# BIOCHEMICAL AND BIOENERGETIC ASPECTS OF DENITRIFICATION IN *RHODOPSEUDOMONAS SPHAEROIDES* FORMA SP. *DENITRIFICANS*

by

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# PREFACE

Part of the work described in this thesis has been presented in scientific meetings (Australian Biochemical Society, 1983, 1984, 1985). Some of the results have been published or submitted for publication in the journals listed below:

1). Purification and properties of a dissimilatory nitrite reductase from a phototrophic denitrifying bacterium.
B. Kundu and D.J.D.Nicholas.
*Proc. Aust. Biochem. Soc.* 15: 57 (1983).

2). Proton translocation during denitrification in *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*.
B. Kundu and D.J.D. Nicholas.
*Proc. Aust. Biochem. Soc.* 16: 21 (1984).

3). Proton motive force during nitrate reduction in a photodenitrifier, *Rhodopseudomonas sphaeroides* f. sp. *denitrificans*.
B. Kundu and D.J.D. Nicholas.
*Proc. Aust. Biochem. Soc.* 17: 73 (1985).

4). Proton translocation during denitrification in *Rhodopseudomonas sphaeroides* f. *denitrificans*.
B. Kundu and D.J.D. Nicholas.
*Arch. Microbiol.* 140: 358-364 (1985).

5). Denitrification in *Rhodopseudomonas sphaeroides* f. *denitrificans*.
B. Kundu and D.J.D. Nicholas.
*Arch. Microbiol.* 141: 57-62 (1985).

6). Effects of $Na^+$ on light-dependent proline uptake in washed cells of *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*.
B. Kundu and D.J.D. Nicholas.
*Biochem. Int.* 12: 215-223 (1986).

7). Demonstration of a $Na^+/H^+$ exchange activity in a phototrophic denitrifier, *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*.
B. Kundu and D.J.D. Nicholas.
*Biochem. Int.* 12: 403-412 (1986).

8). Demonstration of a $K^+/H^+$ exchange activity in a photodenitrifier, *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*.
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*FEMS Microbiol. Lett.* 34: 245-249 (1986).

9). Active transport of proline into washed cells of *Rhodopseudomonas sphaeroides* f. sp. *denitrificans*
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10). Proton motive force in a photodenitrifier, *Rhodopseudomonas sphaeroides* forma *specialis denitrificans*.
B. Kundu and D.J.D. Nicholas.
*Can. J. Biochem. Cell. Biol.* 64: 328-336 (1986).

11). $Na^+$ and $K^+$ transport in a photodenitrifier, *Rhodopseudomonas sphaeroides* forma *specialis denitrificans*.
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## DECLARATION

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

If accepted for the award of Ph.D. degree, this thesis will be available for loan and photocopying.

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# ABBREVIATIONS

The abbreviations for chemicals, symbols and units in general follow either the tentative rules of IUPAC-IUB Commission on Biochemical Nomenclature (*Biochem. J.* (1966) 101: 1-7) or the instruction to authors for the *Biochimica et Biophysica Acta* (*Biochim. Biophys. Acta* (1982) 715: 1-23.

## Chemicals

| | |
|---|---|
| ADP | adenosine 5'-diphosphate |
| ATP | adenosine 5'-triphosphate |
| ATPase | adenosine triphosphatase |
| BSA | bovine serum albumin |
| BPB | bromophenol blue |
| BV | benzyl viologen |
| BVH | benzyl viologen (reduced) |
| CCCP | carbonyl cyanide-m-chlorophenyl hydrazone |
| DBMIB | dibromo-methyl-isopropyl-*p*-benzoquinone |
| DCCD | N,N'dicyclohexylcarbodiimide |
| $DCPIP_{red}$ | dichorophenolindophenol (reduced) |
| DESB | diethylstilbestrol |
| DIECA | diethyl dithiocarbamate (sodium salt) |
| DNP | 2,4 dinitrophenol |
| EDTA | ethylenediamine tetra acetic acid |
| HEPES | 4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid |
| HOQNO | 2-n-heptyl-4-hydroxyquinoline-N-oxide |
| 8-HQ | 8-hydroxyquinoline |
| $NAD^+$ | nicotinamide adenine dinucleotide (oxidised) |
| NADH | nicotinamide adenine dinucleotide (reduced) |
| $NADP^+$ | nicotinamide adenine dinucleotide phosphate (oxidised) |
| NADPH | nicotinamide adenine dinucleotide phosphate (reduced) |
| NEM | N-ethylmaleimide |
| $N_2O$-R | nitrous oxide reductase |
| *p*CMB | *p*-chloromercuribenzoate |
| $PMS_{red}$ | phenazine methosulphate (reduced) |
| POPOP | 1,4-bis-[2,(4-methyl-5-phenyloxazolyl)]-benzene |
| PPO | 2,5-diphenyloxazole |
| SDS | sodium-dodecylsulphate |
| TCA | trichloroacetic acid |
| Tris | 2-amino-2-hydroxymethyl propane-1,3-diol. |
| $TPP^+$ | tetraphenyl phosphonium cation |

## Symbols and Units

| | |
|---|---|
| A | absorbance |
| amu | atomic mass unit |
| °C | degree centrigrade (celcius) |
| Ci | curie |
| cm | centimeter |
| $\Delta p$ or $\tilde{\mu}H^+$ | proton motive force or proton electrochemical gradient |
| $\Delta pH$ | transmembrane pH gradient |
| $\Delta\Psi$ | transmembrane potential difference (membrane potential) |

| | |
|---|---|
| F | free energy or Faraday's constant |
| g | gram or unit of gravitational field |
| h | hour(s) |
| $\rightarrow H^+/O$ | no. of protons translocated per $2e^-$ transferred to O |
| K | kilo |
| $K_m$ | Michaelis constant |
| L | litre |
| M | molar |
| m | meter |
| mCi | millicurie |
| mg | milligram |
| µCi | microcurie |
| min | minute(s) |
| ml | millilitre |
| mmol | millimole(s) |
| mM | millimolar |
| ug | microgram |
| ul | microlitre |
| umol | micromole(s) |
| uM | micromolar |
| N | normal |
| nm | nanometer |
| nmol | nanomole(s) |
| % | percent |
| $pH_{in}$ | intracellular pH |
| $pH_{out}$ | extracellular pH |
| R | gas constant |
| s | second(s) |
| S | substrate concentration |
| t | time |
| $t_{\frac{1}{2}}$ | half time |
| T | transmission or thermodynamic temperature (Kelvin) |
| v | volume |
| V | velocity of the reaction |
| $V_{max}$ | rate of enzyme catalyzed reaction at infinite concentration of substrate |
| wt | weight |

Others

| | |
|---|---|
| approx. | approximately |
| atm. | atmosphere |
| conc. | concentration |
| cpm | counts per minute |
| e.g. | for example |
| *et al.* | *et alia* (and others) |
| GC/MS | gas chromatography linked to mass-spectrometry |
| i.e. | that is |
| max. | maximum |
| min. | minimum |
| m-, p-, O- | meta-, para-, ortho- |
| No. | number |
| / | per |
| p. (plural pp.) | page |
| PAGE | polyacrylamide gel electrophoresis |
| soln. | solution |

| | |
|---|---|
| *viz.* | namely |
| vs. | versus |
| v/v | volume:volume |
| w/v | weight:volume |
| < | less than |
| ≤ | less than or equal to |
| > | greater than |
| ≥ | greater than or equal to |
| ≃ | approximately equal |

# TABLE OF CONTENTS

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# SUMMARY

## SUMMARY

1. This thesis embodies results of an investigation on some aspects of denitrification and energy conservation in a phototrophic bacterium, *Rhodopseudomonas sphaeroides* forma sp. *denitrificans*.

2. This photodenitrifier grows anaerobically with $NO_3^-$ as the terminal electron acceptor both in light and in the dark. In washed cells, the stoichiometry for the reduction of $^{15}NO_3^-$ or $^{15}NO_2^-$ to $^{15}N_2$ was 2:1 and that of $N_2O$ to $N_2$, 1:1. NO was not detected during denitrification of $NO_3^-$ and $NO_2^-$. The pathway of denitrification in this bacterium is as follows: $NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$.

3. Oxygen inhibited the *de novo* synthesis of the denitrifying enzymes (e.g. nitrate reductase, nitrite reductase and nitrous oxide reductase) as well as the activity of preformed enzymes in washed cells.

4. Nitrate reductase (E.C.1.9.6.1) of this bacterium is a molybdenum protein containing thiol groups. The purified enzyme was present in aggregated forms (70 Kd, 100 Kd and 112 Kd). This enzyme converted $NO_3^-$ to $NO_2^-$ with reduced benzyl viologen as the electron donor.

5. Nitrite reductase (E.C.1.7.2.1) was purified and shown to be a Cu-protein with a molecular weight of 82 Kd. It has two subunits, 40 Kd each. The enzyme contains two Cu atoms, one in each subunit. This enzyme reduced $^{15}NO_2^-$ to $^{15}N_2O$ with a 2:1 stoichiometry utilizing reduced viologen dyes as electron donors.

6. Oxidant pulse experiments were carried out with washed cells of the photodenitrifier. These cells actively translocated protons with $NO_3^-$, $NO_2^-$, $N_2O$ and $O_2$ respectively, as the terminal acceptor. Proton translocation was inhibited by carbonylcyanide-m-chlorophenyl hydrazone. The proton extrusion stoichiometry ($^{\rightarrow}H^+/2e^-$ ratio) in valinomycin-treated cells in light were as follows: $NO_3^- \rightarrow 0.5N_2$, 4.82; $NO_2^- \rightarrow 0.5\ N_2$, 5.43; $N_2O \rightarrow N_2$, 6.20; and $O_2 \rightarrow H_2O$, 6.43. In the dark the comparable values were 3.99, 4.10, 4.17 and 3.95. Thus, illuminated cells produced higher $^{\rightarrow}H^+/2e^-$ values than those in the dark, indicating a close link between photosynthesis and denitrification in the generation of proton gradients across the bacterial membranes. The stoichiometries for proton consumption (BVH as the electron donor), $^{\rightarrow}H^+/2e^-$ ratios, without a permeant ion were $NO_3^- \rightarrow NO_2^-$, -1.95; $NO_2^- \rightarrow 0.5\ N_2O$, -3.03; and $N_2O \rightarrow N_2$, -2.02. These data indicate that these events occur on the periplasmic side of the membrane.

7. Washed cells of the photodenitrifier developed a $\Delta p$ of about -175 mV to -200 mV during denitrification in the dark and -200 to -245 mV in light. With $NO_2^-$ as the terminal acceptor, $\Delta p$ was less than that with $NO_3^-$, $N_2O$ or $O_2$. The values of $\Delta\Psi$ in the dark were about -150 mV for $NO_3^-$ and $N_2O$ and -140 mV for $NO_2^-$.

