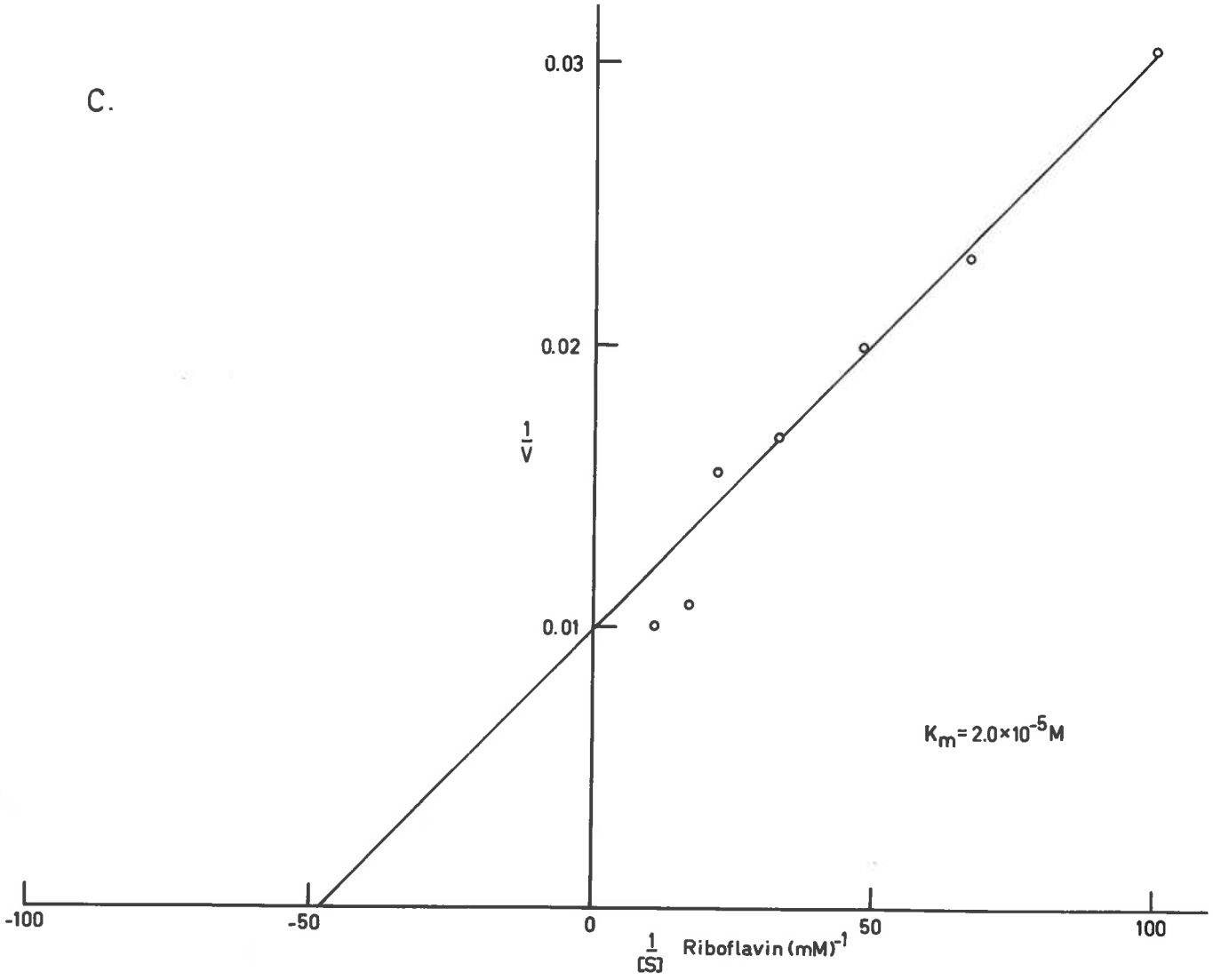
B. FAD

C. Riboflavin


A.
0.02
1/V
0.01
$K_m = 1.7 \times 10^{-5} M$
-100
-50
0
50
100
1/[S] FMN (mM)$^{-1}$


B.
0.02
0.01
1/V
$K_m = 1.1 \times 10^{-5}$ M
-100
-50
0
50
100
1/[S] FAD (mM)$^{-1}$

C.


0.03
0.02
0.01
1/V
$K_m = 2.0 \times 10^{-5} M$
-100
-50
0
50
100
1/[S] Riboflavin (mM)$^{-1}$

stimulation up to a concentration of 2×10^{-4} M. Michaelis constants of nitrite reductase for each of the three co-factors were calculated from the double reciprocal plots (Lineweaver and Burk, 1934) given in Figure 32. Apparent Km values were: FMN, 1.7×10^{-5} M; FAD, 1.1×10^{-5} M; riboflavin, 2.0×10^{-5} M.

I. Michaelis Constants.

The apparent K_m of nitrite reductase for its substrate was calculated to be in the order of 10^{-4} M nitrite from the double reciprocal plot shown in Figure 33. where the reciprocal of the velocity of nitrite reduction is plotted against the reciprocal of nitrite concentration.

J. Stoichiometry.

Table XXVI. compares the amount of $NADH_2$ supplied with the amount of nitrite reduced. The ratio of nitrite reduced to $NADH_2$ supplied was close to one. These data indicate that one mole of $NADH_2$ is oxidized per mole of nitrite reduced.

In Table XXVII. the amount of nitric oxide recovered in alkaline permanganate solution by the method of Anderson (1965) is compared with the amount of nitrite utilized. For each mole of nitrite reduced, close to one mole of nitric oxide was recovered.

TABLE XXVI.

STOICHIOMETRY OF NITRITE REDUCTION WITH $NADH_2$ AS DONOR

Standard assay mixture as given in text, but varying amounts of NADH were used as shown. All samples incubated for 30 min.

NADH supplied (μmoles)	NO_2^- reduced (μmoles)	NO_2^-/NADH
1	0.7	0.7
1	0.67	0.67
0.5	0.51	1.0
0.5	0.56	1.1
0.2	0.26	1.3
0.2	0.32	1.6
0.1	0.11	1.1
0.1	0.14	1.4

TABLE XXVII.

NITRIC OXIDE PRODUCTION BY NITRITE REDUCTASE

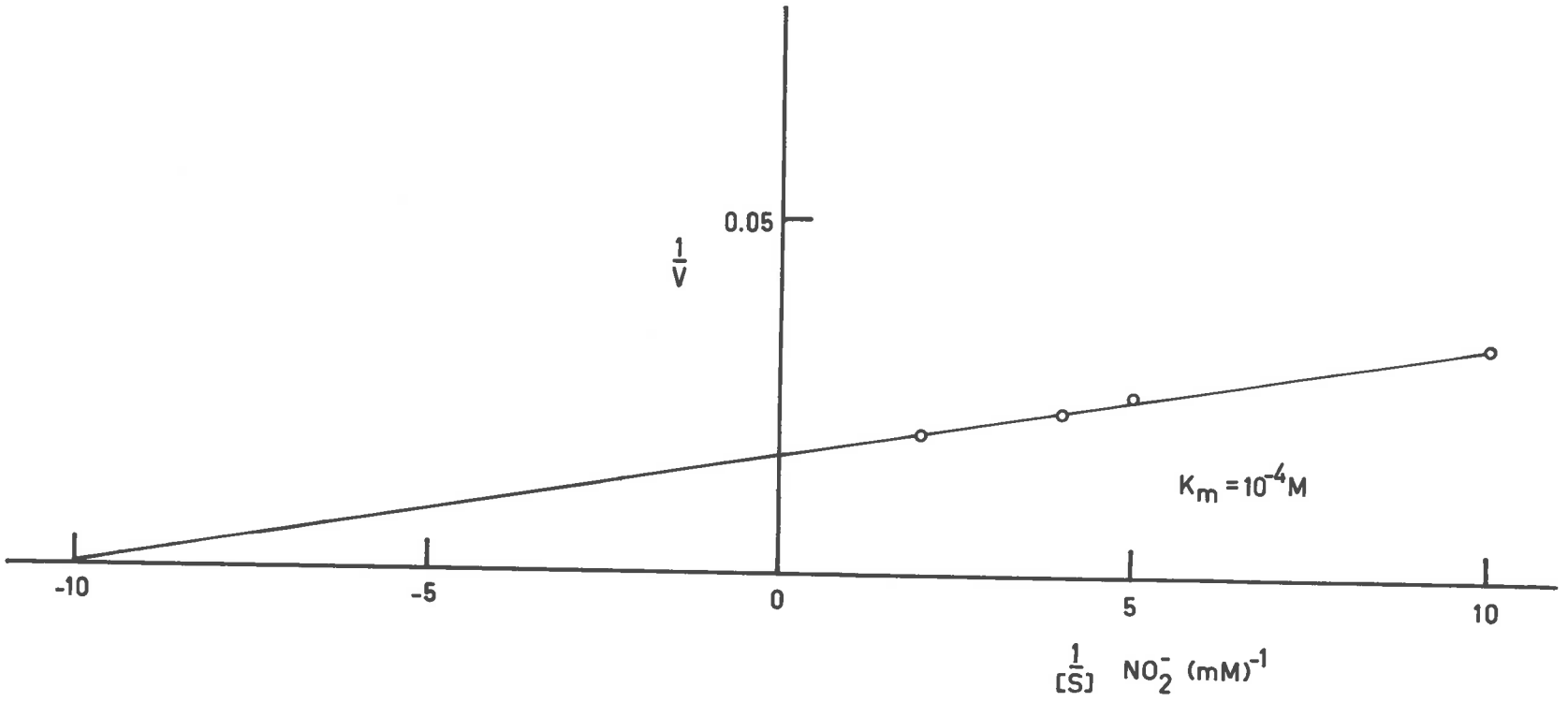
Nitric oxide produced by purified nitrite reductase (0.15 mg protein) using reduced benzyl viologen as electron donor was estimated by the alkaline permanganate method described on p. 61.

Nitrite reduced (μmole)	Nitric oxide produced (μmole)	$\frac{NO_2^-\text{ reduced}}{NO\text{ produced}}$
1.045	0.990	0.94
1.032	0.900	0.87

FIGURE 33.

MICHAELIS CONSTANT OF NITRITE REDUCTASE FOR NITRITE

Double reciprocal plot (Lineweaver and Burk, 1934) of rate of nitrite reduction versus concentration of sodium nitrite. Standard assay system as given in the text.


0.05
1/V
$K_m = 10^{-4}M$
-10
-5
0
5
10
1/[S] NO_2^- $(mM)^{-1}$

K. Inhibitor Studies.

Table XXVIII. shows the effects of a range of inhibitors on the purified enzyme when $NADH_2$ or reduced benzyl viologen was used as the hydrogen donor. Enzyme activity was strongly inhibited by metal chelating agents such as sodium diethyldithiocarbamate, potassium cyanide, 2,2'-dipyridyl, o-phenanthroline, and hydroxyquinoline when either $NADH_2$ or reduced benzyl viologen was the hydrogen donor. When $NADH_2$ was the electron donor, sodium diethyldithiocarbamate (5 mM) inhibited nitrite reduction by 70 per cent. Although p-chloromercuribenzoate inhibited nitrite reduction by 80 per cent when $NADH_2$ was the electron donor, it did not inhibit when reduced benzyl viologen was used. The inhibition of $NADH_2$-nitrite reduction was reversed by about 70 per cent by adding an equal concentration of reduced glutathione to the reaction mixture. Mepacrine (1 mM) inhibited nitrite reduction by 50 per cent when $NADH_2$ was the electron donor, but had no effect with reduced benzyl viologen as donor. Hydroxylamine hydrochloride, adjusted to pH 7.0 immediately before use, inhibited nitrite reduction by 20 per cent. Nitrate, chlorate, thiosulfate, sulfate, sulfite, and arsenite each at 5 mM had no effect on nitrite reduction. These data suggest that a metal(s) and sulfhydryl groups are of

TABLE XXVIII.

INFLUENCE OF INHIBITORS ON THE PURIFIED NITRITE REDUCTASE

The effect of inhibitors on enzyme activity was assayed using the standard techniques as described in Methods. The inhibitor was incubated with the enzyme for 15 min before starting the reaction.

Inhibitor	Final concn. (mM)	% Inhibition	
	Hydrogen donor:	$NADH_2$–PMS	BVH
Sodium diethyl-dithiocarbamate	5	100	83
KCN	5	60	93
	10	86	93
2,2'-Dipyridyl	5	27	47
o-Phenanthroline	5	40	35
8-Hydroxyquinoline	5	21	32
p-Chloromercuribenzoate	1	82	0
p-Chloromercuribenzoate + glutathione	1	27	-
Mepacrine	1	30	0
Hydroxylamine · HCl (pH 7.0)	5	20	-
Iodo-acetamide	1	0	-
NaF	1	0	0
Quinine	5	0	0
Amytal	1	0	0
Ethyl carbamate	5	0	0

importance in nitrite reduction by this enzyme.

L. $NADH_2$ Oxidase Activity.

The level of $NADH_2$ oxidase activity in the various fractions during purification was assayed. The ability of the purified system to oxidize $NADH_2$ aerobically was one-tenth that of the crude extracts while the nitrite reducing activity was increased 160-fold.

M. Metal-deficient Cultures.

Since the inhibitor studies indicated the participation of a metal or metals in nitrite reductase activity, the effect of growing the organism in metal-deficient cultures was investigated. Table XXIX. shows the effect of iron deficiency on the enzyme activity. Crude extracts prepared from cells that had been sub-cultured five times in iron depleted culture media were assayed for reductase activity with $NADH_2$ or reduced benzyl viologen as electron donors. Although the total yield of cell mass was much reduced in the deficient cultures, little effect was observed on the specific activity of the enzyme when $NADH_2$ was the electron donor. When reduced benzyl viologen was the hydrogen donor, the specific activity of the enzyme was higher in the iron-deficient cells than in normal ones.

TABLE XXIX.

EFFECT OF IRON DEFICIENCY ON NITRITE REDUCTASE ACTIVITY

Cells were subcultured 5 times in iron deficient medium (depleted of iron as described in Methods). Cells from an 18 hr culture were collected, washed and broken by ultrasonication. The activity was assayed in the crude extract.

Sample	Electron donor	NO_2^- reduced μmoles/ml/ 30 min	Protein mg/ml	Specific activity μmoles/ mg protein/30 min
Fe-deficient	NADH	600	5.6	110
Complete	NADH	1000	11.0	90
Fe-deficient	BVH	3600	5.6	635
Complete	BVH	3700	11.0	340

N. Effect of Dialysing Against Metal Chelating Compounds.

Some metal-protein complexes can be dissociated by dialysis against KCN or other metal chelating agents. Accordingly, nitrite reductase preparations were dialysed for 24 hours against 5 mM KCN or 5 mM sodium diethyldithiocarbamate. The enzyme activity was inhibited after such dialysis, but when these preparations were further dialysed against 0.1 M phosphate buffer (pH 7.0) activity was restored. The metal chelator could be dialysed away without affecting the activity of the enzyme.

The procedure for the removal of metal prosthetic groups according to Rubowitz (1938) was also carried out as described in Methods. After treatment with KCN followed by dialysis against 0.05 M Tris-HCl buffer (pH 7.0), nitrite reductase activity was decreased by 50 per cent when the $NADH_2$-FMN system acted as electron donor and 20 per cent with reduced benzyl viologen as electron donor. Addition of metals (Cu^{+1}, Cu^{+2}, Fe^{+2}, Fe^{+3}, Mo^{+6}, or Zn^{+2}) to the treated enzyme did not, however, restore activity.

O. Effect of Organo-Nitro Compounds.

A number of aromatic nitro-compounds were tested for inhibitory activity on nitrite reductase. Table XIX. shows that a number of these compounds were strongly inhibitory. In Figure 34. double reciprocal plots of the

TABLE XXX.

EFFECT OF AROMATIC NITRO-COMPOUNDS ON NITRITE REDUCTION

Standard $NADH_2$-FMN nitrite reductase system was used as described on p. 55; 0.1 ml of a saturated aqueous solution of each compound was added to the reaction mixture.