8. During photodenitrification with either $NO_3^-$, $NO_2^-$, $N_2O$ or respiration to $O_2$ in light, $\Delta\Psi$ values varied between -152 and -167 mV. Like $\Delta\Psi$, the $\Delta pH$ was higher in light than in the dark resulting in a 20 to 30 mV increase in $\Delta p$ during illumination with $NO_3^-$, $NO_2^-$ or $N_2O$ as the acceptor. Both $\Delta\Psi$ and $\Delta pH$ were reduced at higher pH values ($\geqslant$7.5). Changes in pH in response to $O_2$ in light were less than those in the dark, indicating light inhibition of $O_2$ respiration.

9. The cells maintained a reasonably high $\Delta p$ without addition of a substrate or when inhibitors were used; the cells retained a fairly high $\Delta\Psi$ even in the presence of an inhibitor. However, $\Delta pH$ was appreciably lowered and it was almost abolished in the presence of either KCN, rotenone, $NaN_3$, CCCP, DNP, DCCD, antimycin A or HOQNO. The combination of an uncoupler (e.g. CCCP) and an electron transport effector (e.g. antimycin A) further reduced the $\Delta pH$. Antimycin A and HOQNO were more effective in inhibiting photosynthetic electron transport to either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ than the dark respiration to these substrates. DBMIB, a quinone antagonist, markedly reduced $\Delta pH$ in light with either $NO_3^-$, $NO_2^-$ or $N_2O$ as the terminal acceptor, indicating that photosynthetically generated electrons are used for denitrification in this bacterium.

10. Washed cells, prepared from cultures grown anaerobically in light with $NO_3^-$ as the terminal acceptor, readily incorporated [$^{14}$C]-proline both in light and in the dark. The proline uptake was coupled to the reduction of either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$. Light stimulated the accumulation of proline in these cells. The addition of $NO_3^-$ to washed cells in light decreased the $K_m$ for proline from 40 µM to 5.7 µM.

11. Proline transport was inhibited by antimycin A and HOQNO, both in light and in the dark in the presence of $NO_3^-$ indicating that electron transfer from both denitrification and photosynthesis are involved in this uptake. Inhibition by CCCP and DNP indicate that proline transport is energy dependent.

12. The $H^+$/proline stoichiometry increased from 1 to 2.5 (in light) wher the external pH was increased from 6.0 to 8.0. Under these conditions $\Delta\mu$ pro increased but $\Delta p$ decreased markedly at pH values >7.0.

13. The addition of either $Na^+$ or $NO_3^-$ to washed cells in light stimulated the uptake of proline. The $K_m$ value for proline was reduced from 50 µM to 6.7 µM on adding 20 mM NaCl to cells washed in a $Na^+$ free buffer. The addition of KCl and $NaNO_3$ to $Na^+$-loaded cells in light resulted in an additive effect on the uptake of proline indicating that $Na^+/K^+$ antiporter is involved in restoring the $Na^+$ gradient required for an increased proline uptake. Proline transport was inhibited by valinomycin whereas nigericin stimulated its uptake. These results show that both $\Delta p$ and the establishment of a $Na^+$ gradient are involved in the uptake of proline in this bacterium.

14. Washed cells, grown under photodenitrifying conditions, showed a $\Delta pH$-dependent $^{22}Na^+$ uptake. The accumulated $Na^+$ was rapidly released from the cells after treatment with gramicidin. These cells suspended in 150 mM KCl also acidified the external medium in response to $Na^+$ pulses in light and in the dark both aerobically and anaerobically. This acidification was partially inhibited by about a half on adding CCCP. The $Na^+/H^+$ antiporter, which was electrogenic, had a reversible $Na^+/H^+$ exchange activity.

15. Washed cells from photodenitrifying cultures exhibited a $\Delta pH$-dependent $K^+$ uptake. These cells also acidified the suspension medium in response to $K^+$ pulses both aerobically and anaerobically in light and in the dark. The results indicate that the photodenitrifier has a reversible $K^+/H^+$ exchange activity which reflects its role in regulating the intracellular $K^+$ concentration, as well as intracellular pH.

16. The acidification of the external medium resulting from $K^+$ pulses was inhibited by CCCP indicating that the antiporter is energy-dependent. Addition of KCl to washed cells depolarized the

$\Delta\Psi$ with a concomitant increase in $\Delta pH$, suggesting that the $K^+/H^+$ antiporter is electrogenic.

17. The $K^+$-depleted cells, prepared by treatment with diethanolamine, contained less than 5 mM $K^+$. These cells accumulated $^{22}Na^+$ only when amine was not present in the external buffer (50 mM Tris-HCl, pH 7.5). Studies with $^{22}Na^+$-loaded cells indicate that the photodenitrifier had antiporters for $Na^+/H^+$, $K^+/Na^+$ and $K^+/H^+$ and lacked a respiration (denitrification)-dependent $Na^+$ pump.

18. The $K^+/Na^+$ antiporter was electrogenic and required proton motive force for its operation. Thus, the addition of either NaCl or KCl to $K^+$-depleted cells resulted in a depolarization of $\Delta\Psi$, both in light and in the dark, by 10-35 mV, which was partially compensated for by a concomitant increase (5-13 mV) in $\Delta pH$.

# 1. INTRODUCTION

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## 1.1 THE PHOTOSYNTHETIC BACTERIA

The phototrophic bacteria include various types of gram-negative aquatic bacteria. They are found in a wide range of environments including marine and fresh water. They are important components of these ecosystems because of their capacity to utilize solar energy for the fixation of carbon dioxide ($CO_2$) and dinitrogen ($N_2$). These bacteria, under some circumstances, produce copious amounts of $H_2$ gas which has stimulated an interest in the use of solar energy for the production of this non-polluting fuel (Gest and Kamen,1949; Benemann and Weare, 1974). $N_2$-fixing photosynthetic bacteria are considered to be a potential source of fertilizer nitrogen (Weare and Shanmugam, 1976) and as a source of single cell protein (Shipman *et al.*, 1977). The photosynthetic bacteria have also been successfully employed in sewage disposal and in the treatment of polluted water (Kobayashi and Haque, 1971).

The morphological characteristics of photosynthetic bacteria are varied, since cells may either be spherical, rod-, vibrio-, or spiral-shaped. Their sizes range from 0.3 to over 5 µm (Pfenning and Truper, 1974). The phototrophic bacteria are classified primarily on their physiological features (Pfenning and Truper, 1974). They are divided into two main groups, the green and the purple photosynthetic bacteria and are further divided into four families:

(i) *Chlorobiaceae*; green and brown sulphur bacteria

(ii) *Chloroflexaceae*; filamentous green gliding bacteria

(iii) *Chromatiaceae*; purple sulphur bacteria

(iv) *Rhodospirillaceae*; purple non-sulphur bacteria.

Purple non-sulphur bacteria (*Rhodospirillaceae*) are characterized by their inability to grow well on elemental sulphur and sulfide as photosynthetic electron donors. These bacteria are highly versatile since they obtain energy *via* photosynthesis or respiration. In general, most species are microaerophilic. They grow under either light anaerobic conditions or in the dark in air. Many species grow in air in light or in the dark. Indeed, every major type of energy conversion and substrate utilization has been observed in these bacteria (van Niel, 1944; Uffen and Wolfe, 1970; Uffen, 1976; Satoh *et al.*, 1976; Madigan and Gest, 1979; Madigan *et al.*, 1980), *viz;* photoheterotrophic growth, photoautotrophic growth, dark chemoheterotrophic growth, dark chemoautotrophic growth, dark anaerobic fermentative growth. In strains able to grow under microaerophilic and aerobic conditions, the photopigment content and the intracytoplasmic membrane system decrease as the concentration of $O_2$ increases. The formation of photopigments is depressed at low $O_2$ concentrations. Most purple non-sulphur bacteria requiie vitamins and amino acids for growth. These bacteria have the following characteristics:

(i) They are predominantly photoheterotrophic.

(ii) They can photoassimilate a wide range of organic substrates e.g. fatty acids and other organic acids, primary and secondary alcohols, carbohydrates and even aromatic compounds.

(iii) They are sensitive to $H_2S$; their growth is usually inhibited by low concentrations of sulphide, even though some can oxidise very low concentrations of sulphide anaerobically in light.

(iv) All purple non-sulphur bacteria can grow photoautotrophically in the presence of $H_2$.

(v) The main photosynthetic pigment is bacteriochlorophyll *a*.

(vi) The predominant carotenoids are spirilloxanthin, spheroidenone, rhodopin, rhodopinal and lycopene.

(vii) They are either strictly anaerobic or facultative anaerobic.

(viii) They all fix $N_2$.

## 1.2 THE DENITRIFYING BACTERIA

Microorganisms utilize $NO_3^-$ for two purposes:

(a) as a sole source of nitrogen (nitrate assimilation, which occurs both under aerobic and anaerobic conditions) and

(b) as a terminal electron acceptor (nitrate respiration and denitrification, occurs under anaerobic conditions).

During assimilation, $NO_3^-$ is reduced to $NH_4^+$ and incorporated into cell-nitrogen compounds whereas in denitrification $NO_3^-$ serves as an alternative terminal acceptor to $O_2$ and is reduced to $NO_2^-$ and thence to nitrogenous gases. The reduction of $NO_3^-$ to $N_2$ gas is associated with the following changes in the free energy (Thauer *et al.*, 1977).

$$NO_3^- + H_2 \rightarrow NO_2^- + H_2O \quad -39.0 \text{ Kcal/mol} \qquad (1)$$

$$2NO_2^- + H_2 + 2H^+ \rightarrow 2NO + 2H_2O \quad -35.1 \text{ Kcal/mol} \qquad (2)$$

$$2NO + H_2 \rightarrow N_2O + H_2O \quad -73.2 \text{ Kcal/mol} \qquad (3)$$

$$N_2O + H_2 \rightarrow N_2 + H_2O \quad -81.6 \text{ Kcal/mol} \qquad (4)$$

The energy released during the reduction of $NO_3^-$ to $NO_2^-$ and $NO_2^-$ to $N_2$, is utilized for the production of ATP (Thauer *et al.*, 1977).

Denitrifying bacteria are widely distributed in nature and are biochemically and taxonomically very diverse. These bacteria are predominantly prototrophic heterotrophs that oxidize a variety of organic substrates ranging in complexity from methanol to aromatic compounds (Payne, 1976). Some of them grow autotrophically on $H_2$ and $CO_2$ or reduced sulphur compounds and the other group is phototrophic.

1.2.1 · Non-photosynthetic

Nitrate may be dissimilated either to $NO_2^-$ only ($NO_3^-$-respiration) or reduced further to $N_2$ gas *via* $N_2O$ (denitrification). Nitrate respiration occurs in *Escherichia coli* (Taniguchi *et al.*, 1956) and in *Hemophilus parainfluenzae* (White, 1962). Some denitrifiers do not utilize $NO_3^-$ as a terminal electron acceptor but can reduce $NO_2^-$ *via* $N_2O$ to $N_2$ e.g. *Alcaligenes odorans* (Chatelain, 1969; Pichinoty *et al.*, 1978); some strains of *Bacillus* (Pichinoty *et al.*, 1979) and *Nisseria sicca*, *N. flavescens* and *N. subflava* (Grant and Payne, 1981). A few denitrifying bacteria lack $N_2O$-reductase and therefore yield $N_2O$ as the final product e.g. *Corynebacterium nephridii* (Hart *et al.*, 1965; Renner and Becker, 1970), *Propionibacterium acidipropionici* (Payne and Riley, 1969), *Pseudomonas fluorescens* (Greenberg and Becker, 1972, 1977). The following bacteria denitrify $NO_3^-$ to $N_2$ gas: *Propionibacterium pentosaceum* (Gent-Ruijters *et al.*, 1975), *Paracoccus (Micrococcus) denitrificans* (Lam and Nicholas, 1969a; Sapshead and Wimpenny, 1972) *Pseudomonas aeruginosa* (Fewson and Nicholas, 1961), *Ps. denitrificans* (Radcliffe and Nicholas, 1970) and *Thiobacillus denitrificans* (Baalsrud and Baalsrud, 1954; Aminuddin and Nicholas, 1973). *Paracoccus denitrificans* oxidises $H_2$ while reducing $NO_3^-$ to $N_2$ gas (John and Whatley, 1975). *Hyphomicrobium* strains use methane as the reductant for denitrification (Sperl and Hoare, 1971; Meiberg *et al.*, 1980; Nurse, 1980). *Thiobacillus denitrificans* utilize reduced sulphur compounds for denitrification (Baldensperger and Garcia, 1975; Sawhney and Nicholas, 1978). Some bacilli can grow anaerobically with NO as a terminal electron acceptor (Pichinoty *et al.*, 1978, 1979). *Azospirillum brassilens (Spirillum lipoferum)*, a $N_2$-fixing bacterium, can also denitrify $NO_3^-$ (Neyra *et al.*, 1977). Soybean bacteroids reduce $NO_3^-$ to $NO_2^-$; thus some strains of *Rhizobium* reduce $NO_3^-$ and $NO_2^-$ to $N_2O$ or $N_2$ or both (Zablotowicz *et al.*, 1978; Bhandari and Nicholas, 1984). The microbiological, physiological

and biochemical aspects of denitrification have been reviewed by Payne (1973, 1976), Stouthamer (1976), Haddock and Jones (1977), Thauer *et al.*, (1977) and Knowles (1982).

### 1.2.2 Photosynthetic

Many photosynthetic bacteria assimilate dinitrogen or combined nitrogen compounds into cell nitrogen (Pfenning and Truper, 1974). Recently strains of purple non-sulphur bacteria have been described which denitrify $NO_3^-$ to $N_2$ gas. Malofeeva *et al.*(1974) found that *Rhodopseudomonas palustris* could grow with $NO_3^-$ as the sole source of nitrogen under anaerobic conditions ($H_2$ atmosphere) in light but the bacterium did not grow with $NO_3^-$ in the dark. Three strains of *Rhodopseudomonas sphaeroides* forma sp. *denitrificans* have been reported to denitrify $NO_3^-$ to $N_2$ gas (Satoh *et al.*, 1976). These cells grew well anaerobically in the dark with $NO_3^-$ but did not assimilate $NO_3^-$ into cell nitrogen aerobically, either in light or in the dark. Recently, Klemme *et al.*, (1980) reported the existence of two strains of *Rhodopseudomonas palustris* which displayed nitrate and nitrite reductase activities of a dissimilatory type. A spontaneous mutant of *Rhodopseudomonas capsulata* possessed a dissimilatory nitrate reductase activity (McEwan *et al.*, 1982).

## 1.3 BIOCHEMISTRY OF DENITRIFICATION

### 1.3.1 Pathway of denitrification

In view of the importance of denitrification several research groups have attempted to elucidate the pathway and have characterized the enzymes involved. This work has shown the formation of $NO_2^-$ and $N_2O$ as free obligatory intermediates in the overall process (Payne, 1973; St. John

and Hollocher, 1977).

Oxidation states of N atom → +5 +3 +2 +1 0

$$NO_3^- \xrightarrow{-2e^-} NO_2^- \xrightarrow{-e^-} (NO)? \xrightarrow{-e^-} N_2O \xrightarrow{-2e^-} N_2$$

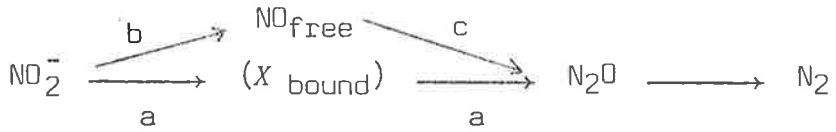
The first step is mediated by nitrate reductase and involves a 2-electron reduction of $NO_3^-$ to $NO_2^-$. Dissimilatory nitrate reductase has been purified and characterized from many bacteria. Involvement of NO in the reduction of $NO_2^-$ is still a matter of conjecture. It was found that purified nitrite reductases often released NO as a gaseous product (Miyata and Mori, 1969; Iwasaki and Matsubara, 1972; Cox and Payne, 1973) whereas washed cells do not. Semipurified nitric oxide reductase preparations which reduce NO to $N_2O$ have been prepared from *Ps. aeruginosa* (Fewson and Nicholas, 1960), *Ps. denitrificans* (Miyata *et al.*, 1969) and *Ps. perfectomarinus* (Payne *et al.*, 1971). These results indicate that NO cannot be ruled out as a possible intermediate in the denitrification process. On the other hand *in vivo* $^{15}N$ isotope-exchange studies with cells of *Ps. aeruginosa* indicated that there was not a pool of free $^{15}NO$ in cells supplied with $NO_2^-$ (St. John and Hollocher, 1977). The evidence for the participation of an NO-binding complex in the reduction of $NO_2^-$ to $N_2O$ is conflicting (Rowe *et al.*, 1977, Legall *et al.*, 1979; Zumft *et al.*, 1979). Three possible schemes for $NO_2^-$ to $N_2O$ reduction have been proposed (Knowles, 1982):

Scheme I

$$NO_2^- \longrightarrow N_2O \longrightarrow N_2$$
$$\uparrow$$
$$NO$$

Scheme II

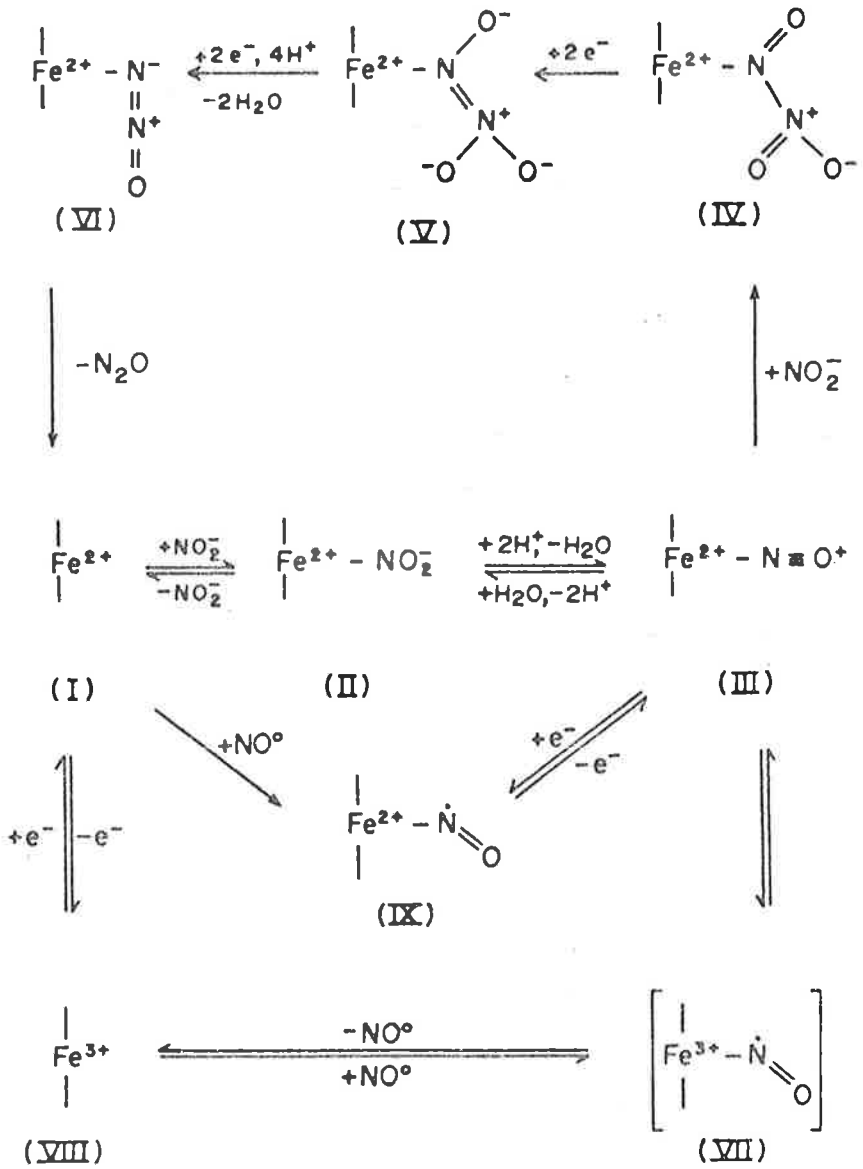

Scheme III




Scheme I was based on studies with *Pa. denitrificans* and *Ps. aeruginosa* whereas Scheme II is derived from results for *Ps. stutzeri* (X being a common mononitrogen intermediate). Scheme III accommodates results for *Ps. denitrificans* in which 'a' was the major pathway and 'b' was the main pathway for *Ps. aureofacience* (Garber and Hollocher, 1981).

A key feature that distinguishes denitrification from the 6-electron reduction of $NO_2^-$ to $NH_4^+$ is the formation of a N-N bond in the former pathway. Averill and Tiedje (1982) have suggested a chemical route to a N-N bond intermediate which is applicable to both haem and copper containing nitrite reductases (Fig. 1). The scheme involves an initial binding of nitrite (II) to an axial position of a ferrous porphyrin (I) followed by protonation and dehydration to the ferrous-nitrosyl complex (III). Complex III can either lose NO, forming the ferric porphyrin (VIII) thus providing a facile route for exchange of isotope between NO and $NO_2^-$ or undergo attack by free $NO_2^-$ to form $N_2O_3$ which is co-ordinated to the iron (IV). Two electron reduction of (IV) will produce co-ordinated oxyhyponitrite (V) and further reduction by 2 electrons, protonation and dehydration will yield a species containng coordinated $N_2O$ (VI), loss of which regenerates the starting ferrous porphyrin (I).

**FIG. 1. Possible routes of reduction of $NO_2^-$ to $N_2O$**



Ferrous-nitrosyl complexes such as (III) are known to be relatively unstable, decomposing readily to the ferric porphyrin (VIII) and free NO (Wayland and Olson, 1974), presumably *via* internal electron transfer (III $\longrightarrow$ VII). The ferrous-NO complex (IX) is not directly involved in either reduction of $NO_2^-$ to $N_2O$ or in isotope exchange between $NO_2^-$ and NO.