Inhibitor	Inhibition (%)
2,4-Dinitrophenol	49
o-Nitrophenol	44
m-Nitrophenol	30
p-Nitrophenol	44
2,6-Dinitrophenol	44
m-Dinitrobenzene	44

FIGURE 34

EFFECT OF 2,4-DINITROPHENOL AND 2,6-DINITROPHENOL ON NITRITE REDUCTASE ACTIVITY

Double reciprocal plot (Lineweaver and Burk, 1934) of rate of nitrite reduction at varying concentrations of Na NO_2. Nitrophenol (10^{-3} M) was added to each assay of one series. $NADH_2$ was electron donor. Details of assay are given in the text.

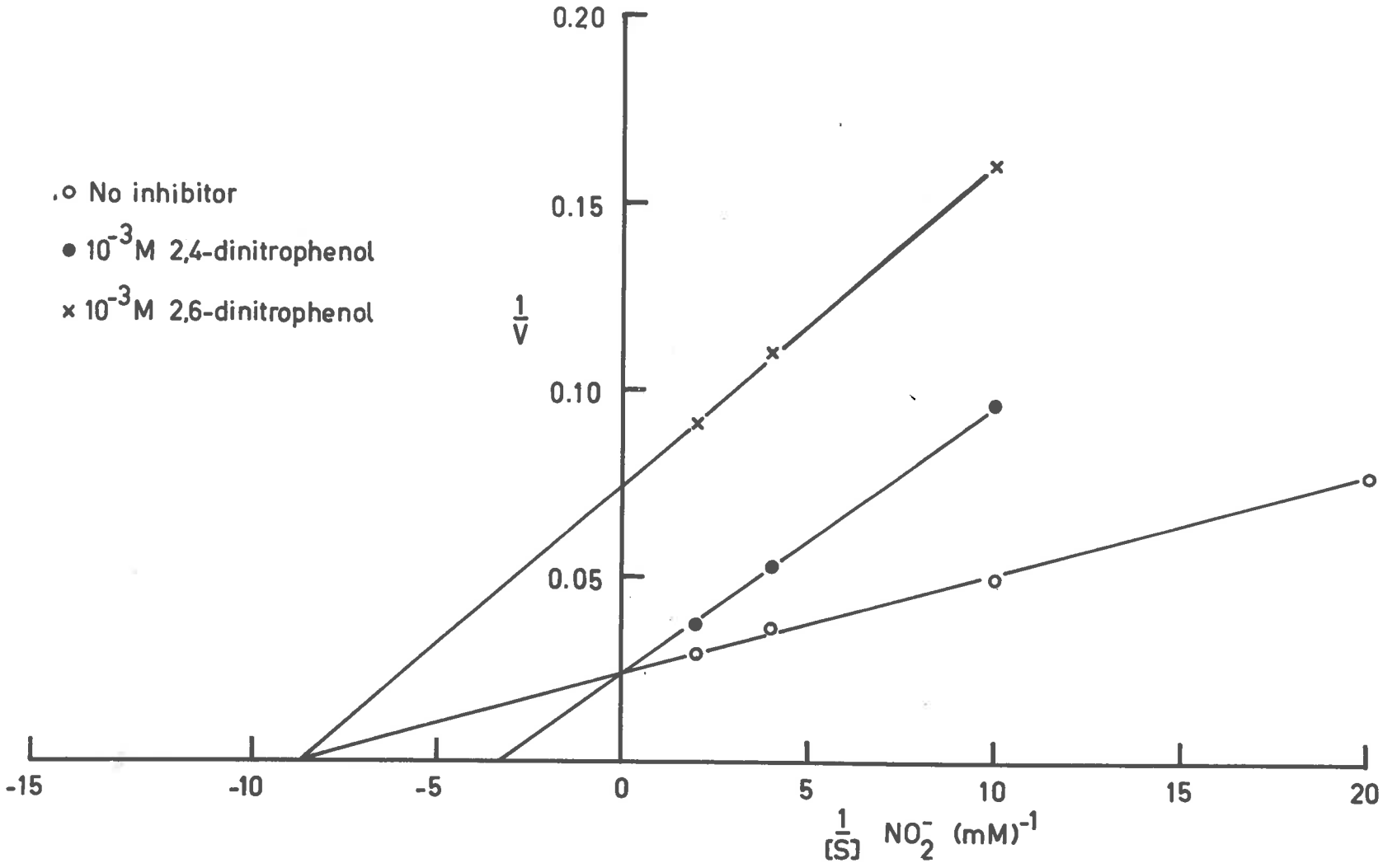

o No inhibitor
• 10^{-3}M 2,4-dinitrophenol
× 10^{-3}M 2,6-dinitrophenol
0.20
0.15
0.10
0.05
$\frac{1}{V}$
-15
-10
-5
0
5
10
15
20
$\frac{1}{[S]}$ NO_2^- $(mM)^{-1}$

FIGURE 35.

EFFECT OF m-DINITROBENZENE ON NITRITE REDUCTASE ACTIVITY

Double reciprocal plot (Lineweaver and Burk, 1934) of rate of nitrite reduction at varying levels of Na NO_2. m-Dinitrobenzene was added at two levels. Details of assay are given in text. $NADH_2$ was electron donor.

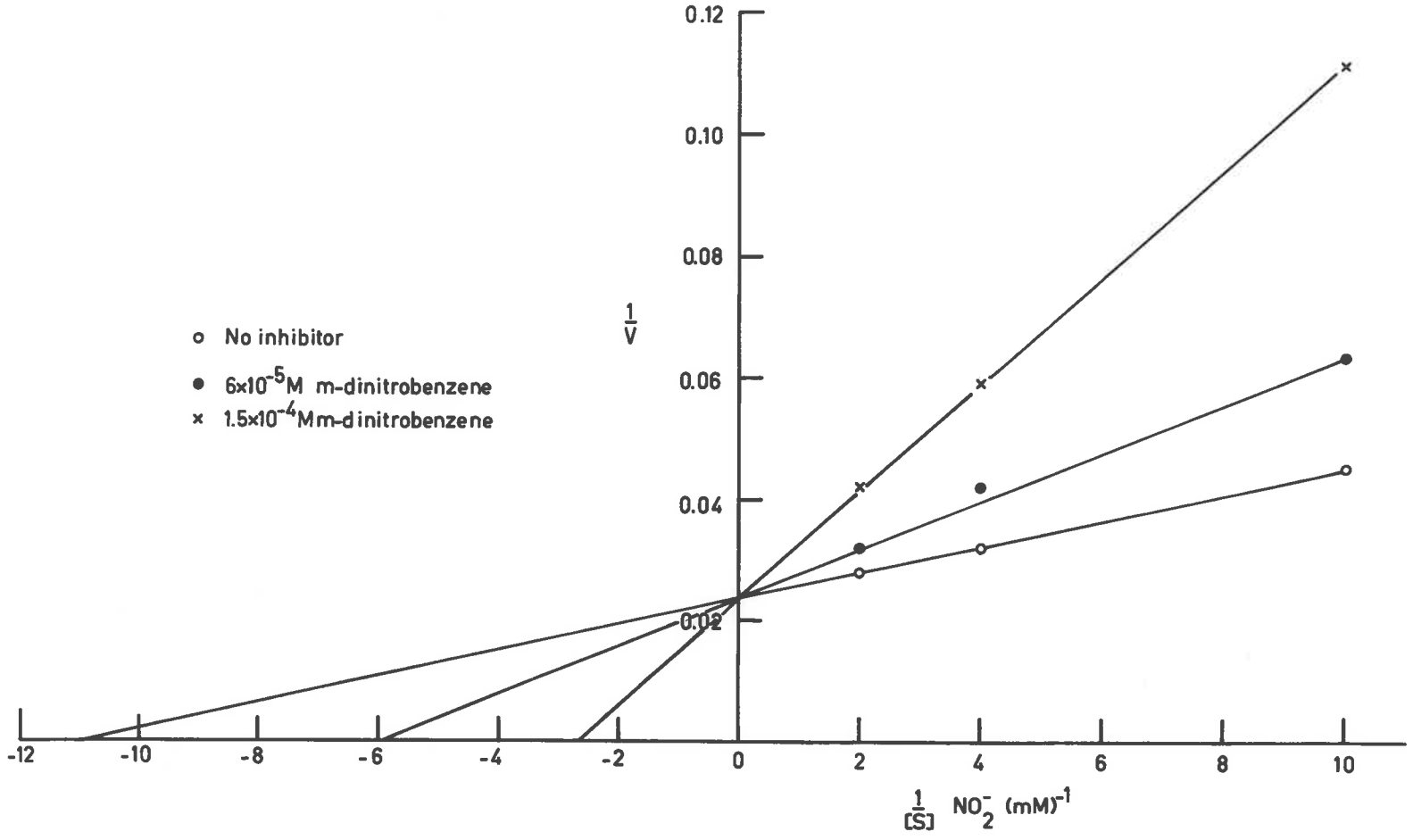

0.12
0.10
0.08
0.06
0.04
0.02
1/V
o No inhibitor
• 6x10⁻⁵M m-dinitrobenzene
× 1.5x10⁻⁴Mm-dinitrobenzene
-12
-10
-8
-6
-4
-2
0
2
4
6
8
10
1/[S] NO₂⁻ (mM)⁻¹

FIGURE 36

EFFECT OF 2,4-DINITROPHENOL DERIVATIVES ON NITRITE REDUCTASE ACTIVITY

Double reciprocal plots (Lineweaver and Burk, 1934) of rate of nitrite reduction at varying levels of Na NO_2. Aliphatic derivatives of 2,4-dinitrophenol (5×10^{-4} M) were added at each level of nitrite. Standard assay as given in text. $NADH_2$ was the donor.

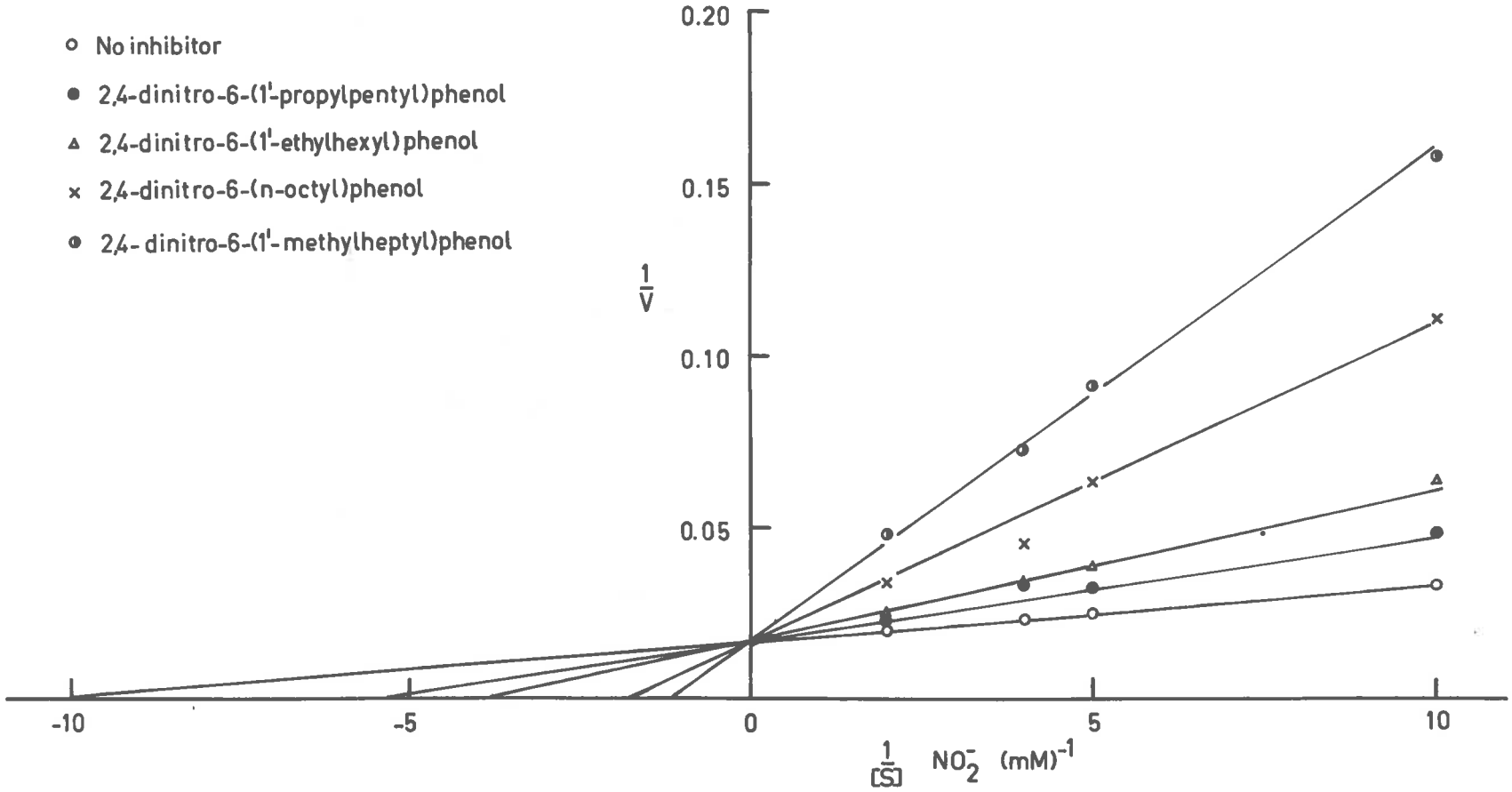

No inhibitor
2,4-dinitro-6-(1'-propylpentyl)phenol
2,4-dinitro-6-(1'-ethylhexyl)phenol
2,4-dinitro-6-(n-octyl)phenol
2,4-dinitro-6-(1'-methylheptyl)phenol
0.20
0.15
0.10
0.05
1/V
-10
-5
0
5
10
1/[S] NO2- (mM)-1

FIGURE 37.

EFFECT OF 2,6-DINITROPHENOL DERIVATIVES
ON NITRITE REDUCTASE ACTIVITY

Double reciprocal plots (Lineweaver and Burk, 1934) of the rate of nitrite reduction at varying levels of Na NO_2. Standard assay mixture with $NADH_2$ as electron donor was used. Aliphatic derivatives of 2,6-dinitrophenol (5×10^{-4} M) was added to one series of assays.