Although many bacteria under denitrifying conditions do not release $N_2O$ it is the end product in *C. nephridii* (Renner and Becker, 1970), in some rhizobia (Daniel *et al.*, 1980) and pseudomonads (Firestone *et al.*, 1979; Greenberg and Becker, 1977). It is also the product of NO reduction in certain fractions of *Ps. perfectomarinus* (Payne *et al.*, 1971). Furthermore $N_2O$ reductase is inhibited by acetylene resulting in an accumulation of $N_2O$. During the reduction of $^{15}NO_3^-$ or $^{15}NO_2^-$ in *Ps. aeruginosa* in the presence of a pool of unlabelled $N_2O$, the $^{15}N$ was located in $N_2O$ (St. John and Hollocher, 1977). On the other hand, Allen and Niel (1952) and Sacks and Barker (1952) reported that azide and cyanide inhibited the reduction of $N_2O$ to $N_2$ while not affecting the reduction of $NO_2^-$ to $N_2$. Thus, from this evidence, it appeared that $N_2O$ could not be an intermediate. However, it was shown that $NO_2^-$ can counteract the azide inhibition of $N_2O$-reductase and permit the reduction of $N_2O$ to $N_2$, thus explaining the lack of accumulation of $N_2O$ during the reduction of $NO_2^-$ in the presence of azide (Sidransky *et al.*, 1978).

### 1.3.2 Nitrate reductase

Dissimilatory nitrate reductase functions as a respiratory enzyme in bacteria growing under anaerobic conditions. The synthesis and functioning of this enzyme is regulated by the partial pressure of $O_2$ and the concentration of $NO_3^-$ in the environment (Pichinoty and D'Ornano, 1961;

Van't Riet *et al.*, 1968). The enzyme reduces chlorate and bromate but not iodate and it has been classified by Pichinoty (1964) as type A $NO_3^-$ reduction. Dissimilatory nitrate reductases are generally associated with cell membranes (except in *Spirillum itersonii*, Gauthier *et al.*, 1970; and in *Clostridium perfringens*, Chiba and Ishimoto, 1973) since they are involved in energy conservation. The catalytic site of this enzyme is located on the cytoplasmic side of the membrane in *Pa. denitrificans* (John, 1977; Kristjansson *et al.*, 1978; Jones *et al.*, 1980). Garland *et al.*, (1975), however, found that $NO_3^-$ is reduced on the outer side of the cytoplasmic membrane of *E. coli*. A periplasmic location of this enzyme was demonstrated in *R. sphaeroides* f. *denitrificans* (Sawada and Satoh, 1980).

Properties of dissimilatory nitrate reductase from various bacteria have been reviewed by Thauer *et al.* (1977). Dissimilatory reduction of $NO_3^-$ to $NO_2^-$ is linked to a particulate electron-transport chain consisting of dehydrogenases and electron carriers with nitrate reductase as the terminal enzyme. Although NADH serves as an electron donor for nitrate reductase in *E. coli* (Cole and Wimpeny, 1968), *Ps. aeruginosa* (Fewson and Nicholas, 1961), *Ps. denitrificans* (Radcliffe and Nicholas, 1970), *B. stearothermophilus* (Kiszkiss and Downey, 1972) and in *Aerobacter aerogenes* (Knook *et al.*, 1973), the reduction of $NO_3^-$ may also be linked to various organic acids e.g. formate (in *E. coli*, Taniguchi and Itagaki, 1960; in *Proteus mirabilis*, DeGroot and Stouthamer, 1970; and in *Ps. denitrificans*, Radcliffe and Nicholas, 1970). FAD, FMN and ubiquinones were shown to be electron carriers for $NO_3^-$ reduction in denitrifying bacteria (Nason, 1962; Forget and Pichinoty, 1964; Scholes and Smith, 1968; Clegg, 1976). Nitrate respiring bacteria contain at least one *b* type

cytochrome which is involved in $NO_3^-$ reduction (Lam and Nicholas, 1969b; Radcliffe and Nicholas, 1970; Stouthamer, 1976). Under anaerobic conditions, the electrons from the respiratory chain are diverted to $NO_3^-$ at the level of cytochrome *b* (Payne, 1976). In *T. denitrificans*, activity of nitrate reductase is coupled to the oxidation of inorganic sulphur compounds (Adams *et al.*, 1971; Aminuddin and Nicholas, 1973). Dissimilatory nitrate reductases e.g. *E. coli* (Forget, 1974), *Pa.* (*M.*) *denitrificans* (Forget, 1971) and *Pa.* (*M.*) *halodenitrificans* (Rosso *et al.*, 1973) contain iron, labile sulphide and molybdenum (Stouthamer, 1976). Growth of *E. coli* on tungstate produced an inactive enzyme but activity was restored by the *in vivo* incorporation of Mo into the cells (Scott *et al.*, 1979). Forget and Dervertanian (1972) observed that electron spin resonance (ESR) signals due to Mo (V) and Fe (III) were eliminated on reduction of the enzyme and this was accompanied by the appearance of the signals due to Fe (II-S). Addition of $NO_3^-$ to the reduced enzyme restored the ESR signals attributed to Mo (V) and Fe (III). These investigations indicated that Mo and Fe function as electron carriers in the reduction of $NO_3^-$ by nitrate reductase.

### 1.3.3 Nitrite reductase

Dissimilatory nitrite reductase catalyzes the reduction of $NO_2^-$ to yield gaseous products. The synthesis of this enzyme is also controlled by the concentrations of $O_2$ and $NO_3^-$ in the growth medium (Delwiche and Bryan, 1976). This enzyme has been purified from several bacteria and it appears that there are two main types: (1) Haemoproteins of cytochrome type *cd* which also has a cytochrome oxidase activity (Lam and Nicholas, 1969c; Sawhney and Nicholas, 1978). This type occurs in *A. faecalis*

(Matsubara and Iwasaki, 1971), *Pa. denitrificans* (Lam and Nicholas, 1969c; John and Whatley, 1975), *Ps. aeruginosa* (Horio *et al.*, 1961; Shimada and Orii, 1975), *Ps. perfectomarinus* (Zumft *et al.*, 1979; Zumft and Vega, 1979) and *T. denitrificans* (Radcliffe and Nicholas, 1968; Sawhney and Nicholas, 1978; LeGall *et al.*, 1979). (2) The copper containing nitrite reductases are found in *Achromobacter cycloclastes* (Iwasaki *et al.*, 1975), *Ps. denitrificans* (Iwasaki *et al.*, 1963) and in denitrifying strains of *R. sphaeroides* (Sawada *et al.*, 1978; Michalski and Nicholas, 1985). Haem containing nitrite reductases are associated with membranes e.g. *Pa. denitrificans* (Kristjansson *et al.*, 1978), *Ps. denitrificans* (Koike and Hattori, 1975), *B. stearothermophilus* (Reiling and Zuber, 1980), *T. denitrificans* (Sawhney and Nicholas, 1978) and copper proteins are usually soluble enzymes e.g. in *A. cycloclastes* (Iwasaki and Matsubara, 1972). In *Pa. denitrificans* proton translocation experiments indicated an inner membrane face location for the enzyme (Kristjansson *et al.*, 1978) whereas in the same bacterium (Meijer *et al.*, 1979) and in *Ps. aeruginosa* (Wood, 1978) similar studies indicated a periplasmic location. A membrane location is consistent with the *in vivo* evidence of phosphorylation associated with $NO_2^-$ reduction (Naik and Nicholas, 1966) in *Ps. denitrificans* (Koike and Hattori, 1975) and *B. stearothermophilus* (Reiling and Zuber, 1980).

The products of $NO_2^-$ reduction in washed cells of *Pa. denitrificans* (Hollocher, 1982), *Ps. aeruginosa* (St. John and Hollocher, 1977; Kim and Hollocher, 1983), *R. japonicum* (Bhandari and Nicholas, 1984) are primarily $N_2O$ and $N_2$. With enzyme preparations of varying purity NO is produced, as in *Ps. perfectomarinus* (Cox and Payne, 1973), *A. faecalis* (Iwasaki and Matsubara, 1971) and *A. cycloclastes* (Iwasaki and Matsubara, 1972).

### 1.3.4 Nitric oxide and nitrous oxide reductases

Semipurified nitric oxide reductase fractions from *Ps. aeruginosa* (Fewson and Nicholas, 1960), *Ps. denitrificans* (Miyata *et al.*, 1969) and *Ps. perfectomarinus* (Payne *et al.*, 1971) reduced NO to $N_2O$. Only limited information is available about the properties of this enzyme. In *Ps. perfectomarinus* (Cox and Payne, 1973) nitric oxide reductase was reported to be a soluble enzyme whereas in *A. faecalis* (Matsubara and Iwasaki, 1971) and *Ps. denitrificans* (Miyata *et al.*, 1969) the enzyme was associated with cell membranes.

Electron transport to nitrous oxide reductase, which reduces $N_2O$ to $N_2$, generates a membrane potential slightly smaller than that associated with $O_2$ utilization (McCarthy *et al.*, 1981). It appears to involve cytochromes *b* and *c* (Matsubara, 1975; Boogerd *et al.*, 1980). These cytochromes are membrane bound in *Ps. denitrificans* (Matsubara, 1975) and continuous-culture studies of energy yields in this bacterium grown on various N-oxides indicate that energy conservation is associated with $N_2O$ reductase (Koike and Hattori, 1975). It is likely that this enzyme is located in cell membranes; indeed, a membrane bound enzyme was found in *Ps. perfectomarinus* (Payne *et al.*, 1971). Active preparations of this enzyme were recently prepared by gentle osmotic lysis of spheroplasts of *Pa. denitrificans* (Kristjansson and Hollocher, 1980). Further studies have achieved a 60-fold purification with respect to the crude lysate. The enzyme is sensitive to $O_2$ and has a molecular weight of about 85 kd. The enzyme contains Cu (Matsubara and Mori, 1968; Kristjansson and Hollocher, 1981). Zumft *et al.* (1982, 1985) have recently purified $N_2O$ reductase from *Ps. perfectomarinus*. Partially purified $N_2O$-reductase from *R. capsulata* N22DNAR$^+$ (McEwan *et al.*, 1985) appears to have properties that are similar

to the enzyme from *Ps. perfectomarinus*. A multi-copper $N_2O$ reductase has been purified to homogeneity from *R. sphaeroides f. denitrificans* (Michalski *et al.*, 1986). It is a monomeric enzyme and one or two disulphide bridges appear to be involved in its tertiary structure. The enzyme contains four copper atoms per molecule and the EPR spectrum is quite distinct from that of nitrite reductase and other Cu containing enzymes.