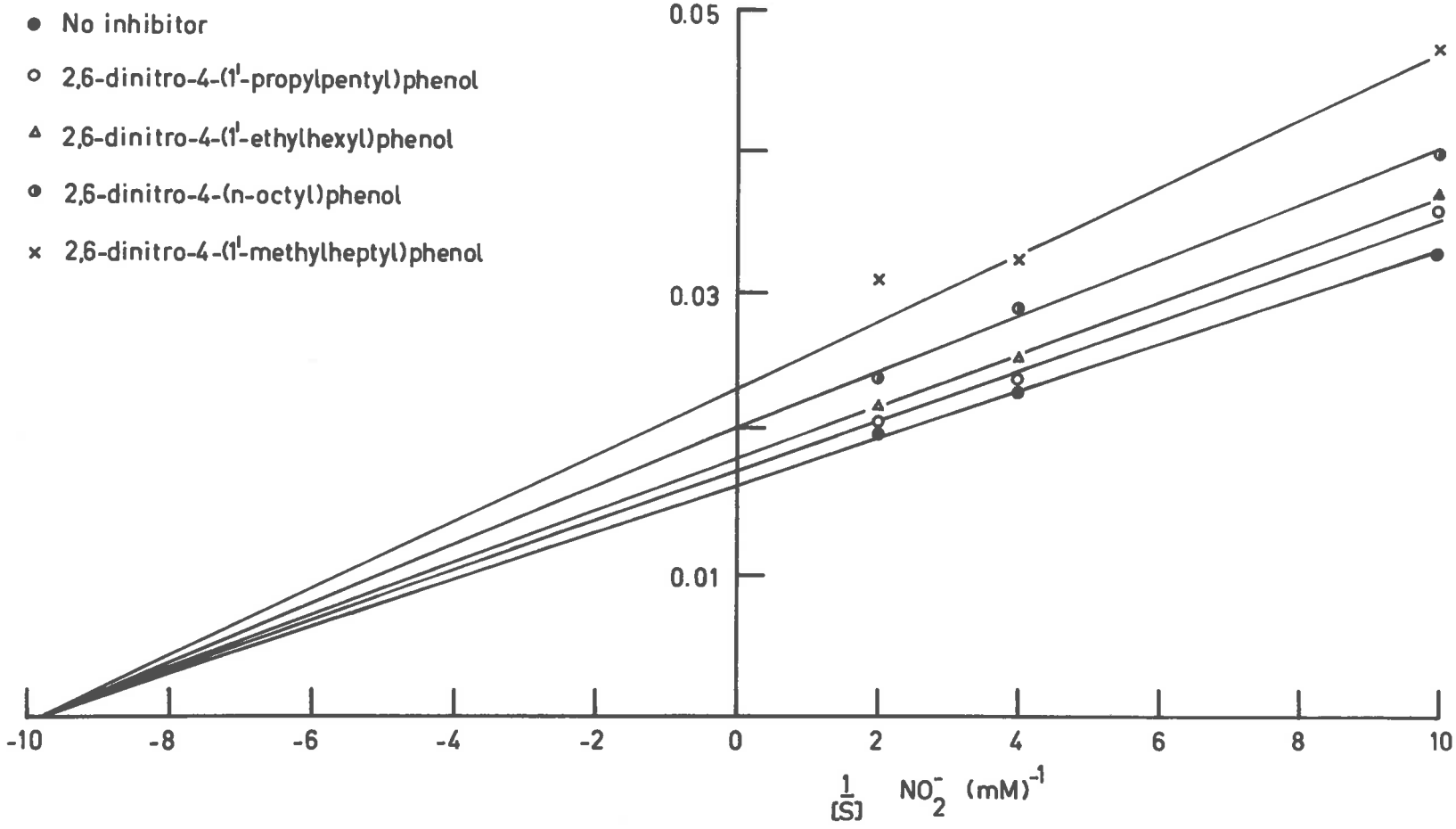
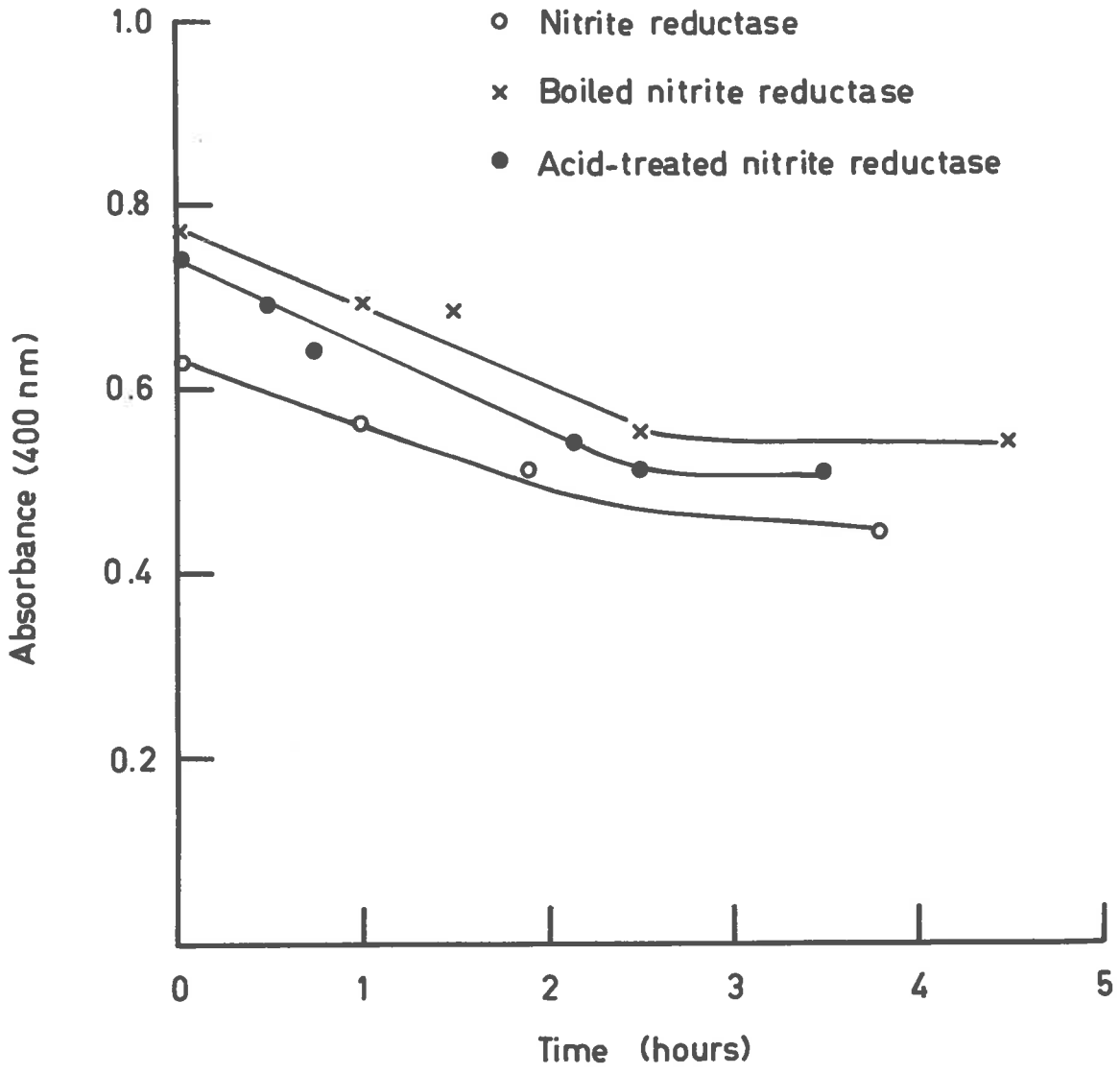

● No inhibitor
○ 2,6-dinitro-4-(1'-propylpentyl)phenol
△ 2,6-dinitro-4-(1'-ethylhexyl)phenol
◐ 2,6-dinitro-4-(n-octyl)phenol
× 2,6-dinitro-4-(1'-methylheptyl)phenol
0.05
0.03
0.01
-10
-8
-6
-4
-2
0
2
4
6
8
10
1/[S] NO2- (mM)-1

FIGURE 38.

REDUCTION OF 2,4-DINITROPHENOL

Rate of reduction of 2,4-dinitrophenol was followed spectrophotometrically at 400 mm. Reaction mixture contained 0.5 mole $NADH_2$, 0.02 mole FMN, 0.15 mole 2,4-dinitrophenol, 0.1 M phosphate buffer (pH 8.0) to 3.0 ml, and nitrite reductase that had been untreated, boiled, or acid denatured. The reaction was carried out anaerobically in Thunberg-type cuvettes.

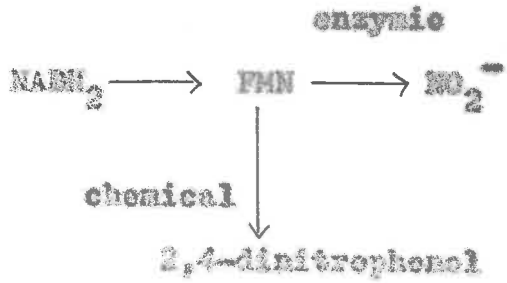

Nitrite reductase
Boiled nitrite reductase
Acid-treated nitrite reductase
Absorbance (400 nm)
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
Time (hours)

effect of 2,4-dinitrophenol and 2,6-dinitrophenol on the rate of nitrite reduction at varying levels of nitrite are presented. The 2,4-dinitrophenol inhibited the enzyme competitively while 2,6-dinitrophenol was a non-competitive inhibitor. A similar plot for m-dinitrobenzene at two concentrations is given in Figure 35. which shows a competitive type of inhibition. In Figures 36. and 37. the inhibitory effects of nitrophenol compounds with aliphatic substituents are shown. These derivatives exhibited competitive and non-competitive inhibitions of the enzyme in the same way as the parent compounds.

Experiments were performed to ascertain if the 2,4-dinitrophenol was reduced by the enzyme system. Figure 38. shows that it was reduced in the system, but that active nitrite reductase was not necessary for the reaction to take place. The reaction rate did not decrease when the enzyme used had been denatured by boiling or acid-treatment. Under anaerobic conditions in the light, $NaBH_4$ (0.15 mM) reduced FMN (0.014 mM) chemically at an initial rate of 4.8 umoles/minute at 25° in 0.1 M phosphate buffer (pH 6.0). The 2,4-dinitrophenol acted as an acceptor for electrons for FMN which had been reduced either chemically or enzymically.

Thus the inhibition of nitrite reductase can be

attributed to a competition for reducing power between nitrite which is reduced enzymically, and 2,4-dinitrophenol which is reduced chemically. This is shown in the scheme below:



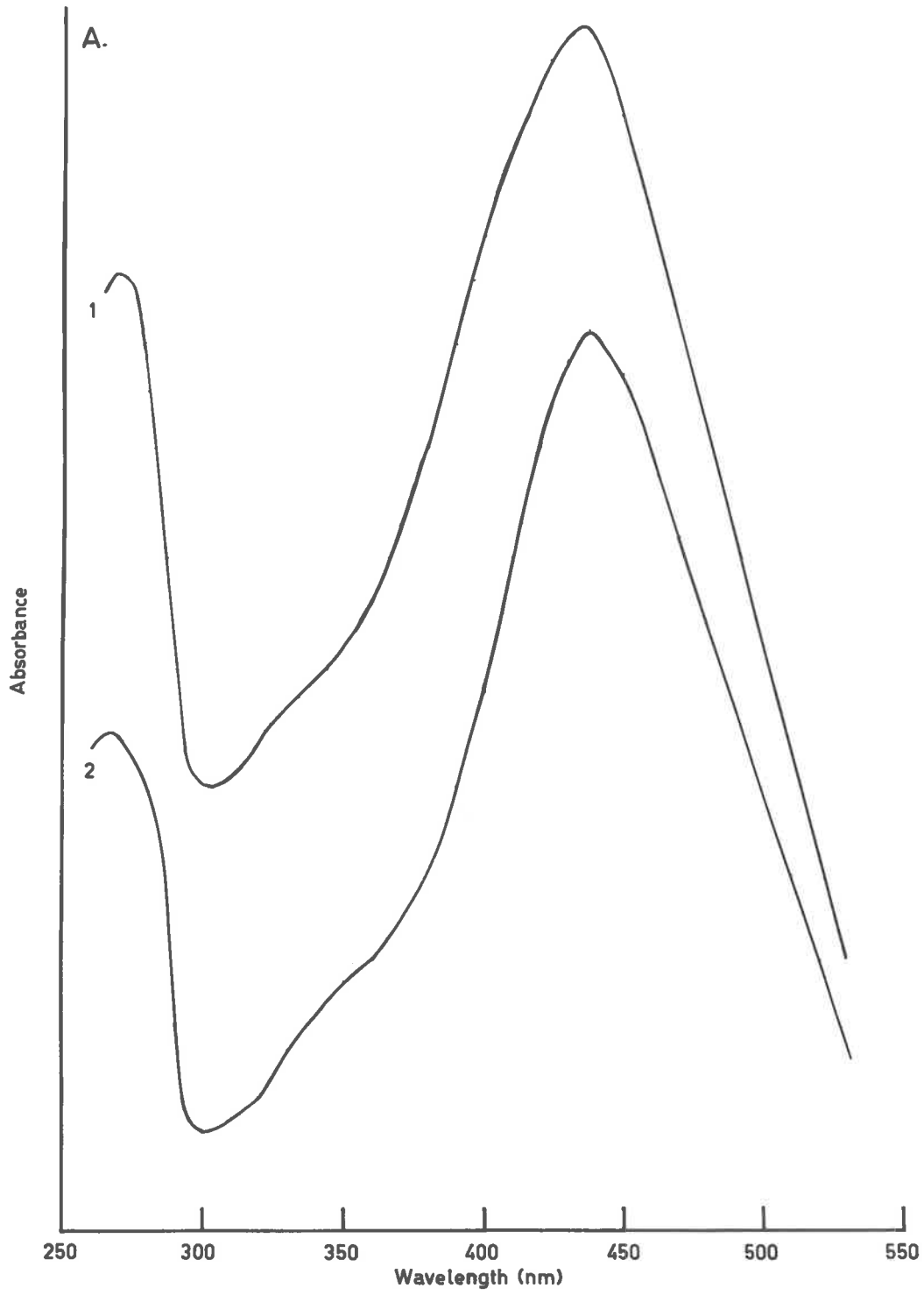
The product of the reduction of 2,4-dinitrophenol by $FMNH_2$ was determined by reacting the nitrophenol anaerobically with FMN that had been reduced with hydrogen and palladised asbestos. The resulting mixture was separated by thin-layer chromatography on silica gel plates using benzene-ethanol (9:1 by volume) as the solvent. In this system the FMN remained at the origin and two products migrated at different rates. These spots were scraped off the plates and dissolved in 0.1 M phosphate buffer (pH 7.5) and their absorption spectra compared with those of authentic 2-amino-4-nitrophenol and 2-nitro-4-amino-phenol. The ultraviolet absorption spectra are shown in Figure 39. The spectrum of the product and also its R_F value (0.66) were identical with that of authentic 2-amino-4-nitrophenol.

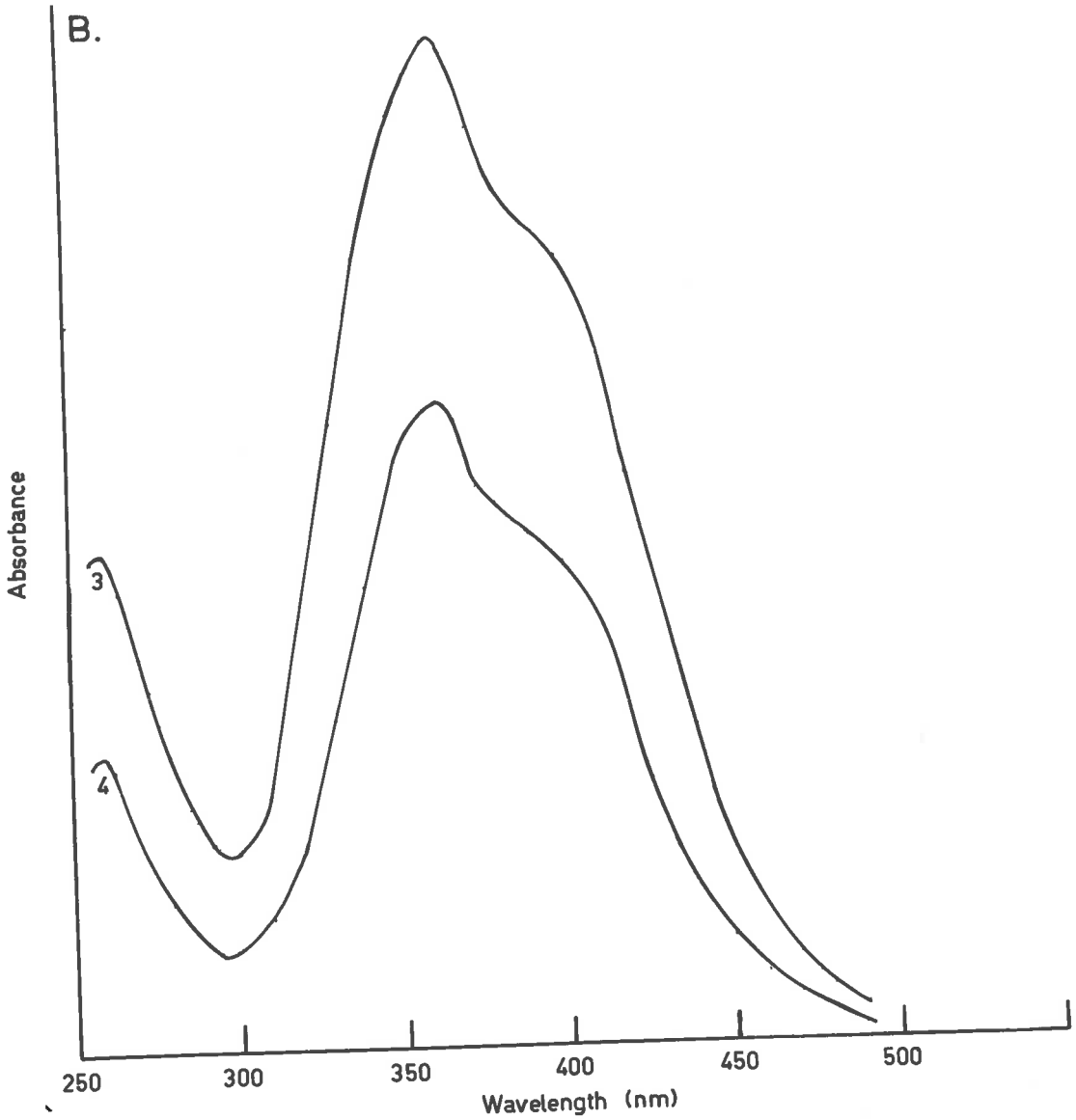
FIGURE 39.