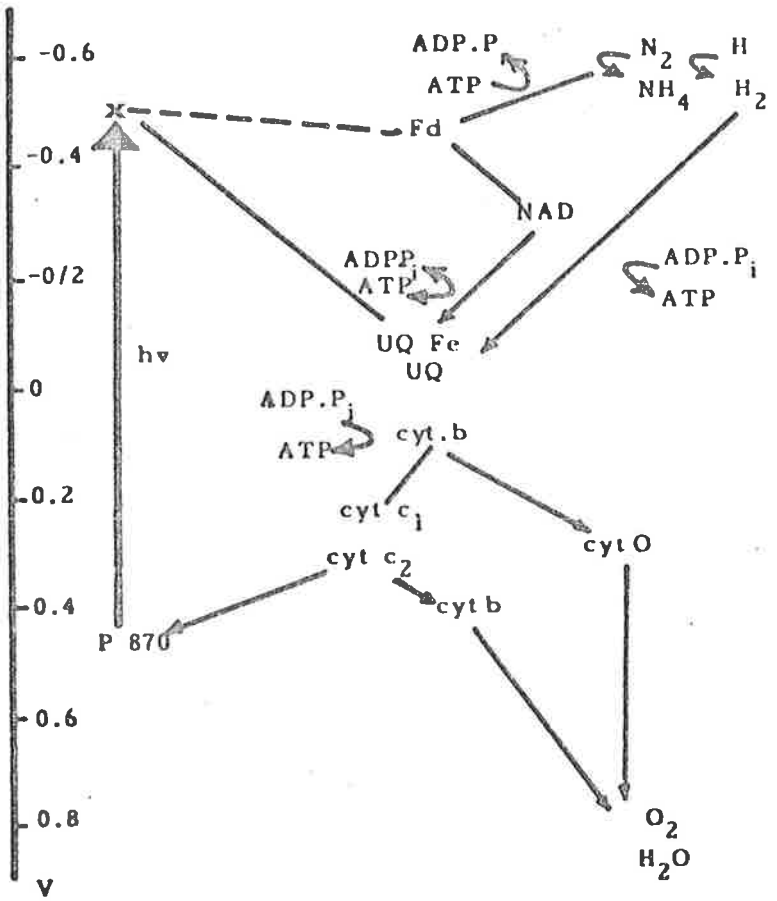
## 1.4 ENERGY CONSERVATION

### 1.4.1 Electron transport

The electron transfer pathway of a denitrifying bacterium e.g. *Pa. denitrificans* is similar to the electron transport system in mitochrondria (Whatley, 1981). In photosynthetic bacteria the respiratory and photosynthetic chains share common redox components. The pathways of photosynthetic electron flow in purple non-sulphur bacteria (Fig. 2) are thought to involve either a non-cyclic or a cyclic passage of electrons *via* a complex assembly of redox carriers. These bacteria lack photosystem II and therefore cannot undertake the photolysis of $H_2O$, as do green plants. However, the photosystem I of these bacteria utilizes either organic compounds or $H_2$ as electron donors.

Non-cyclic electron flow involves a light-induced excitation of electrons from the level of bacteriochlorophyll (P-870) to a highly negative acceptor X (Fig. 2). This carrier (X) which is a non-haem iron protein then directly reduces ferredoxin and then NAD(P)H, thus providing reducing equivalents essential for cellular metabolism. A reduction of such an extremely electronegative species (X , -600 mV) has been observed during illumination (Blankenship and Parson, 1978).

**FIG.2. A scheme of photosynthetic and respiratory electron transport in Rhodopseudomonas capsulata and their interaction with hydrogenase and nitrogenase**



The alternative pathway of photosynthetic electron transport involves a more favoured cyclic electron flow (Fig. 2). A light-activated bacteriochlorophyll promotes the reduction of a primary electron acceptor X, which in turn reduces an ubiquinone. This ubiquinone transfers electrons *via* cyt. *b* to cyt. $c_1$ and cyt. $c_2$ and finally to bacteriochlorophyll (P-870) during which phosphorylation of ADP to ATP occurs. Light energy activates the bacteriochlorophyll again thus reactivating the cycle.

In addition to this anaerobic photosynthetic metabolism purple non-sulphur bacteria can respire to $O_2$ in the dark. Indeed, light is known to inhibit $O_2$ respiration in the *Rhodospirillaceae* (Clayton, 1955). Two kinds of hypothesis have been advanced to explain this phenomenon:

(i) the respiratory and photosynthetic chains share common redox components, so that a light-induced change in the redox state of a shared carrier inhibits an intermediate reaction in the respiratory chain (Clayton, 1955; Horio and Kamen, 1962; Nishimura, 1963; Fork and Goedheer, 1964; Keister and Minton, 1971);

(ii) the two electron transport chains share the same coupling membrane so that $\Delta p$ (proton motive force) generated exerts a thermodynamic control upon the rate of respiration (Cotton *et al.*, 1982; McCarthy and Ferguson, 1982).

Cyt. $c_2$ functions as both the primary electron donor to the reaction centre in photosynthetic electron transport and as an electron donor for Cyt. $b_{413}$ in respiration. The respiratory system also produces ATP due to electron transfer from electronegative species, such as NADH, *via* a sequence of redox carriers to $O_2$.

Reducing equivalents are probably generated by an energy-dependent reversed electron flow, as occurs in energy-linked electron transfer reactions in mammalian mitochrondria (Ernster and Lee, 1964; Gest, 1972) and in chemoautotrophic bacteria (Aleem *et al.*, 1963). In the phototrophic bacteria the ATP produced from either light-induced cyclic electron flow, or respiration to $O_2$, can be utilized to generate reductant [Fd or NAD(P)H] *via* an ATP-dependent reverse electron flow in the presence of a suitable substrate (e.g. an organic acid).

### 1.4.2 Energy coupling

Dissimilatory $NO_3^-$ reduction and denitrification are energy yielding types of respiration, but the sites of coupling of electron transport to phosphorylation have not been determined. One step reductions of $NO_3^-$ to $NO_2^-$ and $N_2O$ to $N_2$ are known to support the growth of various bacteria (Naik and Nicholas, 1967; Payne and Riely, 1969; Matsubara, 1971). Nitrate reduction coupled to oxidative phosphorylation has been demonstrated in *Ps. aeruginosa* (Yamanaka *et al.*, 1962), *E. coli* (Ota *et al.*, 1964) and in *Pa. denitrificans* (Imai *et al.*, 1968). Esterification of $^{32}P$ labelled phosphate concomitant with reduction of $NO_3^-$ to $NO_2^-$ by extracts of sonicated *E. coli* has been demonstrated with a variety of organic acids or NADH as electron donors (Ota *et al.*, 1964). Phosphorylation coupled to $NO_3^-$ and $NO_2^-$ reduction by a particulate fraction from *M. (Pa.) denitrificans* is also achieved with organic acids or NADH serving as electron donors (Naik and Nicholas, 1967; Imai *et al.*, 1968). There is no esterification with NO, $N_2O$ or $NH_2OH$ as potential electron

acceptors. This lack of coupling with $N_2O$ reduction may be lost during the preparation of cell extracts. *M. (Pa.) denitrificans* can grow anaerobically with $N_2O$ as the terminal oxidant (Pascal *et al.*, 1965), thus confirming ATP production at this step.

### 1.4.3 Proton translocation

Kristjansson *et al.* (1978) determined the yield of protons accumulated by washed cells of denitrifiers (*Pa. denitrificans* and *Ps. denitrificans*) in response to small oxidant pulses provided by $O_2$, $NO_3^-$, $NO_2^-$ and $N_2O$ respectively. In the presence of valinomycin which permits a full recovery of the total proton yield by exchanging potentially "bound" protons with potassium, they showed that the yields of $H^+$ per two electrons oxidised were: for O = 7.5, $NO_3^-$ = 4.3, $NO_2^-$ = 3.7 and for $N_2O$ = 4.5. Garland *et al.* (1975) also found that proton translocation was associated with respiratory nitrate reductase activity in *E. coli*. They concluded that the $^{\rightarrow}H^+/NO_3^-$ ratio was greater than 2. $^{\rightarrow}H^+$/electron acceptor ratios have been determined, by the oxidant pulse method, in *Pa. denitrificans* when oxidising endogenous substrates during the reduction of $O_2$, $NO_2^-$ or $N_2O$. Thus, under optimal proton translocation conditions the ratios, $^{\rightarrow}H^+/N_2O(\rightarrow N_2)$, $^{\rightarrow}H^+/NO_2^-(\rightarrow N_2)$, $^{\rightarrow}H^+/NO_2^-(\rightarrow N_2O)$ and $^{\rightarrow}H^+/O$ were 4.02, 5.79, 3.37 and 6.0 to 6.3 respectively, (Boogerd *et al.*, 1981). Garber *et al.* (1982) demonstrated proton translocation linked to the reduction of NO in *Pa. denitrificans*. Minimum $^{\rightarrow}H^+/2\bar{e}^-$ ratios for both $NO \rightarrow \frac{1}{2} N_2O$ and $NO \rightarrow \frac{1}{2} N_2$ reactions were about 3.7. Respiration of NO resulted in transient proton translocation in anaerobically grown cells of *Pa. denitrificans*, *R. sphaeroides* sub sp.*denitrificans*, *A. cycloclastes* and

*Rhizobium japonicum* gave respectively, $^{\rightarrow}H^+/NO$ ratios of 3.65, 4.96, 1.94 and 1.12 (Shapleigh and Payne, 1985).

### 1.4.4 The proton motive force

Mechanism of oxidative phosphorylation is best explained by Mitchell's chemiosmotic couplding hypothesis. A vectorial organization of the oxidation-reduction reactions in the membrane is proposed which results in electrogenic transport of $H^+$ from one side of the membrane to the other (Mitchell, 1966, 1977). According to this hypothesis electron transport causes $H^+$ ions to be pumped outward, across the inner membrane of the chloroplast, mitochondrion or bacterium, thus generating an acid-outside (inside alkaline) gradient of $H^+$ ($\Delta\mu H^+$) between two bulk aqueous phases separated by the inner membrane. This $H^+$ gradient is the energy-rich state into which electron-transport energy is transformed. The chemiosmotic hypothesis further proposes that the $H^+$ ions ejected by electron transport flow back into the cell matrix through a specific $H^+$ channel or 'pore' in the $F_1F_o$ ATPase molecule, driven by the concentration gradient of $H^+$. The free energy released as $H^+$ flows back through the ATPase causes the coupled synthesis of ATP by removal of HOH from ADP and phosphate. The relatively high internal $OH^-$ concentration (inside alkaline) "pulls" $H^+$ from the active site of $F_1$ ATPase and the relatively high external $H^+$ concentration "pulls" $OH^-$ in the outward direction. In *in vivo* systems ATP is continuously removed for cytosolic ATP-consuming reactions, while $\Delta\mu H^+$ is continuously replenished by the respiratory or photosynthetic electron-transfer chains. The free energy stored in $\Delta\mu H^+$ has been called proton motive force ($\Delta p$) and consists of a membrane potential ($\Delta\Psi$) and a transmembrane pH gradient ($\Delta pH$). These two parameters are related to each other by the following equation:

$$\Delta p = \Delta\Psi - \frac{2.3\ RT}{F}\ \Delta pH \qquad (1)$$

$$\text{or in millivolts } \Delta p = \Delta\Psi - 59\Delta pH \qquad (2)$$

Measurement of ΔpH and ΔΨ in microscopic systems (e.g. bacteria) is frequently based on determination of the equilibrium distributions of radioactively labelled permeant weak acids or bases and permeant lipophilic cations, respectively (Rottenberg, 1975, 1979). Electrophysiological techniques have been applied to giant cells of *E. coli* produced by growth in 6-amidinopennicillanic acid (Felle *et al.*, 1978). The ΔΨ values measured agree to within 10 mV with those obtained from [$^3$H] tetraphenyl phosphonium cation ([$^3$H]TPP$^+$) distribution studies (Porter *et al.*, 1979). It has also been demonstrated that the distribution of this lipophilic cation provides an excellent quantitative assessment of ΔΨ in cultured neuroblastoma/glioma NG 108-15 hybrid cells (Lichstein *et al.*, 1979 a,b) and in isolated guinea pig synaptosomes (Ramos *et al.*, 1979).