ABSORPTION SPECTRA OF REDUCTION PRODUCTS
OF 2,4-DINITROPHENOL

Products were eluted from thin-layer chromatogram as described in the text and dissolved in 0.1 M phosphate buffer (pH 7.5).

1. Authentic 2-amino-4-nitrophenol
2. Fast moving band from thin-layer chromatogram
3. Authentic 2,4-dinitrophenol
4. Slow moving band from thin-layer chromatogram


A.
1
2
Absorbance
250
300
350
400
450
500
550
Wavelength (nm)


B.
Absorbance
3
4
250
300
350
400
450
500
Wavelength (nm)

DISCUSSION

I. THE EFFECT OF CULTURAL CONDITIONS ON GROWTH.

P. denitrificans utilized nitrate as the sole electron acceptor as shown by its good growth on nitrate medium under anaerobic conditions. The organism cannot grow by fermentative processes, however; thus there is no growth under anaerobic conditions in the absence of nitrate.

Nitrite cannot substitute for nitrate as the sole terminal electron acceptor and will not support anaerobic growth even with very long incubation periods. The possibility that the lack of growth on nitrite might be due to its toxic action on the cells rather than to its inefficiency as terminal electron acceptor must be considered. This seems unlikely, however, since nitrite is excreted into the medium at high concentrations during anaerobic growth on nitrate without affecting the growth rate of the cells. When the cultures were grown aerobically in the absence of nitrate, sodium nitrite added to the medium had no effect on growth up to a concentration of 2 gm/l, a concentration twice that used in the anaerobic cultures that yielded no growth. This observation is in contrast with observations on *Micrococcus denitrificans* which grows under anaerobic conditions

with nitrite as the sole terminal electron acceptor (Lam, 1969).

Preservation of long term stock cultures in a dehydrated state on anhydrous silica gel proved to be an efficient way of maintaining viable cultures. An added advantage of this technique was that it avoided the necessity of frequent subcultures and the attendant risk of strain variation over extended periods of time due to possible mutations.

When the cultures were grown anaerobically in deep cultures without bubbling with nitrogen, vigorous gas production was observed. Experiments were carried out to identify these gaseous products. When ^{15}N-labelled nitrate was the electron acceptor for actively growing cultures, nitrogen gas was the major product along with small quantities of nitrous oxide and traces of nitric oxide.

Nitrous oxide has been reported as a major product of denitrification in soil (Vijler and Delwiche, 1954) although it is probably not utilized further in bacterial metabolism (Sacks and Barker, 1952; Fewson and Nicholas, 1961c). The data reported herein establish that a small, but appreciable quantity of nitrous oxide is formed from nitrate by actively growing cultures of P. denitrificans.

This production of nitrous oxide from nitrate may help to account for its occurrence in soils.

Verhoeven (1956) questioned the role of nitric oxide as a normal intermediate in denitrification. He observed production of nitric oxide only from cells supplied with nitrite or from aged cells given nitrate or nitrite. Freshly prepared suspensions of P. denitrificans produced nitric oxide; actively growing cultures produced traces of nitric oxide from ^{15}N-labelled nitrate as detected by the sensitive mass spectrometric assay. Nitric oxide has been established as the sole product of nitrite reductase in P. denitrificans as discussed in a later section. Both nitric oxide and nitrous oxide thus appear to occur as normal but minor products of nitrate reduction in this bacterium.

II. EFFECT OF CULTURE CONDITIONS ON THE ELECTRON TRANSPORT SYSTEM.

The organism was observed to grow well with either oxygen or nitrate as the terminal electron acceptor. Growth in air gave about 25 per cent greater yield of cells than did anaerobic growth with nitrate.

It has been shown that carbohydrate metabolism during denitrification differs little from that occurring during aerobic growth (Spangler and Gilmour, 1966). Thus if we assume complete combustion of glucose in nitrate respiration, the energy yield to be expected from nitrate respiration and oxygen respiration are as shown in the following equations (Takahashi, Taniguchi, and Egami, 1963):

Nitrate respiration:

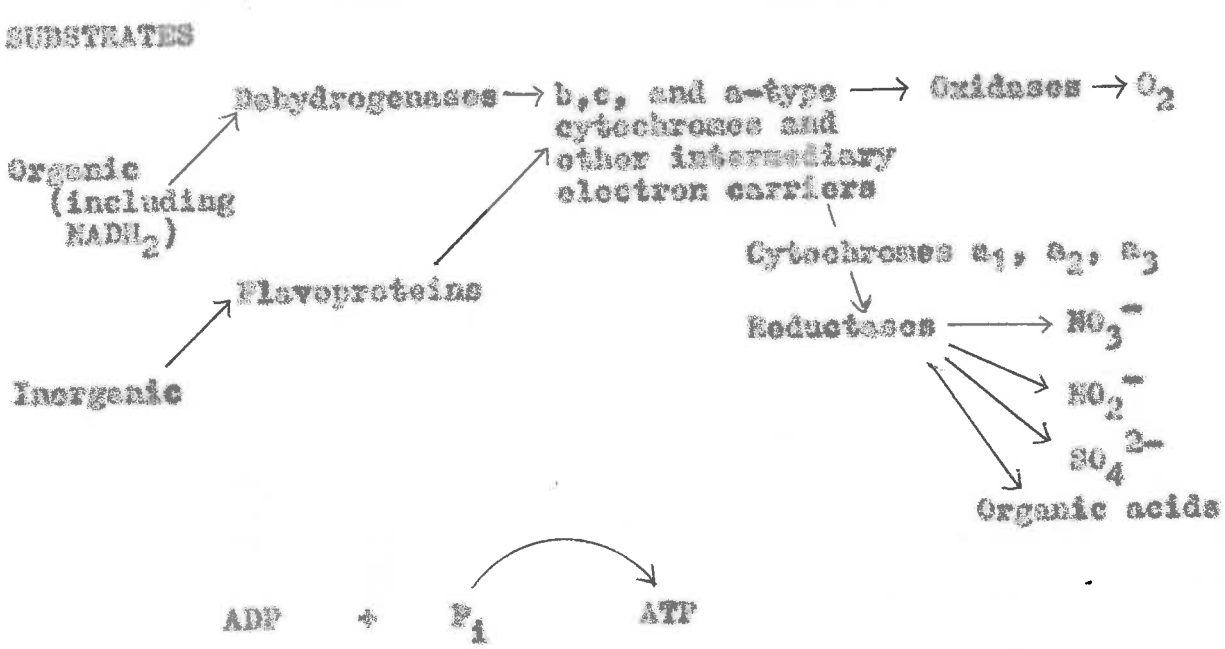
$$C_6H_{12}O_6 + 12KNO_3 \rightarrow 6CO_2 + 6H_2O + 12KNO_2 + 422 \text{ kcal.}$$

Oxygen respiration:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O + 674 \text{ kcal.}$$

A lower yield of cells can be therefore expected from growth utilizing nitrate respiration. The decreased yield from cultures grown anaerobically on nitrate is also in line with the preference observed in denitrifiers for oxygen respiration over nitrate respiration (Chang and Morris, 1962; Skerman and MacRae, 1961).

It is of interest to observe the effect of the terminal electron acceptor on the composition of the electron transfer chain. The electron transfer system in cells grown under either anaerobic or aerobic conditions was bound to the cell membrane fraction and contained cytochromes, flavin, and quinone as intermediary electron carriers. This observation is in agreement with those in other bacteria. Smith (1968) gives the following generalized scheme for electron transport and phosphorylation in bacteria:



These enzymes and carriers are bound to membraneous structures in such a way that electron and energy transfer reactions occur rapidly.

In common with many other bacteria (Smith, 1968) a number of cytochrome components are contained in the cell membranes. Cytochromes of the a, b, c, and o types have been observed in difference spectra of whole cells and their extracts. Comparison of the difference spectra of cell membrane preparations showed that the electron transport systems present in aerobically and anaerobically grown cells differed in composition both qualitatively and quantitatively. Cytochrome a was present in cells grown in air, but could not be detected in nitrate-grown cells. Total cytochrome content was increased about 30 per cent when cells were grown on nitrate. Cytochrome b and cytochrome c increased by 70 per cent and 20 per cent respectively in cells grown anaerobically with nitrate. Flavoprotein content was also increased by anaerobic growth on nitrate, while quinone content was about the same in cells grown with or without air.

In the mammalian respiratory chain the cytochrome content is constant in proportions of about 1 : 1 (Klingenberg, 1968). The bacterial electron transport system appears to be less rigidly organized and changes in the composition of the electron transfer chain accompanying variations in growth conditions have been reported in other microorganisms. In contrast to the results for P. denitri-

ficans, oxygen is necessary for the production of cytochromes in some facultative microorganisms. The cytochrome oxidase and cytochrome c contents of anaerobically grown baker's yeast are very low (Dolin, 1961). In Bacillus subtilis (Downey, 1964) and Pasteurella pestis (Englesberg, Levy, and Gibor, 1954) cytochrome content was depressed by anaerobic conditions. Schaeffer (1952) observed that in aerobically grown Bacillus cereus, cytochromes a, b, and c were present while in anaerobic cells, cytochrome b was reduced to 1/30 of the amount in cells grown in air, and cytochromes a and c were completely absent. The biosynthesis of cytochromes is depressed in anaerobic cultures of Staphylococcus epidermidis. Aerobically grown cells contain cytochromes a, b_1, and c while those grown without air contain only traces of cytochromes b_1 and c (Jacobs and Conti, 1965). Staph. epidermidis cells grown in air reduced nitrate, while those grown anaerobically did not, even though nitrate reductase itself was present and was active with artificial electron donors such as reduced benzyl viologen (Jacobs, Johantgen, and Deibel, 1963). This observation was explained by the hypothesis that cytochrome carriers are necessary for nitrate reduction. Added hemin stimulated cytochrome production and also the ability to reduce nitrate. Exogenous hemin had no effect

however on nitrate reduction by P. denitrificans, Esch. coli, Corynebacterium diphtheriæ, or Bacillus polymyxa (Jacobs et al., 1964). Bacillus stearothermophilus grown on nitrate contains less cytochrome oxidase than when grown aerobically (Downey, 1966).

In other microorganisms anaerobic growth leads to an increase in cytochrome content. White (1962) reported that cytochrome content in Hemophilus parainfluenzae was increased when grown anaerobically on nitrate. In P. denitrificans total cytochrome, cytochrome b and cytochrome a were increased by anaerobic growth on nitrate (Ferra and Lascelles, 1965; Lam, 1969).

High concentrations of ubiquinone were found in cell-free extracts of P. denitrificans. This observation is in agreement with that of Page et al. (1960) who found 1.20 μmole of ubiquinone/gm dry weight of aerobically grown cells which they identified as ubiquinone Q_{10}.

Little difference was observed in the ubiquinone content of cells grown aerobically or anaerobically with nitrate. This is in agreement with observations on P. aeruginosa, Proteus vulgaris and some strains of Esch. coli in which ubiquinone content was about the same whether the cells were grown with or without air (Bishop, Pandya, and King, 1962). In other microorganisms such as Saccharomyces

cerevisiae and Esch. coli B4, ubiquinone content is suppressed during anaerobic growth (Lester and Crane, 1959).

Bacteria may thus be divided into two groups: (a) those which when grown anaerobically switch to a cytochrome independent system of energy metabolism, and (b) those which when grown anaerobically on nitrate utilise the same sort of cytochrome containing electron transport system that is present in aerobic metabolism, and indeed synthesise larger amounts of these carriers. Nitrate then appears to serve in P. denitrificans as the terminal acceptor for the same sort of electron transfer chain that occurs in aerobically grown cells, and the organism may therefore be placed in group (b).

Cytochrome b content of the cells grown anaerobically on nitrate was increased 70 per cent while cytochrome c was increased only 20 per cent. It has been suggested that in some microorganisms cytochrome c is involved in nitrite reduction (Cole, 1968; Lam, 1969), and in these cases the cytochrome content of cells grown with nitrate was greatly increased. In M. denitrificans grown anaerobically, for example, cytochrome c content was more than double that found in aerobically grown cells (Lam and Nicholas, 1969). The conclusion that cytochromes are not involved in nitrite reduction in P. denitrificans, which is discussed more

fully later, is supported by the relatively small increase in cytochrome *g* content observed when the organism is grown anaerobically on nitrate.

The level of $NADH_2$-nitrate reductase activity in cells grown with oxygen as terminal acceptor in the absence of nitrate was reduced to less than one-sixth of that in cells grown anaerobically on nitrate. Formate-nitrate reductase activity was reduced to one-ninth of that in anaerobically grown cells. Formate dehydrogenase activity was reduced to very low levels in aerobically grown cells.

Nitrate did not appear to be required for the induction of nitrate reductase although its production was strongly repressed by oxygen. *Esch. coli* K-12 exhibited a similar response to oxygen (Showe and DeMoss, 1968). Synthesis of nitrate reductase was initiated by anaerobic conditions in the presence or absence of nitrate, and was repressed by oxygen. Specific activity of nitrate reductase in anaerobic cultures was about six-times higher in the presence than in the absence of nitrate. Since *P. denitrificans* cannot grow anaerobically without nitrate, it was not possible to investigate the effect of anaerobiosis in the absence of nitrate on the level of nitrate reductase activity. The regulation of nitrate reductase synthesis has been suggested to depend upon redox-sensitive repressors.

Wimpenny and Cole (1967) also suggested that regulation of nitrate reductase production was more dependent on the oxidation-reduction potential of the medium than on a specific inducer. It has been established that aerated cultures are not necessarily completely saturated with oxygen (Sherman and MacRae, 1961) and the requirements for full respiration by all the cells may not be fulfilled. Thus the observation that the synthesis of nitrate reductase in P. denitrificans is repressed, but not completely eliminated, by aerobic growth in medium without nitrate can be accounted for by its partial repression by oxygen. Synthesis of the enzyme appears to be dependent upon redox potential as well as on a specific inducer.

Aerobically grown cells contained little formate dehydrogenase activity, while high activity was found in cells grown anaerobically on nitrate. Formate was an effective electron donor for nitrate reduction by cell-membrane preparations, and its physiological significance will be discussed more fully in a later section.

Succinate oxidising activity in cell-membrane preparations was very low in nitrate grown cells, while oxidation of succinate proceeded at a rapid rate in extracts of oxygen-grown cells. In this respect, it is of interest that succinate did not function as an electron donor for nitrate

reduction in nitrate grown cells. This is in contrast with particulate preparations from nitrate grown cultures of M. denitrificans that oxidise succinate utilising either oxygen or nitrate as terminal acceptor (Lam, 1969).

The respiratory chain found in the cell-membrane fraction resembled the mammalian respiratory chain (Klingenberg, 1963) in that oxidation of $NADH_2$ was inhibited by amytal, rotenone, antimycin A, and HOQNO. The sensitivity of bacterial respiratory systems to these inhibitors varies from species to species. Thus the respiration of cell-free extracts of Bacillus subtilis, Staphylococcus aureus, Esch. coli, and Proteus vulgaris was inhibited by HOQNO (Lightbown and Jackson, 1956). In Staph. aureus HOQNO blocked electron transfer after cytochrome b_1 (Jackson and Lightbown, 1958). White and Smith (1962, 1964) reported that HOQNO inhibited the oxidation of $NADH_2$ and formate in Hemophilus parainfluenzae between cytochromes b and c. Respiration of Rhodospirillum rubrum was also restricted by HOQNO.

Van Demark and Smith (1964) reported that antimycin A inhibited $NADH_2$ oxidation by Mycoplasma hominis. Oxidation of succinate and $NADH_2$ was inhibited by antimycin A in Rhodospirillum rubrum.

Amytal inhibited respiration by Mycoplasma hominis (Van Demark and Smith, 1964), Rhodospirillum rubrum (Taniguchi and Kamen, 1963), Staph. aureus (Taber and Morrison, 1964) and Hydrogenomonas ruhlandii (Packer, 1958).

In contrast, antimycin A had no effect on glucose oxidation in Acetobacter suboxydans (King and Cheldelin, 1957). Neither amytal, HOQNO, nor antimycin A inhibited the oxidation of ferrous iron by extracts of Ferrobacillus ferrooxidans (Blaylock and Kamen, 1963). Antimycin A had no effect on respiration by extracts of Staph. aureus (Taber and Morrison, 1964) or Azotobacter vinelandii (Brummer et al., 1957).

Although the addition of either nitrate or nitrite had no effect on the oxidation of $NADH_2$ as measured spectrophotometrically, the addition of nitrate, but not nitrite, inhibited oxygen uptake by the particles prepared from nitrate grown cells as assayed in the oxygen electrode. Nitrate has a similar effect on $NADH_2$ oxidation by cell-membrane fractions from P. denitrificans, but in that system nitrite also inhibits oxygen uptake (Lam and Nicholas, 1969). Nitrate appears to be able to compete successfully with oxygen as a terminal acceptor of electrons from the respiratory chain of P. denitrificans.

Experiments with ^{15}N-labelled nitrate, nitrite, and ammonia showed that P. denitrificans does not utilize these compounds as a source of nitrogen for cellular constituents. This observation confirms that of Daniels (1966) who reported that P. denitrificans did not grow on a basal medium containing ammonium chloride as the sole nitrogen source. In this respect, P. denitrificans differs from some other denitrifiers such as N. denitrificans which can assimilate nitrate, nitrite, or ammonia into cellular nitrogen (Lam, 1969).

III. NITRATE REDUCTASE.

The nitrate reducing system that utilises $NADH_2$ or formate as electron donor is localized in the cell-membrane fraction (P-144) from P. denitrificans that also contains the respiratory electron transfer chain. The association of the nitrate reducing system with the electron transfer apparatus of the cell is a characteristic feature of nitrate reductase of the respiratory type as observed in Esch. coli (Taniguchi et al., 1957), P. aeruginosa (Pearson and Nicholas, 1961b), and M. denitrificans (Lam, 1969). P. denitrificans conforms to the general pattern of localisation of respiratory nitrate reducing systems since breakage of the cells by ultrasonication failed to dissociate the nitrate reductase from the cell-membrane fraction. The next step, that of reducing nitrite, is catalyzed by a soluble protein as discussed in Section IV.

Suspensions of whole cells reduced nitrate to nitric oxide, nitrous oxide and nitrogen gas. When the particulate fraction (P-144) alone was used with $NADH_2$ as the donor no gaseous products were detected suggesting that nitrite was reduced only as far as a non-volative intermediate. One mole of nitrite was recovered per mole of nitrate reduced thus confirming that the particles are capable of reducing system in P. denitrificans differs from that of

M. denitrificans which produces traces of gaseous products from nitrate. In the Micrococcus system stoichiometric recovery of nitrite was only observed in the purified, soluble preparations using reduced viologen dyes as the electron donors (Lam, 1969).

$NaBH_4$ and sodium formate were the most effective electron donors for nitrate reduction by the particulate preparation whereas succinate, lactate or $NaBH_2$ were relatively ineffective. Succino-oxidase activity was greatly depressed in cells grown anaerobically with nitrate, and this effect probably accounts for the lack of activity of succinate as an electron donor for nitrate reduction. This observation contrasts with observations on M. denitrificans (Lam, 1969) in which succinate is active as an electron donor for nitrate reduction.

Formate is an efficient electron donor in Esch. coli (Taniguchi, Sato, and Egami, 1956; Taniguchi and Itagaki, 1959, 1960). The physiological significance of formate as an electron donor for nitrate reduction in Esch. coli has been established by Cole and Wimpenny (1968) who have shown that cell-free extracts can, in their system, produce formate from pyruvate. This reaction takes place by way of the phosphoroclastic reaction in which CO_2 acts as acceptor for the protons liberated from pyruvate, thus

forming formate. The reaction can be represented thus:

$$CH_3COCOOH + CoASH + H^+ \longrightarrow \text{Acetyl CoA} + HCOOH$$

Itagaki, Fujita, and Sato (1962) observed that cultures of Esch. coli grown in formate-nitrate medium contained high levels of formate dehydrogenase. When the cells were grown in the absence of nitrate, the formate dehydrogenase activity was low even in the presence of formate. The level of succinate dehydrogenase was less than ten per cent of the formate dehydrogenase in cells grown anaerobically in nitrate medium.

In P. denitrificans, formate dehydrogenase activity was increased 9-fold by growing the cells anaerobically in nitrate medium while the succinate dehydrogenase activity was depressed to an undetectable level. Although it was not possible to demonstrate the presence of the metabolic pathway from pyruvate to formate in P. denitrificans, it is known to be widely distributed in anaerobically grown microorganisms (Cole and Wimpenny, 1968; Shuster and Lynen, 1960; Hoening, 1963). It has been observed that in some microorganisms such as Streptococcus faecalis this system is extremely labile and sensitive to oxygen (Wood, 1966). It is possible that despite all precautions taken to exclude oxygen the formate-pyruvate exchange system was denatured during the preparation of the extracts. The

observation that oxygen inhibits formate-nitrate reductase while having little effect on $NADH_2$-nitrate reductase suggests that oxygen may be detrimental to the functioning of the entire formate system.

The presence of an active formate dehydrogenase system in the cells grown anaerobically on nitrate and the much lower level of activity in cells grown on oxygen suggest that this system is an integral part of the nitrate respiration system and that formate is of physiological importance as a hydrogen donor for nitrate reduction *in vivo* in *P. denitrificans* as well as in *Esch. coli*.

Nitrate acts as an alternative acceptor to oxygen for electron transfer via the cytochrome chain. Antimycin A and HOQNO, inhibitors that block electron transport between cytochrome *b* and cytochrome *c* in animal mitochondria (Klingenberg, 1968) had no effect on nitrate reduction with either formate or $NADH_2$ as electron donor. However these compounds inhibited $NADH_2$ oxidation with oxygen as terminal acceptor. These observations suggest that nitrate reductase derives its electrons from the cytochrome chain at a site prior to cytochrome *c*.

Nitrate reduction with $NADH_2$ as the donor was not inhibited by oxygen, although adding nitrate to particles aerobically oxidizing $NADH_2$ decreased the rate of oxygen

uptake. When nitrate was added it was reduced to nitrite in amounts stoichiometric with the degree of inhibition of oxygen uptake. This observation also suggests that nitrate reductase derives its electrons at an earlier point in the electron transfer chain than does cytochrome oxidase and is therefore able to deplete the reducing equivalents available to oxygen.

Nitrate reduction with $NADH_2$ as the electron donor was inhibited by mepacrine, amytal, Piericidin A, and rotenone. When formate was the electron donor, mepacrine inhibited to the same extent as when $NADH_2$ was the donor. Piericidin A and amytal restricted nitrate reduction to a lesser extent than when $NADH_2$ was the donor and rotenone was without effect. Amytal and rotenone inhibit at the $NADH_2$ dehydrogenase level of the electron transfer chain, and mepacrine acts as a flavin antagonist (Klingenberg, 1968).

Metal chelating agents, especially dithiol, which binds molybdenum (Meriwether, Marzluff, and Hodgson, 1966), inhibited nitrate reduction with either formate or $NADH_2$ as an electron donor. The participation of iron in nitrate reduction was implicated by the reduction in nitrate reductase activity in cells grown in iron-deficient medium.

The participation of a ubiquinone-type carrier in the electron transfer chain is suggested by the inhibition of $NADH_2$-nitrate reductase by Piericidin A at 0.2 mM. This inhibition was reversed by about 30 per cent through adding ubiquinone Q_{10} at a concentration of 2 mM. Piericidin A has been shown to inhibit electron transfer at or near the ubiquinone site (Imai, Asano, and Sato, 1968). P. denitrificans contained a high level of ubiquinone (1.2 ug/mg protein) when grown with either oxygen or nitrate as the terminal acceptor. Downey (1962) has shown that irradiation of the particulate fraction with 360 nm light destroys Vitamin K-type electron carriers. This treatment had no effect on nitrate reductase activity nor on the level of ubiquinone in particles from P. denitrificans.

Figure 40. shows a possible sequence for electron transfer in nitrate reduction in P. denitrificans.

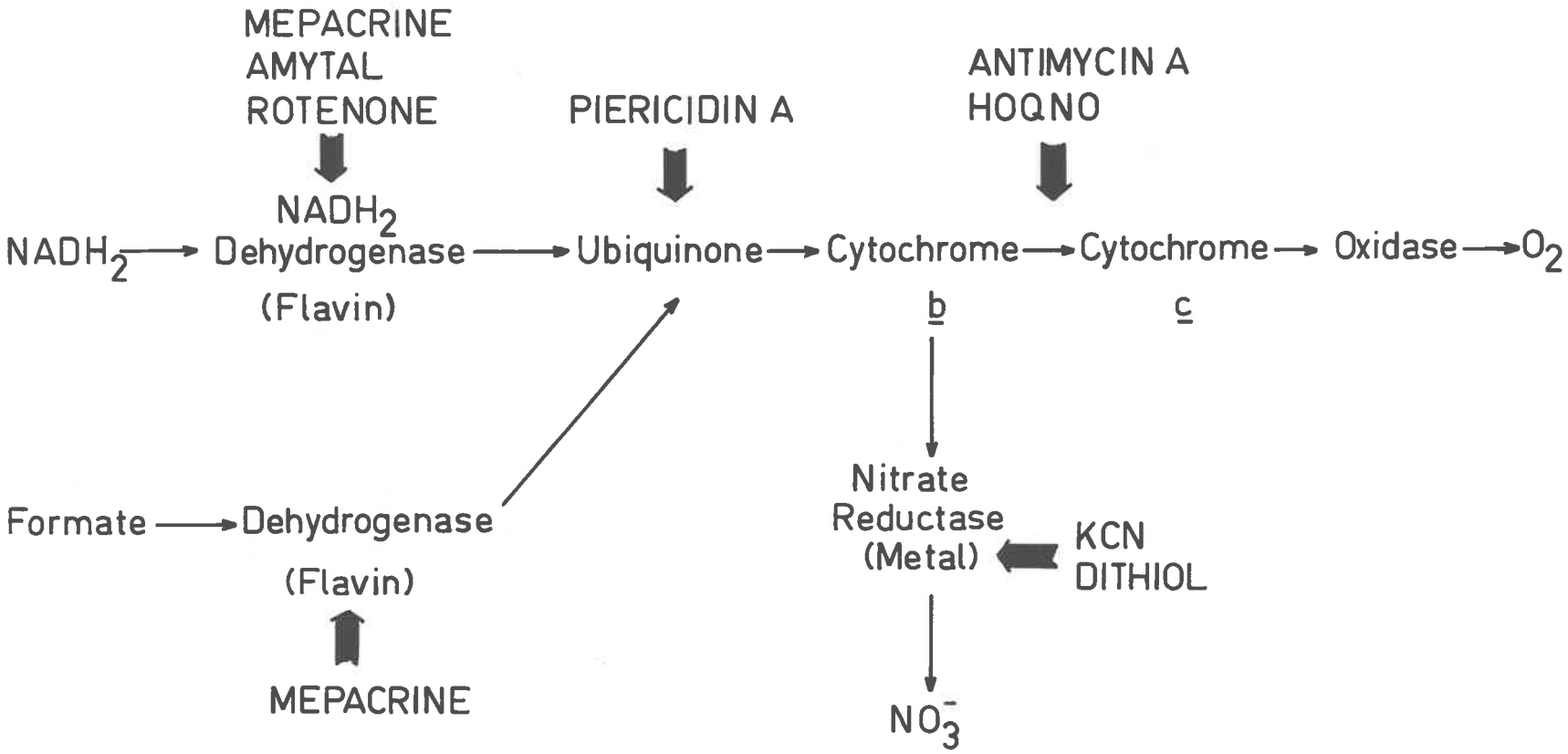
Reduced cytochrome b appears to be a physiological electron donor for nitrate reduction as indicated by the following observations:

(a) HOQNO and antimycin A, compounds that block electron transfer between cytochrome b and cytochrome c in animal mitochondria and some bacteria, inhibited $NADH_2$ oxidation by oxygen but not by nitrate.

FIGURE 40.

ELECTRON TRANSPORT SCHEME FOR NITRATE REDUCTION

Suggested electron transport scheme for the reduction of nitrate by formate or $NADH_2$ in P. denitrificans. Heavy arrows indicate sites of inhibitor action.


MEPACRINE
AMYTAL
ROTENONE
PIERICIDIN A
ANTIMYCIN A
HOQNO
NADH2
NADH2
Dehydrogenase
(Flavin)
Ubiquinone
Cytochrome
b
Cytochrome
c
Oxidase
O2
Formate
Dehydrogenase
(Flavin)
MEPACRINE
Nitrate
Reductase
(Metal)
KCN
DITHIOL
NO3-

(b) Ascorbate plus TMPD reduced cytochrome *c*, but failed to act as an electron donor for nitrate reduction in either the particulate or solubilized preparations.

(c) Cytochrome *b* level was increased 70 per cent by growth on nitrate, but cytochrome *c* content was little changed.

(d) Cytochrome *c* was lost upon purification of the soluble BVH-nitrate reductase which retained a cytochrome *b* type absorption spectrum.

The electron transfer scheme suggested for nitrate reduction in *P. denitrificans* is similar to that proposed by Taniguchi (1961) for nitrate reduction in *Esch. coli*. The *Esch. coli* system also contained flavin, and cytochrome b_1 was established as the penultimate electron carrier for the reduction of nitrate by $NADH_2$ or formate. The reduced cytochrome b_1 was oxidized either by nitrate reductase or by an oxidase in the presence of oxygen.

Downey (1966) found that nitrate reductase from *Bacillus stearothermophilus* transferred electrons from cytochrome b_1 to nitrate, and that cytochrome c_1 did not appear to be involved in nitrate reduction.

Cytochrome *b* has also been established as the penultimate electron carrier in respiratory nitrate reduction in *M. denitrificans* (Lam, 1969). In this organism

$NADH_2$ oxidation by oxygen was inhibited by antimycin A and HOQNO, while electron transfer to nitrate was unaffected. The rate of nitrate reduction by particles from this organism was also unaffected by nitrate, while the rate of oxygen uptake was decreased by adding nitrate to the reaction mixture.