Several species of photosynthetic bacteria possess membrane-located pigments, particularly carotenoids, which are electrochromic, i.e. their absorption spectra are sensitive to electric fields (Jackson and Crofts, 1969). Quantitative evaluation of membrane potentials in chromatophores from *R. capsulata* (Baccarini-Melandri *et al.*, 1977) and *R. sphaeroides* (Jackson and Crofts, 1969) by the electrochromic method has given higher values than those obtained by permeant ion redistribution in chromatophores from *R. sphaeroides* (Michels and Konings, 1978; Ferguson *et al.*, 1979) and *R. rubrum* (Schuldiner *et al.*, 1974). In *R. capsulata* electrochromic method indicated larger membrane potentials in the intact cells than did ion distribution during both respiratory and photosynthetic electron flow (Clark and Jackson, 1981).

In a number of bacterial systems, it has been found that ΔpH varies

with external pH (Ramos *et al.*, 1976; Padan *et al.*, 1976; Bakker *et al.*, 1976; Ramos and Kaback, 1977a; Tokuda and Kaback, 1977; Krulwich *et al.*, 1978; Guffanti *et al* ., 1978). With intact *E. coli* cells and right-side-out membrane vesicles from this bacterium (Ramos *et al.*, 1976; Ramos and Kaback, 1977a) and *Salmonella typhimurium*, ΔpH exhibits maximal values of about 2 pH units (i.e., -120 mV) at pH 5.5 to 6.0 and decreases to zero at about pH 7.5. On the other hand ΔΨ has been reported in some instances to increase slightly with external pH (Padan *et al.*, 1976; Ramos *et al.*, 1976; Ramos and Kaback, 1977a; Tokuda and Kaback, 1977) and in others (Zilberstein *et al.*, 1979; Porter *et al.*, 1979) to increase much more markedly with pH. In washed cells of *R. japonicum* and bacteroids from *Glycine max*, ΔΨ and ΔpH were linearly related to external pH. Thus, in cells ΔΨ increased from -136 mV at pH out 6.1 to -200 mV at pH out 8.0 whereas in bacteroids it varied from -150 mV at pH out 6.1 to -182 mV at pH out 8.0. In both cases (cells and bacteroids) ΔpH was dependent on external pH (Bhandari and Nicholas, 1985). Oxygen-induced Δp has been demonstrated in photosynthetic bacteria but no information is available about the involvement of Δp during denitrification in these bacteria.

### 1.4.5 Amino acid transport

Aerobically grown bacteria catalyze the active transport of a wide variety of metabolites (Konings and Freese, 1972; Kaback and Hong, 1973; Kaback, 1974). Studies on active transport of amino acids has been mainly concerned with non-photosynthetic microorganisms and mammalian systems. Green and purple bacteria have been shown to incorporate small amounts of glutamate during growth (Sadler and Stanier, 1960). *Chromatium vinosum*, a photosynthetic purple sulfur bacterium, accumulates leucine, valine and

alanine during growth (Gibson, 1984) by an active transport system (Knaff, 1978; Knaff *et al.*, 1979, 1980). In this bacterium the uptake of alanine and its analogue, α-amino-isobutyrate (AIB) was found to be light-dependent and sensitive to protonophores (Knaff and Davidson, 1982). Active transport of alanine by the non-sulphur purple bacterium *R. sphaeroides* is dependent on respiration in the dark and on photosynthesis in light (Hellingwerf *et al.*, 1975; Elferink *et al.*, 1983).

The active transport of amino acids into bacteria is linked to coupling of either high energy phosphorylated compounds or ion gradients (Booth and Hamilton, 1980). In the latter case transport has been found to be predominantly linked to proton gradients (Booth and Hamilton, 1980). Light-dependent, uncoupler sensitive alanine transport systems of *R. sphaeroides* and *C. vinosum* were dependent on proton gradients (Hellingwerf *et al.*, 1975; Knaff, 1978). The electrochemical proton gradients across the cytoplasmic membranes develop a proton motive force which is known to drive energy consuming processes such as ATP synthesis and secondary solute transport (Harold, 1977a,b). The relationship between proton motive force and the rate of energy consuming processes has been studied in detail in several bacterial systems (Robertson *et al.*, 1979; Lanyi and Silverman, 1979; Melandri *et al.*, 1980).

The involvement of Δp in amino acid transport has been studied mainly in aerobic bacteria. In membrane vesicles of *E. coli* grown anaerobically with $NO_3^-$, it was shown that active transport can also be energized by anaerobic respiration (Konings and Kaback, 1973). Active transport of proline was supported by NO in *Pa. denitrificans* (Garber *et al.*, 1982). There is little information on amino acid transport on denitrifying bacteria including photodenitrifiers.

Alanine transport in *C. vinosum* occurred *via* an alanine/$Na^+$ symport (Knaff *et al.*, 1981; Pettitt *et al.*, 1982). In a number of bacteria the uptake of amino acids e.g. α-amino-isobutyric acid, glutamic acid, leucine, proline is also coupled to $Na^+$ gradient (Halpern *et al.*, 1973; Hirata *et al.*, 1974; Lanyi *et al.*, 1976a,b; Pearce *et al.*, 1977; Tsuchiya *et al* ., 1977; Tokuda *et al.*, 1982). A light-dependent uptake of proline and glutamate in a purple sulphur bacterium, *Ectothiorhodospira halophila*, was stimulated by $Na^+$ (Rinehart and Hubbard, 1976).

### 1.4.6 $Na^+$, $H^+$ and $K^+$ antiporters

The ion transport processes in biological systems have been considered in great detail in bacteria (Harold, 1974, 1977a,b; Reed *et al.*, 1981), algae (Raven, 1975, 1976), fungi (Jennings, 1974, 1976), higher plants (Anderson, 1974; Lucas *et al.*, 1980) and in animals (Gupta *et al.*, 1977). In general, cations which are usually less permeant than anions require energy for transport into cells. The proton motive force is important for energy transduction in bacteria, chloroplasts and mitochrondria (Mitchell, 1966; Harold, 1972, 1977; Kell, 1978). Bacteria possess several genetically distinct cation transport systems in addition to pumps which extrude protons (Harold and Altendorf, 1974; Rhoads *et al.*, 1976; Epstain and Laimins, 1980; Padan *et al.*, 1981). There are three different mechanisms of energy coupling for transport systems which derive their energy from previously formed electrochemical gradients (Mitchell, 1966, 1973), *viz.* symports, uniports and antiports. Among several ion-transport systems, $Na^+$ and $K^+$ play an important role in maintaining osmotic balance, regulating intracellular pH (Krulwich *et al.*, 1979; Beck and Rosen, 1979; Brey *et al.*, 1980; Plack and Rosen, 1980; Tokuda *et al.*,

1981; Davidson and Knaff, 1982) and in the active transport of nutrients e.g. sugars and amino acids (Stock and Roseman, 1971; Thomson and MacLeod, 1971; Lanyi *et al.*, 1976a,b; Tokuda and Kaback, 1977; Eddy, 1978; Lanyi, 1979). Energy-dependent accumulation of $K^+$ has been reported in a number of bacteria (Harold and Baarda, 1967; Eisenstadt, 1972; Hussan and McLeods, 1975; Rhoads *et al.*, 1977) and an energy-dependent system has been observed in *R. capsulata* (Jasper, 1978). The uptake of $K^+$ by *R. sphaeroides* is driven by $\Delta\Psi$ *via* an electrogenic transport system which does not utilize either $\Delta$pH or ATP as an energy source (Hellingwerf *et al.*, 1982). Light-driven, uncoupler-sensitive $Na^+$ uptake has been demonstrated in chromatophores from *C. vinosum*, *R. rubrum* and *R. sphaeroides* respectively (Knaff *et al.*, 1981; Hellingwerf *et al.*, 1982) but the mechanism involved has not been elucidated.

The $Na^+/H^+$ antiporter in bacterial membrane is an example of a cation transport system which is involved in two important physiological functions; namely, generation of a transmembrane $Na^+$ gradient (Harold and Altendorf, 1974; Lanyi, 1979) and regulation of cytoplasmic pH (Beck and Rosen, 1979; Krulwich *et al.*, 1979; Brey and Rosen, 1980; Plack and Rosen, 1980; Padan *et al.*, 1981; Tokuda *et al.*, 1981; Davidson and Knaff, 1982). Operation of cationic exchanges in bacterial membranes has been postulated in the chemiosmotic coupling hypothesis (Mitchell, 1968, 1970, 1973) where the electrochemical proton gradient is converted into an electrochemical cation gradient by symport or by antiport systems.

The presence of $Na^+/H^+$ antiporters has been demonstrated in many pro-Karyotes (Lanyi, 1979; Padan *et al.*, 1981; Krulwich, 1983) and eukaryotes (Moleenaar *et al.*, 1981; Rindler and Saier, 1981; Aronson *et al.*, 1982; Frelin *et al.*, 1983). In *E. coli* (West and Mitchell, 1974; Zilberstein

*et al.*, 1982),it was shown that the $Na^+/H^+$ antiporter is driven by the pH gradient ($\Delta pH$) whereas Schuldiner and Fishkes (1978) proposed that the membrane potential ($\Delta\Psi$) drives the exchange reaction at alkaline pH. The uphill $Na^+$ efflux in bacteria has also been reported to be driven by both $\Delta pH$ and $\Delta\Psi$ (Lanyi, 1979; West, 1980). In photosynthetic bacteria e.g. *Chromatium vinosum*, movements of $Na^+$ have been studied in cells and spheroplasts (Knaff *et al.*, 1980, 1981; Davidson and Knaff, 1982) especially in relation to the uptake of sugars and amino acids. The mechanism by which these $Na^+$ movements occur is not fully understood, but the available data indicate that a $Na^+/H^+$ antiporter is involved (Davidson and Knaff, 1982).

The $K^+/H^+$ exchange reactions catalyzed by the $K^+/H^+$ antiporter present in bacterial membranes have similar functions as the $Na^+/H^+$ antiporter. The $K^+/H^+$ antiporter maintains the internal $K^+$ concentration at some suitable level, thereby sustaining osmotic balance as well as regulating internal pH (Brey *et al.*, 1980; Padan *et al.*, 1981; Knaff and Davidson, 1982; Davidson and Knaff, 1982). $K^+/H^+$ antiporters have been found in *E. coli* (Brey *et al.*, 1978, 1980) and in *C. vinosum* (Davidson and Knaff, 1981).