In some other microorganisms, nitrate reductase was linked with cytochrome c. Pewsen and Nicholas (1961a, b) found that the nitrate reducing system from P. aeruginosa required cytochrome c for full activity and also suggested that cytochrome c was involved in nitrate reduction by M. denitrificans. A purified nitrate reductase from Achromobacter fischeri (Photobacterium sepia) exhibited a cytochrome c type absorption spectrum (Sadana and McElroy, 1957).

The rate of nitrate reduction by the particulate preparation was stimulated more than 4-fold by adding 2 mM menadione to the reaction mixture. This menadione activated nitrate reduction was unaffected by rotenone and it thus appears to provide a by-pass around the rotenone sensitive site in the electron transfer chain. Dicoumarol had no effect on the normal nitrite reducing system, but inhibited the menadione stimulated system by about 60 per cent at a concentration of 0.1 mM.

It has been shown (Klingenberg, 1968) that menadione can act as a bypass from the $NADH_2$ level of the electron transport chain to the cytochrome *b* level in animal mitochondria. In *Rsch. coli*, Heredia and Medina (1960) demonstrated that menadione could provide an additional pathway for the reduction of nitrate by $NADH_2$ separate from the usual electron transfer system which contained flavin and cytochrome b_1. This bypass system was also present in *P. denitrificans*.

It was observed that chlorate, perchlorate, bromate, and iodate inhibited nitrate reduction by both the particulate and the solubilised nitrate reductase preparations. Chlorate and bromate proved to be competitive inhibitors of $NADH_2$-nitrate reductase in the particles with K_i values of 1.7×10^{-3} M (chlorate) and 1.25×10^{-3} M (bromate). Chlorate and bromate acted as acceptors for the formate dehydrogenase system in the particles. The Michaelis constant for chlorate in this system was calculated to be 2.8×10^{-3} M which is close to the value of the K_i of chlorate as an inhibitor of nitrate reduction. When chlorate was the acceptor for the formate dehydrogenase system, activity was completely inhibited by 0.17 mM dithiol. Potassium cyanide and o-phenanthroline, each at 5 mM, inhibited 92 per cent and 84 per cent respectively, while mepacrine (1 mM), PCMB (2 mM), and potassium

thiocyanate (5 mM) inhibited 40 per cent, 70 per cent and 30 per cent respectively. Antimycin A, BOQNO, or rotenone had no effect. These inhibitions are similar in pattern to those observed for nitrate reduction by the particulate preparation with formate as the electron donor. Chlorate also acted as a competitive inhibitor for nitrate reduction by the solubilized enzyme. A K_i value of 1.8×10^{-3} M was found.

The competitive nature of the inhibition by chlorate of nitrate reduction by either the particulate or the purified nitrate reductase suggest that chlorate may act as an alternative electron acceptor to nitrate. Other evidence supporting this hypothesis includes the similarity of K_m and K_i values for chlorate as an acceptor in the formate dehydrogenase system and as an inhibitor of the nitrate reductase system respectively, and also the similar pattern of inhibition for the formate to nitrite system and the formate to chlorate system.

Chlorate inhibition has been observed in a number of other bacterial nitrate reductases. Nackenthal and Nackenthal (1963) reported that nitrate reductase from Bacillus cereus was competitively inhibited by chlorate. Chlorate and perchlorate were shown to be alternative substrate for nitrate reductase (Nackenthal, 1965).

Stouthamer (1967) found that mutants of *Aerobacter aerogenes* lacking nitrate reductase were also unable to reduce chlorate. The nitrate reductase from *P. aeruginosa* was competitively inhibited by chlorate, and it was suggested that chlorate may act as an alternative substrate for the enzyme (Fewson and Nicholas, 1961b).

The nitrate reductase from *Esch. coli* was competitively inhibited by chlorate, bromate, and iodate. Chlorate and bromate acted as substrate for the enzyme (Taniguchi, 1961). Pichinoty (1964) reported that the nitrate reductase from *M. denitrificans* also reduced chlorate. Paull, Wallace, and Nicholas (1969) found that chlorate acted as a competitive inhibitor of the nitrate reductase from *Nitrobacter agilis*.

Nitrate reductase from *P. denitrificans* seems then to conform to a general pattern of being inhibited by chlorate and bromate which compete with nitrate for the active site of the enzyme.

IV. NITRITE REDUCTASE

It is generally considered that assimilatory-type nitrite and nitrite reductase systems consist of soluble proteins, while dissimilatory nitrate and nitrite reducing enzymes are bound to the cellular membranes that contain the respiratory electron transfer chain. These membrane structures are sedimented when subjected to high speed centrifugation.

The physical distribution of nitrite reductase in P. denitrificans was studied. In contrast to most dissimilatory nitrite reductase systems more than 90 per cent of the enzyme activity was found in the soluble fraction left after centrifuging the crude extracts at 144,000 x g for 2 hours. Subsequent centrifuging at 325,000 x g resulted in the sedimentation of only a small fraction (about 20 per cent) of the active protein.

It has been observed that some methods of breaking bacterial cells result in greater dissociation of the respiratory enzymes from the electron transport system than do others (Smith, 1968). Therefore different methods of cell-disruption were compared to determine if any difference in the distribution of the nitrite reductase could be observed. These methods included repeated freezing and thawing, ultrasonication and disruption in the French

pressure cell. No differences in enzyme distribution were observed between the harsher method of cell disruption by ultrasonication, and the two more gentle procedures, repeated freezing and thawing and the French press. This can be interpreted as indicating that the nitrite reducing system from this organism is only loosely associated with the cell-membranes, since harsh treatment is not required to separate it from the respiratory system.

It is interesting to compare this observation with the distribution of nitrite reductase from other denitrifying bacteria. In a halotolerant bacterium studied by Asano (1959), the nitrite reductase was particle-bound and was associated with cytochrome b_4. In Micrococcus denitrificans, Lam and Nicholas (1969) found that about 70 per cent of the nitrite reductase activity was in the supernatant fraction when the enzyme was assayed with reduced benzyl viologen or phenazine methosulfate as the electron donor. When $NADH_2$ was used as the hydrogen donor, however, activity was localised in the particulate fraction. This apparent anomaly can be explained by the fact that most of the $NADH_2$-oxidase activity is concentrated in the particulate fraction. Thus the apparent localization of enzyme activity follows from the $NADH_2$ donor system rather than the terminal nitrite reductase itself being

associated with the particles.

In the case of *P. denitrificans*, however, nitrite reductase activity was found in the soluble fraction with either $NADH_2$-FMN system or reduced benzyl viologen as the hydrogen donor. It appears therefore that in *P. denitrificans*, nitrate reductase is not associated with a particulate electron transfer system as it is in *M. denitrificans*.

Soluble nitrite reductases are usually associated with the assimilation of nitrate and nitrite into cell nitrogen via ammonia. The nitrite reductase from *N. crassa*, for example, is a soluble enzyme and reduces nitrite to ammonia which is incorporate into cellular constituents. No nitrite assimilation was observed, however, with the system either in studies on the incorporation of ^{15}N-labelled nitrite, nitrate and ammonia, or from growth experiments which showed that the organism did not grow on nitrate or nitrite as the sole source of nitrogen.

The actions of a number of inhibitors on nitrite reduction was studied. The participation of sulfhydryl groups was strongly suggested by the inhibitory action of PCMB on nitrite reduction when $NADH_2$ was the hydrogen donor; the degree of inhibition was reduced by including an equimolar amount of reduced glutathione in the reaction

mixture. Sulfhydryl groups are probably not involved in the reduction of nitrite itself, since sulfhydryl inhibitors had no effect on nitrite reduction when reduced benzyl viologen was the electron donor.

Enzyme activity was also restricted by metal chelating agents and organo-nitro-compounds. Metals have been implicated in the function of a number of nitrite reductases. Walker and Nicholas (1961) found iron and copper to be necessary for the reduction of nitrite by an enzyme from P. aeruginosa. In a cytochrome oxidase preparation with nitrite reductase activity from the same organism Yamanaka and Okunuki (1963b) did not detect copper, but two types of heme, a and a_3 were associated with the enzyme. Nitrite reductase from a halotolerant Micrococcus was inhibited by metal chelating compounds (Asano, 1959).

The strong inhibition of nitrite reductase from P. denitrificans by sodium diethyldithiocarbamate, cyanide, 2,2'-dipyridyl, o-phenanthroline and 8-hydroxylquinoline suggests that metals such as copper and iron may be involved in the action of the enzyme. Cyanide and sodium diethyldithiocarbamate inhibited most strongly.

Accordingly the effect on the enzyme of growing the cells in metal deficient media was determined. Cultures grown on iron deficient medium had decreased cell yield,

but nitrite reductase activity, expressed on a protein basis, was greater than in cells grown on a complete medium. Leuhoff, Nicholas, and Kaplan (1956) reported a similar phenomenon a Pseudomonas fluorescens; cells grown in iron-deficient medium at low oxygen tensions had an eleven-fold increase in nitrite reductase activity over those grown under similar conditions in complete medium. This observation was interpreted to mean that nitrite reduction in this organism did not require the activity of cytochromes. This interpretation is in agreement with the observation on the nitrite reductase reported here, in which cytochromes do not appear to be involved, and the specific activity of the nitrite reductase was increased in cells grown in iron deficient medium.

All attempts to produce copper deficient cultures failed. Cells grew equally well in the complete and low copper media, even when cultures were grown through numerous serial transfers in the deficient medium. This is probably a result of the extremely low levels of copper necessary for normal growth and metabolism.

Some metallo-enzymes can be easily dissociated into the apo-enzyme moiety and the metal prosthetic group by dialysis against metal chelating agents (Frieden, 1962). Accordingly the purified enzyme was dialysed against 5 ml

KCN or against sodium diethyldithiocarbamate (5 mM). Although activity was lost after dialysis against these inhibitors, reactivation occurred upon further dialysis against 0.1 M phosphate buffer (pH 7.0) as the inhibitor was removed. This indicates that the protein has a greater affinity for the metal, if present, than does the inhibitor. The method of Kubowitz (1938) for the removal of metal prosthetic groups by treatment of the reduced enzyme with KCN resulted in a 50 per cent loss of activity which could not be restored by dialysis to remove the KCN or by addition of metals to the treated enzyme.

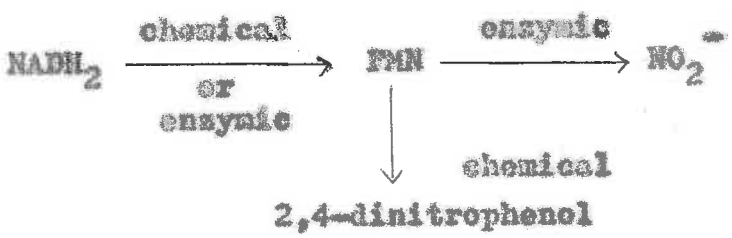
The nitrophenol compounds such as 2,4-dinitrophenol are well known as uncouplers of oxidative phosphorylation (Slater, 1963). The effect of aromatic nitro-compounds on nitrite reductase, however, could not be attributed to this property since nitrite reduction in P. denitrificans is not coupled to oxidative phosphorylation.

It was observed that the inhibition of the nitrite reduction by 2,4-dinitrophenol and p-dinitrobenzene was of the competitive type. It was considered that these compounds which compete with nitrite might themselves be reduced and such proved to be the case. A dissociable compound was formed from p-dinitrobenzene, and 2,4-dinitrophenol was reduced to a compound that was identified

as 2-amino-4-nitrophenol by thin-layer chromatography and comparison of ultraviolet absorption spectra.

This observation led to the speculation as to the possibility of the nitrite reducing enzyme also possessing "nitro-reductase" activity. This did not however prove to be the case since the production of amino-nitrophenol proceeded in the presence of FMN, $NADH_2$, and light, and in the absence of the enzyme. The chemical reduction of FMN by $NADH_2$ in light was observed. This confirms the report of Friedell and MacKenzie (1959). Under anaerobic conditions, the $FMNH_2$ formed was oxidized by 2,4-dinitrophenol. The reduction of 2,4-dinitrophenol was also observed in the presence of $FMNH_2$ which had been produced by reducing FMN with hydrogen and palladized asbestos.

Thus the inhibition was due to the chemical oxidation of $FMNH_2$ by 2,4-dinitrophenol in competition with the enzymic oxidation of the reduced nucleotide by nitrite reductase. These reaction may be schematically represented thus:



Similar observations have been reported by Merkel and Nickerson (1954) who observed that photoreduced riboflavin transferred its electrons to p-dinitrobenzo. Dinitrophenol and p-nitrobenzaldehyde inhibited the photoreduction of riboflavin; this observation could be due to the reoxidation of reduced riboflavin by these compounds. Reoxidation of photochemically reduced FMN by 2,4-dinitrophenol has also been observed by Hadda and Calvin (1963).

A number of the enzymic systems that reduce organonitro-compounds involve the action of flavin co-factors. For example, a nitro-reductase from a Nocardia species and one from Pseudomonas fluorescens that reduced nitrobenzoate to aminobenzoate with $NADH_2$ as electron donor were stimulated by FAD. These systems were described as "non-specific" (Cartwright and Cain, 1959). Dinitro-ortho-cresol was metabolized by a pseudomonad (N.C.I.B. 9771) to 2,3,5-trihydroxytoluene. The first intermediate was 3-amino-5-nitrocresol and reduction to these compounds was stimulated by $FADH_2$, Mn^{2+}, and Fe^{2+}.

Liver homogenates catalyzed the reduction of p-nitrobenzoic acid to p-aminobenzoic acid with $NADPH_2$ as electron donor. The addition of riboflavin, FMN, or FAD accelerated activity (Fouts and Brodie, 1956; Adamson et al., 1965). Flavoprotein was involved in the reduction of the nitro-

group of Furacin (5-nitro-2-furaldehyde semicarbazone) (Asnis, 1957). Sulfhydryl groups, a metal component and FAD were required for the reduction of m-dinitrobenzene to m-nitroaniline by an enzyme system from Neurospora (Nason, 1956).

Vessels (1965) suggested that nitro-groups may be reduced by the non-heme iron component of iron-flavoproteins rather than by a specific nitroreductase. The reduction of nitro-compounds has, in fact, been reported to be catalysed by several enzymes with other specificites. Tsukamura (1954) reported that L-amino acid oxidase or an $NADH_2$-cytochrome c reductase from Mycobacterium avium catalysed the reduction of picric to picramic acid.

Formic dehydrogenase, xanthine oxidase, and lactic dehydrogenase in the presence of redox dyes catalyzed the reduction of a variety of nitrophenol compounds (Grevillo and Stern, 1935). Hydrogenase and ferredoxin from Clostridium mediated the reduction of 2,4-dinitrophenol and amino-nitrophenol to diaminophenol (Del Campo et al., 1966). Kiese, Kessag and Schneider (1957) reported that purified metmyoglobin reductase and old yellow enzyme were each capable of reducing nitrosobenzene.

Competition for reducing power has been demonstrated in some systems. Naik and Nicholas (1966b) found that 2,4-dinitrophenol competed with benzyl viologen and methyl

viologen for reducing equivalents from $NADH_2$ in extracts of Azotobacter vinelandii. Ahmed and Morris (1967, 1968) also found that 2,4-dinitrophenol inhibited nitrite reduction in Ankistrodesmus braunii by means of a similar competition for reducing power.

Mason and Takahashi (1958) suggested that the nitroaryl compounds may, like methylene blue and dichlorophenolindophenol, be simply non-specific electron acceptors for flavoprotein systems such as xanthine oxidase, L-amino acid oxidase, cytochrome c reductase, diaphorases and a number of molybdenum-dependent enzymes.

The observations reported here support the view that 2,4-dinitrophenol acts as a non-specific acceptor. The 2,4-dinitrophenol acted as an acceptor for reduced FMN without the intervention of an enzyme. This chemical oxidation of $FMNH_2$ accounts for the apparent competitive inhibition of nitrite reduction by 2,4-dinitrophenol. Care must therefore be taken in the interpretation of observations that appear to be due to competitive inhibition by organo-nitro-compounds and of apparent enzymic nitro-reductase activity, since it appears that either effect can be due to non-specific chemical interactions of the organo-nitro-compound with reduced electron carriers and co-factors.

Studies were carried out to determine the product of the reaction catalyzed by the soluble nitrite reductase. Manometric experiments showed that a gas was formed during the reduction of nitrite. This gas was completely absorbed in alkaline sulfite or alkaline permanganate solutions which absorb nitric oxide. It was not absorbed by alkali alone. Mass spectrophotometric analysis of the gaseous products of nitrite reduction showed that the gas had a mass number of 30. This peak at mass 30 disappeared when alkaline sulfite or permanganate was included in a side-arm of the reaction vessel. It is unlikely that the mass 30 peak was a result of the dissociation of nitrous oxide since the ionization voltage was kept low. Control spectra of nitrous oxide showed no peaks of dissociation species at mass 30. These data indicate that the product of nitrite reduction was nitric oxide.

The production of nitric oxide from nitrite has been observed in several other denitrifying bacteria such as P. stutzeri (Najjar and Allen, 1954), Thiobacillus denitrificans (Baalsrud and Baalsrud, 1954), P. aeruginosa (Walker and Nicholas, 1961), and M. denitrificans (Lam, 1969. In contrast the halotolerant Micrococcus described by Asano (1959, 1960) reduced nitrite to nitrogen gas, while the nitrite reducing system from P. denitrificans described

by Iwasaki and Mori (1958) produced either nitrogen gas or nitrous oxide depending on the electron donor used.

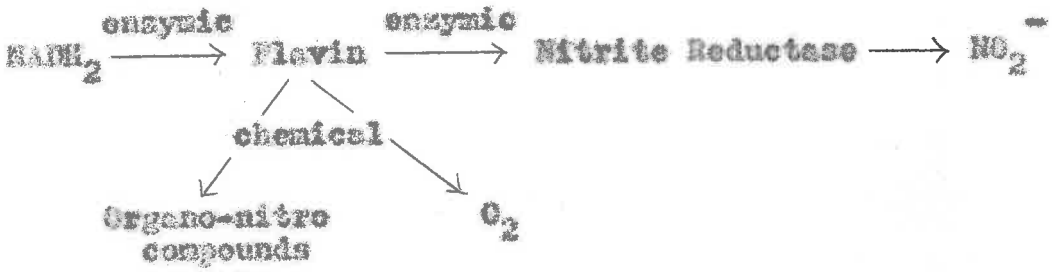
Experiments were carried out to determine if P. denitrificans were capable of growth with nitrite as the sole electron acceptor. No growth occurred anaerobically in 0.1 per cent (w/v) sodium nitrite, even with very long incubation periods to allow for the long lag phase that has been reported in some other organisms for growth on nitrite (Lam, 1969). It seems unlikely that this was due to the toxic effect of nitrite on the cells since high concentrations of nitrite (up to 0.2 per cent (w/v) $NaNO_2$) had no effect on aerobic growth; nitrite also accumulates in the medium up to a level of 9 mM when the cells are grown anaerobically on nitrate without affecting the growth rate.

Naik and Nicholas (1966a) showed that oxidative phosphorylation was coupled to both nitrate and nitrite reduction in M. denitrificans, but only to nitrate reduction in P. denitrificans. This observation in M. denitrificans was extended by Lam (1969) who showed that in this organism similar P/2e ratios of 0.7 were found with either nitrate or nitrite as the terminal hydrogen acceptor. M. denitrificans is capable of anaerobic growth on nitrite which is consistent with the above observations.

The failure of *P. denitrificans* to grow anaerobically with nitrite as the terminal acceptor can be explained by its inability to carry out oxidative phosphorylation concomitant with nitrite reduction. This conclusion is also supported by the soluble nature of the enzyme which does not appear to be associated with the cell membrane fraction that contains the electron transfer chain with its attendant oxidative phosphorylation activity.

Nitrite reductase activity was completely inhibited in air. This property is in contrast with the nitrate reductase from the same organism which is only slightly inhibited by air when $NADH_2$ is the hydrogen donor. The complete inhibition by oxygen may be attributed to the auto-oxidizability of reduced flavins. In the presence of air, $FMNH_2$ would be oxidized to FMN, thereby depriving the nitrite reductase of its reducing equivalents.

The data presented herein establishes that particle-bound cytochromes do not appear to participate in electron transfer to nitrite in *P. denitrificans*. The donor system appears instead to involve the action of a flavin, and a metal appears to be involved in the penultimate electron transfer. The following scheme is proposed:



This electron transfer scheme contrasts with that found in P. aeruginosa (Walker and Nicholas, 1961; Yamanaka, 1964) or M. denitrificans (Lee, 1969).

A comparison of the nitrite metabolism of the nitrate reducing species, Esch. coli, P. denitrificans, and M. denitrificans is of interest. Certain strains of Esch. coli can carry out dissimilatory nitrate reduction, but cannot reduce nitrite; these strains can also grow by fermentative pathways. P. denitrificans can grow anaerobically on nitrate but not on nitrite although nitrite is reduced. M. denitrificans can grow with either nitrate or nitrite as the terminal electron acceptor. Neither P. denitrificans nor M. denitrificans can grow fermentatively. The nitrite metabolism of P. denitrificans may be regarded as an intermediate form between that of the Esch. coli strains that cannot reduce nitrite and that of M. denitrificans in which nitrite can act as terminal electron acceptor. Although the nitrite reducing system of P. denitrificans contributes nothing to the energy metabolism of the cells,

it removes nitrite which can be toxic when present in the medium in high concentrations.

It is possible that two separate enzymes which together constitute nitrite reductase activity are being fractionated together; one a diaphorase-like enzyme that oxidizes $NADH_2$ and reduces FMN, and the second the nitrite reductase itself that utilizes reduced FMN or dyes as the electron donor.

Such a two-enzyme system has been found in spinach (Spinacea oleracea) and maize (Zea mays) by Jay and Hageman (1966). The nitrite reducing systems of these plants consisted of a diaphorase with ferredoxin-$NADPH_2$-reductase activity and the nitrite reductase enzyme itself.

The absolute requirement for the addition of exogenous flavin to the cell-free extracts for nitrite reducing activity might point to such a system operating in P. denitrificans. However a steady decrease in $NADH_2$-oxidase activity with or without added FMN accompanied the increase in $NADH_2$-nitrite reductase activity during purification.

Since the $NADH_2$-oxidase activity and $NADH_2$-nitrite reductase activity do not concentrate together, it appears that the hypothesis that two separate enzymes may be involved is invalid.

A nitrite reducing system has been described by

Iwasaki *et al.* (1963) from a gram-negative denitrifying soil bacterium which was tentatively identified as a strain of *P. denitrificans*. An amine-nitrite reaction was postulated as an important step in denitrification by this organism (Iwasaki and Mori, 1958). Dimethyl-*p*-phenylene diamine and hydroxylamine were both able to function as amine-donors. When dimethyl-*p*-phenylene was the donor, nitrogen gas was produced, while with hydroxylamine as donor, nitrous oxide was the reaction product. The authors postulated that a dismutation-type reaction was involved. With hydroxylamine, the following reaction could occur:

$$HNO_2 + NH_2OH \longrightarrow N_2O + 2H_2O$$

The results of the Japanese workers differ in several important ways from the data presented herein. The soluble nitrite reductase found in the preparation described here reduced nitrite stoichiometrically to nitric oxide, a one electron change, while the enzyme described by the Japanese workers catalysed a two-electron dismutation reaction in which the hydroxylamine-nitrogen donates two electrons and the nitrite-nitrogen gains two electrons. The hydroxylamine-nitrite reductase was assayed in the presence of 5×10^{-2} M hydroxylamine. It was also reported, however, that a 20 minute exposure of the enzyme to 5×10^{-2} M hydroxylamine resulted in its inactivation, but that

nitrite protected the enzyme against this inactivation (Iwasaki and Mori, 1938). Lower concentrations of hydroxylamine apparently had no effect. In the case of the enzyme described herein, however, hydroxylamine was not only ineffective as an electron donor, but also inhibited nitrite reduction at a concentration of 5×10^{-3} M, i.e. one-tenth the level employed as an electron donor in the hydroxylamine nitrite reductase system.

Similarities between the two systems include their common sensitivity to inhibition by cyanide and sodium diethyldithiocarbamate. The metal thereby implicated could be partially removed from the hydroxylamine-nitrite reductase by dialysis against cyanide. The lost activity was restored by adding 10^{-6} M copper to the enzyme (Suzuki and Iwasaki, 1962). In the soluble $NADH_2$-nitrite reducing system described in this thesis, however, extensive dialysis against cyanide or sodium diethyldithiocarbamate failed to remove the metal component since no inhibition was observed after the chelating agent had been removed by dialysis. Treatment of the reduced enzyme with cyanide according to the procedure of Kubowitz (1938) did however result in loss of activity after the KCN had been removed by dialysis. A 50 per cent loss in activity with $NADH_2$-FMN as the electron donor or 20 per cent loss with reduced

benzyl viologen was observed. The addition of exogenous metals including copper failed to reactivate the enzyme. These observations indicate that participation of a metal component which is tightly bound to the apo-enzyme and requires drastic treatment to ensure its dissociation.

It is interesting to consider the physiological significance of the hydroxylamine-nitrite reductase. Although hydroxylamine is considered to be an intermediate in assimilatory nitrate metabolism, it is unlikely to occur in high concentrations in the free state *in vivo* due to its biological toxicity. Takahashi, Taniguchi, and Egami (1963) showed that it formed complexes with heme groups.

The scheme postulated for the functioning of the hydroxylamine-nitrite reductase requires the reduction of part of the nitrite-nitrogen to the -1 oxidation level in order to reduce the remainder to the +1 level (Iwasaki and Mori, 1958). The soluble nitrite reductase system described herein that reduces nitrite to nitric oxide using the more usual $NADH_2$ as the electron donor seems to be a more likely candidate for the role of physiological nitrite reduction in denitrification process.

BIBLIOGRAPHY

Adamson, R.H., R.L. Dixon, F.L. Francis, and D.P. Hall (1965). Proc. Natl. Acad. Sci. (U.S.) 54, 1386-1391.

Ahmed, J. and I. Morris (1967). Arch. Mikrobiol. 56, 219-234.

Ahmed, J. and I. Morris (1968). Biochim. Biophys. Acta 162, 32-38.

Allen, M.B. and C.B. van Niel (1952). J. Bact. 64, 397-412.

Anderson, J.H. (1965). Biochem. J. 94, 236-239.

Asano, A. (1959). J. Biochem. (Tokyo) 46, 781-790.

Asano, A. (1960). J. Biochem. (Tokyo) 47, 678-684.

Asano, A. and A.F. Brodie (1964). J. Biol. Chem. 239, 4280-4291.

Asnis, R.E. (1957). Arch. Biochem. Biophys. 66, 208-216.

Baalsrud, S. and K.S. Baalsrud (1954). Arch. Mikrobiol. 20, 34-62.

Ballentine, R. (1957). In Methods in Enzymology, Vol. 3, p. 984-995. Ed. by Colowick, S.P. and N.O. Kaplan (New York : Academic Press).

Bar-Akiva, A. and J. Sternbaum (1966). Physiologica Plantarum 19, 422-428.

Betts, G.F. and E.J. Hewitt (1966). Nature (London) 210, 1327-1329.

Bishop, D.H.L., K.P. Pandya, and H.K. King (1962). Biochem. J. 83, 606-614.

Blaylock, B.A. and A. Nason (1963). J. Biol. Chem. 238, 3453-3462.

Bovell, C. (1967). Arch. Mikrobiol. 58, 13-19.

Bruemmer, J.H., P.W. Wilson, J.L. Glenn, and P.L. Crane (1957). J. Bacteriol. 73, 113-116.

Candela, N.I., S.G. Fisher, and E.J. Hewitt (1957). Plant Physiol. 32, 280-286.

Cartwright, N.J. and R.B. Cain (1959). Biochem. J. 73, 305-314.

Chang, J.P. and J.G. Morris (1962). J. Gen. Microbiol. 22, 301-310.

Chung, C.V. and V.A. Najjar (1956a). J. Biol. Chem. 218, 617-625.

Chung, C.V. and V.A. Najjar (1956b). J. Biol. Chem. 218, 627-632.

Cole, J.A. (1968). Biochim. Biophys. Acta 162, 356-368.

Cole, J.A. and J.W.T. Wimpenny (1968). Biochim. Biophys. Acta 162, 39-48.

Conn, E.E. and P.K. Stumpf (1963). Outlines of Biochemistry, p. 308 (New York : John Wiley and Sons).

Crane, F.L. (1965). In Biochemistry of Quinones, p. 183-206. Ed. by Morton, R.A. (New York : Academic Press).

Crane, F.L. and R.A. Dilley (1963). Methods of Biochemical Analysis 11, 279-306.

Craven, E. (1931). J. Chem. Soc. 1931, 1603-1606.

Daniels, M.J. (1966). Canad. J. Microbiol. 12, 1095-1098.

Del Campo, F.F., J.M. Ramirez, A. Paneque and M. Losada (1966). Biochim. Biophys. Acta 22, 547-553.

Delwiche, C.C. (1959). J. Bacteriol. 77, 55-59.

Dixon, M. and E.C. Webb (1964). Enzymes, 2nd ed. p. 75-80 (London : Longmans, Green and Co.).

Dolin, M.I. (1961). In the Bacteria, vol. 2, p. 319-363. Ed. by Gunsalus, I.C. and R.Y. Stanier (New York : Academic Press).

Downey, R.J. (1962). J. Bacteriol. 84, 953-960.

Downey, R.J. (1964). J. Bacteriol. 88, 904-911.

Downey, R.J. (1966). J. Bacteriol. 91, 634-641.

Durham, N.N. (1958). Canad. J. Microbiol. 4, 141-148.

Egami, F., M. Mota and H. Sato (1951). Nature (London) 167, 118-119.

Egami, F., K. Ohnishi, K. Lida, and S. Taniguchi (1957). Biokhimiya 22, 122.

Englesberg, E., J.B. Levy, and A. Gibor (1954). J. Bacteriol. 68, 178-185.

Evans, H.J. and C. McAuliffe (1956). In Symposium on Inorganic Nitrogen Metabolism, p. 189-197. Ed. by McElroy, W.D. and B. Glass (Baltimore: Johns Hopkins Press).

Faull, K.F., W. Wallace and D.J.D. Nicholas (1969). Biochem. J. in press.

Fewson, C.A. and D.J.D. Nicholas (1960). Nature (London) 188, 794-796.

Fewson, C.A. and D.J.D. Nicholas (1961a). Biochim. Biophys. Acta 48, 208-210.

Fewson, C.A. and D.J.D. Nicholas (1961b). Biochim. Biophys. Acta 49, 335-349.

Fewson, C.A. and D.J.D. Nicholas (1961c). Nature (London) 190, 2-7.

Fouts, J.R. and B.B. Brodie (1956). J. Pharmacol. Exptl. Therap. 119, 197-207.

Friedan, E. (1962). In Horizons in Biochemistry, p. 451-496. Ed. by Kasha, M. and B. Pullman (New York : Academic Press).

Frisell, W.R. and C.G. MacKenzie (1959). Proc. Natl. Acad. Sci. (U.S.) 45, 1568-1572.

Gayon, V. and G. Dupetit (1886). Mem. Soc. des Sciences Physique et Naturelle de Bordeaux, Series 3, 2, 201-207.

Germanier, R. and K. Wuhrmann (1963). Pathol. Microbiol. 26, 569-578.

Gray, C.T., J.W.T. Wimpenny, D.E. Hughes, and M. Ranlett (1963). Biochim. Biophys. Acta 67, 157-160.

Greville, G.D. and K.G. Stern (1935). Biochem. J. 29, 487-499.

Gunderson, K. and H.L. Jensen (1956). Acta Agriculture Scandinavica 6, 100-114.

Hackenthal, E. (1965). Biochem. Pharmacol. 14, 1313-1324.

Hackenthal, E. and R. Hackenthal (1965). Biochim. Biophys. Acta 107, 189-202.