# 2. MATERIALS AND METHODS

# 2. MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 Chemicals and biochemicals

Carbonyl cyanide-m-chlorophenyl hydrazone (CCCP), N,N'-dicyclohexyl-carbodiimide (DCCD), 2-n-heptyl-4-hydroxyquinoline-N-oxide, antimycin A, dichlorophenyl dimethyl urea (DCMU), gramicidin, dithiothreitol, N-ethylmaleimide, amytal, methyl viologen, chloramphenicol, rifampicin, rotenone, nigericin, pyruvic acid, succinic acid and Tris (hydroxymethyl)-aminoethane were from Sigma Chemical Co., St. Louis, U.S.A. Valinomycin was purchased from Calbiochem Behring Corp., La Jolla, U.S.A. 2,5-diphenyloxazole (PPO) and 1,4-bis [2, (4 methyl-5-phenyl oxazolyl]-benzene (POPOP) were obtained from Packard Instrument Co., Chicago, U.S.A. Phase combining system (PCS) liquid scintillation fluid was from Amersham, Bucks, England.

All other chemicals of the best purity available, were obtained from the following sources: Ajax Chemical Co. (Alburn, Australia), B.D.H. Chemicals Ltd., (Poole, England), May and Baker (Dagenham, England), ICN Pharmaceuticals (Cleveland, U.S.A.), Aldrich Chemical Co., (Milwaukee, U.S.A.) Dow Chemical Co., (Midland, U.S.A.) and Drughouse Ltd., (Adelaide, Australia).

### 2.1.2 Stable isotopes

$K^{15}NO_2$ (27.5 atom % excess) was purchased from L'office National Industriel de l' Azote (ONIA), Marseille, France. $H^{15}NO_3$ (99 atom % excess) was obtained from Isomet Corp., N.J., U.S.A. $K^{15}NO_3$ (99 atom % excess) was prepared by neutralizing $H^{15}NO_3$ with KOH. $K^{15}NO_2$ (97 atom % excess) was prepared by reduction of $K^{15}NO_3$ (99 atom % excess) with lead (Pb) at 420°C in

a silica crucible (Jolly, 1964). The $K^{15}NO_2$ so prepared was dissolved in distilled water, filtered and dried. This solution of $K^{15}NO_2$ contained 10% w/v) $K^{15}NO_3$.

### 2.1.3 Radioisotopes

The radioisotopes, [$^3$H]-tetraphenyl phosphonium bromide ($TPP^+ Br^-$, 23.7 Ci/mmol) and inulin [$^{14}$C]-carboxylic acid (4 mCi/mmol) were from Amersham Pty. Ltd., Sydney, Australia. $^3H_2O$ (1 Ci/mol) and [7-$^{14}$C]-benzoic acid (22.6 mCi/mmol) were supplied by New England Nuclear Corp., (NEN), Boston, Mass., U.S.A. [$^{14}$C]-proline (280 mCi/mmol) and carrier-free $^{22}$NaCl was obtained from Amersham International, Amersham, U.K.

### 2.1.4 Solutions, buffers and solvents

Unless stated otherwise, the aqueous solutions, buffers and reagents used in this study were dispensed in double glass distilled water.

### 2.1.5 Chromatographic materials

Ion exchange materials, namely DEAE-sephacel, DEAE-sephadex and sephadex G-100 for gel filtration were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

### 2.1.6 Enzymes and marker proteins

Carbonic anhydrase was purchased from Sigma Chemical Co., St. Louis, U.S.A. Molecular weight standards were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden.

### 2.1.7 Bacterial strain

*Rhodopseudomonas sphaeroides* forma sp. *denitrificans* strain IL-106 was kindly supplied by Dr. T. Satoh, Department of Biology, Faculty of Science, Tokyo Metropolitan University, Tokyo 158, Japan. This strain was isolated from a trickling filter at the sewage treatment plant in Takada, Japan by Satoh *et al*. (1974).

## 2.2 METHODS

### 2.2.1 Maintenance of stock cultures

Strain IL-106 was maintained in stab cultures in screw-capped Pyrex test-tubes (16 ml), using a medium containing (w/v) yeast extract (0.3%), bacto-peptone (0.3%) and agar (1.5%). The cultures were grown in light with two Philips Philinea tubes (60w, 250v, Ca. 2,000 lux) at 30°C and subsequently stored at room temperature under dim incandescent light. These cultures were viable for approximately two months.

### 2.2.2 Culture media

Cells were grown (10% v/v inoculum) in a basal medium supplemented with 0.2% (w/v) organic compounds and growth factors. The composition of the basal medium as described by Satoh *et al*. (1976) was: $KH_2PO_4$, 0.5g; $K_2HPO_4$, 0.5g; $(NH_4)_2HPO_4$, 0.8g; $MgSO_4,7H_2O$, 0.2g; NaCl, 0.1g; $CaCl_2,2H_2O$, 0.4g; and tap water to 1000 ml. The growth factors were added per litre: niacin, 1 mg; thiamin-HCl, 1 mg; biotin, 0.04 mg. The following modifications were made: the culture solutions were prepared in double glass distilled water and supplemented with a trace element solution (1 ml/L). The composition of the trace element solution (per 250 ml distilled water) was: $H_3BO_3$, 0.7g; $MnSO_4,H_2O$, 398 mg; $NaMoO_4,2H_2O$, 188 mg; $ZnSO_4,7H_2O$, 60 mg;

$Cu(NO_3)_2,3H_2O$, 10 mg (Weaver *et al* ., 1975). Dl-malate (21.4 mM) and $(NH_4)_2$ $HPO_4$ (2.5 mM in N-starvation medium and 12.5 mM in other media) were also included. Denitrifying cultures were grown in media containing $KNO_3$ (20 mM) and $(NH_4)_2HPO_4$ (12 mM) and aerobic cultures in media containing $(NH_4)_2HPO_4$ (12 mM) but no nitrate.

2.2.3 Cultures grown in light

Cultures were grown at 30°C under anaerobic conditions in completely filled 120 ml screw-cap bottles illuminated with two Philips philinea tubes (Ca, 2000 lux). These cultures were supplemented with a limiting concentration of $NH_4^+$ (2.5 mM) so that it was utilized before the cells were harvested.

Photodenitrifying cultures were grown anaerobically in light (Ca, 4000 lux) at 30°C in either 120 ml screw-cap bottles, 250 ml, 1L Duran bottles or 10L bottles. A 10% (v/v) inoculum from N-starved culture was used as the inoculum.

Aerobic phototrophic cultures (2L) were also inoculated with 10% (v/v) inoculum from N-starved cultures. These cultures grown in 4L Erlenmeyer flasks at 30°C were continuously sparged with sterile $O_2$.

2.2.4 Cultures grown in the dark

Anaerobic denitrifying cultures were grown in completely filled Duran bottles (1L) fitted with sterile rubber septa. The septum was pierced with a sterile needle to allow escape of gas ($N_2$) produced during growth. The cultures were incubated in the dark (covered with black polythene sheet) at 30°C.

Dark-aerobic cultures (2L) were grown at 30°C in 4L Erlenmeyer flasks covered with black polythene sheet. These cultures were continuously sparged with sterile $O_2$.

### 2.2.5 Preparation of washed cells

Cultures were harvested during the late-exponential growth phase (18-20h) or as indicated in the text and centrifuged at 20,000 x g for 20 min, in a Sorvall RC-5B refrigerated superspeed centrifuge at 4°C. The cells were washed once and resuspended in 50 mM potassium phosphate buffer (pH 7.0). In the case of denitrifying cultures, the cells were repeatedly washed with the same cold buffer to remove residual $NO_2^-$. Large cultures (10L) were harvested in the same centrifuge fitted with a continuous flow rotor (Ivan Sorvall, Inc., Connecticut, U.S.A.) at a flow rate of 5L/h.

### 2.2.6 Preparation of cell-free extracts

Cell extracts of denitrifying cultures were prepared in a French Pressure Cell. Washed cells were suspended in 0.1M potassium phosphate buffer (pH 7.0) in a 1:2 (w/v) ratio and DNAse (50 µg/ml) and RNAse (5 µg/ml) were added. This cell suspension was passed twice through a French Pressure Cell at 12,000 psi at 4°C. The homogenate was centrifuged at 20,000 x g for 20 min at 4°C and the supernatant ($S_{20}$) was decanted.

### 2.2.7 Preparation of spheroplasts and membrane fractions

The spheroplasts and membrane fractions were prepared as follows: 1g wet weight of washed cells suspended in 40 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 0.45M sucrose, 10 mM $Na_2EDTA$ and 40 mg lysozyme in a 100 ml Erlenmeyer flask were incubated at 30°C in a waterbath reciprocator (60 rev./min) for 1h. Then 2 µg/ml DNAse was added and incubation continued for 10 min. A pellet of spheroplasts was then obtained by centrifuging the suspension of 5,000 x g for 10 min at 4°C. The pellet, washed twice with cold Tris-HCl-sucrose (0.5M) buffer (pH 7.5)

was finally suspended in 5 ml of the same buffer. The spheroplasts thus prepared remained active for more than a week. Membrane fractions were prepared from spheroplasts either by osmotic shock treatment or sonication. In the osmotic shock treatment, 1 ml of the spheroplats were resuspended in 50 ml distilled water. In the other procedure spheroplasts were subjected to ultrasonic treatment with an MSE probe unit at maximum output, with short bursts of 30 sec, over a period of 10 min at 4°C in an icebath. After centrifuging at 5,000 x g for 20 min to remove intact spheroplasts the supernatant fractions were further centrifuged at 240,000 xg for 1 h. The pellet containing membrane fractions was washed several times with cold 100 mM Tris-HCl buffer (pH 7.5) and finally resuspended in the same buffer. Both spheroplasts and membrane fractions were stored at 2°C.

### 2.2.8 Chemical determinations

#### 2.2.8.1 Nitrate

Nitrate concentrations in the samples were determined by nitration of salicylic acid (Cataldo *et al.*, 1975). This method was modified as follows: samples (0 to 100 µl) containing $NO_3^-$ (0 to 300 nmol) were taken in 10 ml test tubes and 0.2 ml, 2% (w/v) 2-hydroxybenzoic acid (prepared in concentrated $H_2SO_4$) was added with constant shaking on a Vortex. Then 10% (w/v) NaOH (3.7 ml) was added slowly to the test tubes and the contents were mixed. The yellow colour developed was measured at 410 nm in a spectrophotometer (Hitachi Perkin Elmer). Controls included samples without $NO_3^-$ or without 2-hydroxy benzoic acid (only conc. $H_2SO_4$). The concentration of $NO_3^-$ was determined from a series of standards which were run at the same time as the unknown samples.