Hadjipetrou, L.P. and A.H. Stouthamer (1965). J. Gen. Microbiol. 38, 29-34.

Hageman, R.H. and F. Flesher (1960). Plant Physiol. 35, 700-708.

Hanson, H.W. and E.E. Kalil'o (1957). Science 125, 1198-1199.

Hart, L.T., A.D. Larson, and C.S. McCloskey (1965). J. Bacteriol. 89, 1104-1108.

Hattori, A. and J. Myers (1966). Plant Physiol. 41, 1031-1036.

Hempfling, W.P., S. Steinberg, and E.V. Estabrook (1964). Int. Congr. Biochem. 5, 779.

Henning, U. (1963). Biochem. Zeitschrift 337, 490-504.

Heredia, C.F. and A. Medina (1960). Biochem. J. 77, 24-30.

Hooper, A.B. (1968). Biochim. Biophys. Acta 162, 49-65.

Imai, K., A. Asano, and R. Sato (1968). J. Biochem. (Tokyo) 63, 207-218.

Ingle, J., K.W. Joy, and R.H. Hageman (1966). Biochem. J. 100, 577-588.

Nagaki, E., T. Fujita, and R. Sato (1962). J. Biochem. (Tokyo) 52, 131-141.

Iwasaki, H. (1960). J. Biochem. (Tokyo) 47, 174-184.

Iwasaki, H. and T. Mori (1958). J. Biochem. (Tokyo) 45, 133-140.

Iwasaki, H., S. Shidara, H. Suzuki, and T. Mori (1963). J. Biochem. (Tokyo) 53, 299-303.

Jackson, F.L. and J.W. Lightbown (1958). Biochem. J. 69, 63-67.

Jacobs, N.J. and S.F. Conti (1965). J. Bacteriol. 89, 675-679.

Jacobs, N.J., R.E. Handy, J.M. Jacobs, K. Chan, and R.H. Doi bio (1964). J. Bacteriol. 87, 1406-1411.

Jacobs, N.J., J. Johantges, and R.H. Doi bio (1963). J. Bacteriol. 85, 782-787.

Jensen, H. (1904). Handbuch der Technischen Mykologie 111, 1-2. (Berlin : Lafar).

Joy, K.W. and R.H. Hageman (1966). Biochem. J. 100, 263-273.

Kateoura, T., K. Ito, T. Kojima, H. Nemoto and F. Egami (1954). J. Biochem. 41, 745-756.

Ke, T.-H., L.L. Gee, and N.N. Durham (1959). J. Bacteriol. 77, 593-598.

Kemp, J.D. and D.E. Atkinson (1966). J. Bacteriol. 92, 628-634.

Kemp, J.D., D.E. Atkinson, A. Ehret, and R.A. Lazzarini (1963). J. Biol. Chem. 238, 3466-3471.

Kessler, E. (1953). Flora 140, 1-38.

Kessler, E. (1956). Arch. Biochem. Biophys. 62, 241-242.

Kielly, H.K. (1956). In Symposium on Inorganic Nitrogen Metabolism, p. 205. Ed. by McElroy, W.D. and B. Glass (Baltimore : Johns Hopkins Press).

Kiese, M., E. Honag, and C. Schneider (1957). Arch. Exptl. Pathol. Pharmakol. 231, 170-175. (In Chem. Abstr. 51, 18039h (1957)).

King, T.E. and V.H. Cheldelin (1957). J. Biol. Chem. 224, 579-590.

Kinsky, S.C. and W.D. McElroy (1958). Arch. Biochem. Biophys. 73, 466-483.

Klingenberg, M. (1968). In Biological Oxidations, p. 3-54. Ed. by Singer, T.P. (New York : Interscience).

Kubowitz, F. (1938). Biochem. Zeitschrift 299, 32-57.

Lam, Y. (1969). Ph.D. Thesis, University of Adelaide.

Lam, Y. and D.J.D. Nicholas (1969). Biochim. Biophys. Acta 172, 450-461.

Lazzarini, R.A. and D.E. Atkinson (1961). J. Biol. Chem. 236, 3330-3339.

Lenhoff, H.M., D.J.D. Nicholas, and N.O. Kaplan (1956). J. Biol. Chem. 220, 983-995.

Lester, R.L. and F.L. Crane (1959). J. Biol. Chem. 234, 2169-2175.

Lightbown, J.W. and F.L. Jackson (1956). Biochem. J. 63, 130-137.

Linoweaver, H. and D. Burk (1934). J. Am. Chem. Soc. 56, 658-666.

Lowry, O.H., N.R. Roberts, and J.I. Kapphahn (1957). J. Biol. Chem. 224, 1047-1064.

Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall (1951). J. Biol. Chem. 193, 265-275.

McNall, E.G. and D.E. Atkinson (1956). J. Bacteriol. 72, 226-229.

Mechsner, K. and K. Wuhrmann (1963). Pathol. Microbiol. 26, 579-591. (In Chem. Abstr. 60, 1172b (1964)).

Medina, A. and D.J.D. Nicholas (1957). Biochim. Biophys. Acta 23, 440-442.

Meiklejohn, J. (1940). Ann. Applied Biol. 27, 558-573.

Meriwether, L.S., V.V. Marshuff, and W.G. Hodgson (1966). Nature (London) 212, 465-467.

Merkel, J.R. and W.J. Nickerson (1954). Biochim. Biophys. Acta 14, 303-311.

Naik, M.S. and D.J.D. Nicholas (1966a). Biochim. Biophys. Acta 113, 490-497.

Naik, M.S. and D.J.D. Nicholas (1966b). Biochim. Biophys. Acta 118, 195-197.

Najjar, V.A. and M.B. Allen (1954). J. Biol. Chem. 206, 209-214.

Nason, A. (1956). In Symposium on Inorganic Nitrogen Metabolism, p. 109-136. Ed. by McElroy, W.D. and B. Glass (Baltimore : Johns Hopkins Press).

Nason, A., H.G. Abraham, and B.C. Averbach (1954). Biochim. Biophys. Acta 15, 159-161.

Nason, A. and H.J. Evans (1953). J. Biol. Chem. 202, 655-673.

Nason, A. and H. Takahashi (1958). Ann. Rev. Microbiol. 12, 203-246.

Nicholas, D.J.D. (1963). Biological Reviews 38, 530-568.

Nicholas, D.J.D. (1966). Annals N.Y. Acad. Sciences 137, 217-231.

Nicholas, D.J.D., D.J. Fisher, W.J. Redmond, and N.A. Wright (1960). J. Gen. Microbiol. 22, 191-205.

Nicholas, D.J.D., A. Medina, G.T.G. Jones (1960). Biochim. Biophys. Acta 37, 468-476.

Nicholas, D.J.D. and A. Nason (1954). J. Biol. Chem. 207, 353-360.

Nicholas, D.J.D. and J.H. Sonvin (1956). Nature (London) 178, 1474-1475.

Ohnishi, T. and T. Mori (1960). J. Biochem. (Tokyo) 48, 406-411.

Ohnishi, T. and T. Mori (1962). Nature (London) 193, 482-483.

Ota, A. (1963). J. Biochem. (Tokyo) 58, 137-144.

Ota, A., T. Yamanaka, and K. Okunuki (1964). J. Biochem. (Tokyo) 55, 131-135.

Packer, L. (1958). Arch. Biochem. Biophys. 78, 54-65.

Page, A.C. Jr., P. Gale, H. Wallick, R.B. Walton, L.E. McDaniel, H.B. Woodruff, and K. Folkers (1960). Arch. Biochem. Biophys. 89, 318-321.

Paneque, A., F.F. Del Campo, J.M. Ramírez, and M. Losada (1965). Biochim. Biophys. Acta 109, 79-85.

Parker, V.H. (1952). Biochem. J. 51, 363-370.

Perkins, D.D. (1962). Canad. J. Microbiol. 8, 591-594.

Fichinoty, F. (1964). Biochim. Biophys. Acta 89, 378-381.

Fichinoty, F. (1965). Ann. Inst. Pasteur, Suppl. 1965 (3), 248-255.

Forre, R.J. and J. Lascelles (1965). Biochem. J. 94, 120-126.

Fumphrey, A.M. and E.E. Redfearn (1960). Biochem. J. 76, 61-64.

Quastel, J.H., M. Stephenson and M.D. Whetham (1925). Biochem. J. 19, 304-317.

Radda, G.K. and M. Calvin (1963). Nature (London) 200, 464-465.

Ramírez, J.M., F.F. Del Campo, A. Paneque, and M. Losada (1966). Biochim. Biophys. Acta 118, 58-71.

Sacks, L.E. and H.A. Barker (1949). J. Bact. 58, 11-22.

Sacks, L.E. and H.A. Barker (1952). J. Bact. 64, 247-252.

Sadana, J.C. and W.D. McElroy (1957). Arch. Biochem. Biophys. 67, 16-34.

Saz, A.K. and E.E. Slie (1953). J. Am. Chem. Soc. 75, 4626-4627.

Saz, A.K. and R.B. Slie (1954). Arch. Biochem. Biophys. 51, 5-16.

Schaeffer, P. (1952). Biochim. Biophys. Acta 9, 261-270.

Schmidt, B. and W.D. Kampf (1962). Arch. Hyg. Bakteriol. 146, 171-182.

Schrader, L.E., G.L. Ritenour, G.L. Eilrich and R.H. Hageman (1968). Plant Physiol. 43, 930-940.

Shove, M.K. and J.A. DeMoss (1968). J. Bacteriol. 95, 1305-1313.

Shuster, C.N. and F. Lynen (1960). Biochem. Biophys. Res. Comm. 3, 350-355.

Silver, W.S. (1957). J. Bacteriol. 73, 241-246.

Simpson, J.R. and W.C. Evans (1953). Biochem. J. 55, xxiv.

Skerman, V.B.D. and I.C. MacRae (1957). Canad. J. Microbiol. 3, 215-230.

Skerman, V.B.D. and I.C. MacRae (1961). Canad. J. Microbiol. 7, 169-174.

Slater, E.C. (1963). In Metabolic Inhibitors, vol. 2, p. 503-516. Ed. by Hochster, R.M. and J.H. Quastel (New York : Academic Press).

Smith, L. (1968). In Biological Oxidations, p. 53-122. Ed. by Singer, T.P. (New York : Interscience).

Smith, L. and D.C. White (1962). J. Biol. Chem. 237, 1337-1341.

Spangler, W.J. and C.H. Gilmour (1966). J. Bact. 91, 245-250.

Spencer, D., H. Takahashi, and A. Nason (1957). J. Bact. 73, 553-562.

Steinberg, R.A. (1956). In Symposium on Inorganic Nitrogen Metabolism, p. 153-158. Ed. by McElroy, W.D. and B. Glass (Baltimore : Johns Hopkins Press).

Stiller, M. (1966). Plant Physiol. 41, 348-352.

Stout, P.R. and D.I. Arnon (1939). Am. J. Botany 26, 144-149.

Stouthamer, A.H. (1967). Arch. Mikrobiol. 56, 68-75.

Stoy, V. (1956). Biochim. Biophys. Acta 21, 395-396.

Suzuki, H. and H. Iwasaki (1962). J. Biochem. (Tokyo) 52, 193-199.

Suzuki, H. and T. Mori (1962). J. Biochem. (Tokyo) 52, 190-192.

Tabak, H.H., C.V. Chambers, and P.W. Kabler (1961). Bacteriol. Proc. 1961, 60.

Taber, H.V. and M. Morrison (1964). Arch. Biochem. Biophys. 105, 367-379.

Takahashi, K., S. Taniguchi, and F. Egami (1963). In Comparative Biochemistry, vol. 5, p. 91-202. Ed. by Florkin, M. and H.S. Mason (New York : Academic Press).

Tang, P.-S. and H.-Y. Wu (1957). Nature (London) 179, 1355-1356.

Taniguchi, S. (1961). Zeitschrift Allgem. Mikrobiol. 1, 341-373.

Taniguchi, S., A. Asano, K. Iida, M. Kono, K. Ohnochi, and F. Egami (1957). Proc. Int. Symp. Enzyme Chem., Tokyo and Kyoto 2, 238-245.

Taniguchi, S. and E. Itagaki (1959). Biochim. Biophys. Acta 31, 294-295.

Taniguchi, S. and E. Itagaki (1960). Biochim. Biophys. Acta 44, 263-279.

Taniguchi, S. and M.D. Kamen (1965). Biochim. Biophys. Acta 96, 395-428.

Taniguchi, S., H. Mitsui, J. Toyada, T. Tamada and F. Egami (1953). J. Biochem. (Tokyo) 40, 175-183.

Taniguchi, S. and K. Ohmachi (1960). J. Biochem. (Tokyo) 48, 50-62.

Taniguchi, S., R. Sato, and F. Egami (1956). In Symposium on Inorganic Nitrogen Metabolism, p. 87-108. Ed. by McElroy, W.D. and B. Glass (Baltimore : Johns Hopkins Press).

Tewfik, M.S. and W.C. Evans (1966). Biochem. J. 99, 31P-32P.

Treadwell, F.P. and W.T. Hall (1942). Analytical Chemistry, 9th ed., vol. 2, p. 727 (New York : Wiley and Sons).

Tsukamura, M. (1954). Igaku to Seibutsugaku (Med. and Biol.) 33, 59-62 and 270-273. (In Chem. Abstr. 50, 10152b (1956)).

Vaidyanathan, C.S. and H.E. Street (1959). Nature (London) 184, 531-533.

Van Demark, P.J. and P.F. Smith (1964). J. Bacteriol. 88, 122-129.

Verhoeven, W. (1956). In Symposium on Inorganic Nitrogen Metabolism, p. 61-86. Ed. by McElroy, W.D. and B. Glass (Baltimore : Johns Hopkins Press).

Verhoeven, W. and J.J.C. Goos (1954). Antonie van Leeuwenhoek 20, 93-101.

Walker, G.C. and D.J.D. Nicholas (1961). Biochim. Biophys. Acta 49, 350-360.

Wallace, W. and D.J.D. Nicholas (1968). Biochem. J. 109, 763-773.

Walters, C.L. and A.McN. Taylor (1964). Biochim. Biophys. Acta 86, 448-458.

Walters, C.L., R.J. Casselden, and A.McN. Taylor (1967). Biochim. Biophys. Acta 143, 310-315.

Waring, W.S. and C.H. Werkman (1942). Arch. Biochem. Biophys. 1, 303-310.

Wessels, J.S.C. (1960). Biochim. Biophys. Acta 38, 195-196.

Wessels, J.S.C. (1965). Biochim. Biophys. Acta 102, 357-371.

White, D.C. (1962a). J. Bacteriol. 83, 851-859.

White, D.C. (1962b). J. Biol. Chem. 238, 3757-3761.

White, D.C. and L. Smith (1963). J. Biol. Chem. 237 1332-1336.

White, D.C. and L. Smith (1964). J. Biol. Chem. 239, 3956-3963.

Wijler, J. and C.C. Delwiche (1954). Plant and Soil 5, 155-169.

Wimpenny, J.W.T. (1968). Biochem. J. 106, 34P.

Wimpenny, J.W.T. and J.A. Cole (1967). Biochim. Biophys. Acta 148, 233-242.

Wimpenny, J.W.T. and A.H.H. Warmsley (1968). Biochim. Biophys. Acta 156, 297-303.

Wood, W.P. (1966). In Methods in Enzymology, vol. 9, p. 718-722. Ed. by Wood, W.A. (New York : Academic Press).

Yamagata, S. (1939). Acta Phytochim. Japan 11, 145-157.

Yamaneka, T. (1964). Nature (London) 204, 253-255.

Yamanaka, T. and K. Okunuki (1963a). Biochim. Biophys. Acta 67, 379-393.

Yamanaka, T. and K. Okunuki (1963b). Biochim. Biophys. Acta 67, 394-406.

Yamanaka, T., A. Ota and K. Okunuki (1960). Biochim. Biophys. Acta 44, 397-398.