2.2.8.2 Nitrite

Nitrite was determined by the method of Nicholas and Nason (1957) and Hewitt and Nicholas (1964). An aliquot (10-1000 µl) containing 50 to 500 nmol $NO_2^-$ was diluted to 1 ml with double distilled water. The red azodye was developed by adding 1 ml 1% (w/v) sulphanilamide in 1.5N HCl followed by 1 ml of 0.02% (w/v) N-(1-naphthyl) ethylene diaminedihydrocholoride. After 15 min, the absorbance was read at 540 nm in 1 cm glass curvettes, in a Hitachi Perkin Elmer spectrophotometer. When $NO_2^-$ was determined in the cell samples, the solution after colour development was centrifuged for 10 min at 2,000 x g to sediment the cells and $NO_2^-$ in the supernatant was measured spectrophotometrically. The concentration of $NO_2^-$ was determined from a standard curve.

2.2.8.3 Protein

Protein was determined by the dye binding method of Bradford (1976) using bovine serum albumin as a standard protein. The absorbance was recorded at 595 nm in 1 cm glass cuvettes in a Hitachi Perkin Elmer spectrophotometer. Protein in the cell samples was first extracted by the method of Meyer *et al.* (1978) and then assayed by the dye binding method.

2.2.9 Enzyme assays

2.2.9.1 *Escherichia coli* nitrate reductase

Extracts of *E. coli* nitrate reductase were prepared as described by McNamara *et al.* (1971). The reaction mixture (3 ml) contained 0.5 ml 0.1M potassium phosphate buffer (pH 7.5), 0.5 ml 0.4M Na-formate, 25 µl *E. coli* nitrate reductase, 10-50 µl $NO_3^-$ containing sample or 0.1 ml 0.1M $KNO_3$ standard and double distilled water to 3 ml. The reaction

mixture was incubated at 45°C for 4h and the reaction was terminated by adding 1 ml 1% (w/v) sulphanilamide in 1.5N HCl. Then 1 ml 0.02% (w/v) N-1-naphthylethylenediamine dihydrochloride was added. After 15 min, 2 ml double glass distilled water was added and centrifuged at 2,000 x g for 10 min. The red colour in the supernatant was read at 540 nm in a Hitachi Perkin Elmer spectrophotometer.

2.2.9.2 Nitrate reductase in washed cells

Nitrate reductase activities were determined in a reaction mixture (1 ml) containing 20 mM Na-malate, 1 mM $KNO_3$ in 50 mM potassium phosphate buffer (pH 7.0) and 0.8 ml washed cells (3-4 mg dry wt.). The assays were carried out in glass tubes (8 x 75 mm) fitted with subaseals. The tubes were evacuated by a water suction pump and flushed with argon for 2 min. The reaction was started by injecting $KNO_3$, previously sparged with argon, into the tubes *via* a gas tight microsyringe. After incubating for 30 min at 30°C, samples (1 ml) were withdrawn. The enzyme activity was determined in two ways: (a) by measuring the $NO_2^-$ produced from $NO_3^-$. In this case the cells were pre-incubated with 2.5 mM KCN to inhibit nitrite reductase. The $NO_2^-$ produced was determined as described in Section 2.2.8.2. and the enzyme activity expressed in nmol $NO_2^-$ produced/30 min/mg dry wt. and (b) by measuring $NO_3^-$ utilization. Nitrate content of the samples were determined by nitration of salicylic acid (Section 2.2.8.1) and the enzyme activity expressed in nmol $NO_3^-$ utilized/30 min/mg dry wt. With either BVH or MVH as the electron donor (2 mM), the reaction was started by injecting 50 µl of 80 mM $Na_2S_2O_4$ in 2% (w/v) $NaHCO_3$ (freshly prepared under argon) and incubated for 10 min.

2.2.9.3 Nitrite reductase in washed cells

Nitrite reductase activity in washed cells was assayed in a reaction mix-

ture (1ml) containing 20 mM Na-malate, 1 mM $KNO_2$ and 0.8 ml cells (3-4 mg dry wt.) in 50 mM potassium phosphate buffer (pH 7.0) in glass tubes ( 8 x 75 mm) fitted with subaseals. The tubes were evacuated by a water suction pump, *via* needles inserted in the caps, and then flushed with argon for 2 min. The reaction was started by injecting $KNO_2$ previously sparged with argon into the tubes *via* a gas-tight micro-syringe. After 30 min incubation at 30°C samples (50 µl) were withdrawn for $NO_2^-$ determination. Nitrite content was determined as described in Section 2.2.8.2 and enzyme activity expressed in nmol $NO_2^-$ utilized/30 min/ mg dry wt. When 2 mM of either BVH or MVH was used as the electron donor, the reaction was started by injecting 50 µl of 80 mM $Na_2S_2O_4$ in 1% w/v $NaHCO_3$ (freshly prepared under argon) and then incubated for 10 min.

2.2.9.4 Nitrous oxide reductase in washed cells

$N_2O$-reductase activity in washed cells was assayed by measuring $N_2O$ utilization from the gas phase by injecting 100 µl samples into a GC/mass spectrometer (Hewlett Packard Model 5992B), fitted with a glass column (1.83 m length, 6 mm outer and 2 mm inner diameter) packed with Tenax GC (60-80 mesh). Helium flow was 2.5 ml/min with the electron multiplier at 1600 V. The assay was done in a reaction vial (6.5 ml) containing 1.5 ml washed cells (7.5 mg dry wt.) and either 0.5 ml of 0.5M Na-malate or 20 mM MVH. The vial was fitted with a rubber septum and the contents were evacuated and flushed with argon as described previously (Section 2.2.9.3). From the gas phase 0.9 ml argon was removed and an equal volume (20% v/v) of $N_2O$ was injected into the vial *via* an airtight micro-syringe, to start the reaction. The cells were incubated at 30°C and samples were withdrawn at intervals from the gas phase (4.5 ml) and analysed by the GC/mass spectrometer using a Selected Ion Monitor programme (SIM).

2.2.9.5 Glucose-6-$PO_4$-dehydrogenase in washed cells

Glucose-6-$PO_4$-dehydrogenase was assayed by following the oxidation of NADH at 340 nm (Conard and Schlegel, 1977). The assay mixture contained 0.1 ml 100 mM $MgCl_2$, 0.1 ml 15 mM NADH, 0.1 ml 120 mM glucose-6-$PO_4$, 0.1 ml cell suspension (20 mg wet wt./ml) and 50 mM Tris-HCl buffer (pH 7.5) to 3 ml. The reaction was carried out for 5 min at 20°C.

2.2.9.6 Nitrate reductase in cell-free extracts

Nitrate reductase was assayed in glass tubes (75 x 8 mm) fitted with subaseals. BVH was used as the electron donor. The reaction mixture in the tube contained 1.8 ml assay mixture (0.2M potassium phosphate buffer, pH 7.0, 0.2 mM BV and 5 mM $KNO_3$), 5-50 µl extract and buffer to 1.9 ml. The tube was then evacuated by a water suction pump *via* a needle inserted in the subaseal and flushed with argon. The reaction was started by injecting 100 µl of freshly prepared $Na_2S_2O_4$ (6 mg/ml) in 1% (w/v) $NaHCO_3$ under argon. After incubation for 10 min at 30°C, the reaction was stopped by vigorous agitation in air. Nitrite produced from $NO_3^-$ by nitrate reductase was measured spectrophotometrically as described in Section 2.2.8.2. Enzyme activity is expressed in nmol $NO_2^-$ produced/min/mg protein.

2.2.9.7 Nitrite reductase in cell-free extracts

This enzyme was also assayed in glass tubes (75 x 8 mm) fitted with subaseals using BVH as the electron donor. The reaction mixture in the tube contained 1.8 ml assay mixture (0.2M potassium phosphate buffer, pH 7.0, 0.2 mM BV and 100 µM $KNO_2$), 5-50 µl extract and buffer to 1.9 ml. The reaction was then carried out anaerobically at 30°C for 10 min as described previously (2.2.9.6). Remaining $NO_2^-$ was measured as described in Section 2.2.8.2. Enzyme activity is expressed in nmol $NO_2^-$ utilized/min/mg protein.

### 2.2.10 Determination of molecular weight of nitrite reductase by gel filtration

The molecular weight determination of nitrite reductase was determined by the method of Andrews (1970) using a Sephadex G-100 column. The column (1.5 x 150 cm) prepared as described in Section 2.2.19.2 was equilibrated with 0.1M Tris-HCl buffer (pH 7.5) and calibrated with BSA dimer (135 Kd), BSA monomer (67 Kd), ovalbumin (43 Kd), chymotrypsinogen A (25 Kd) and ribonuclease A (13.7 Kd). Blue dextran (≈2,000 Kd) was used to determine the void volume. The distribution coefficient (K) was calculated by the formula, $K = \frac{V_e}{V_o}$, where $V_e$ and $V_o$ are elution and void volumes respectively.

### 2.2.11 Native and SDS polyacrylamide gel electrophoresis (PAGE and SDS-PAGE)

Discontinuous, non-denaturing PAGE was carried out in 7.5% (w/v) polyacrylamide tube gells (Davis, 1964). The stacking gel was 3% (w/v) polyacrylamide in 125 mM Tris-HCl buffer (pH 6.8) and the running gel 7.5% (w/v) polyacrylamide in 375 mM Tris-HCl buffer (pH 8.8). The electrode buffer contained 12.5 mM Tris and 96 mM glycine (pH 8.4). Electrophoresis was carried out at 2 mA per gel at constant current. Gels were stained for protein with Coomassie brilliant blue R-250 (Charambach *et al.*, 1967). Activity band for the enzyme was detected by specific staining of the gel with BVH and its reduction by $KNO_2$. The gel was immersed in 10 ml reaction mixture containing 1 mM BV in 0.2M potassium phosphate buffer (pH 7.0) in a 15 ml test tube closed with a subaseal. The contents of the tube were evacuated for 2 min by a water suction pump *via* a needle inserted in the subaseal and then sparged with argon for 2 min. BV in the reaction mixture was then reduced by injecting 500 µl

of $Na_2S_2O_4$ (6 mg/ml in 1% w/v $NaHCO_3$) and the gel was incubated for 20 min at 30°C. The activity band for the enzyme was developed by placing the BVH-stained gel in a solution of 1 mM $KNO_2$ in 0.2M potassium phosphate buffer (pH 7.0). The gels were immediately photographed.

The subunit molecular weight of nitrite reductase was determined by discontinuous gel electrophoresis (12% w/v polyacrylamide) in the presence of 0.1% (w/v) sodium dodecyl sulphate (SDS) using Tris-glycine buffer (pH 8.3) according to the method of Laemmli (1970) and Weber and Osborn (1975). The gels were calibrated with the following SDS-treated protein standards: phosphorylase *b* (94 Kd), albumin (67 Kd), ovalbumin (43 Kd), carbonic anhydrase (30 Kd) and trypsin inhibitor (21.1 Kd). Gels were stained by Coomassie blue method of Charambach *et al.* (1967).

### 2.2.12 Proton translocation

#### 2.2.12.1 Oxidant pulse experiments

These experiments measured changes in proton concentration in the extracellular medium of a dense anaerobic suspension of cells (4.64 mg protein/ml) resulting from injections of small amounts (5 to 50 nmol) of either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$. The method used was a modification (Kristjansson *et al.*, 1978) of that described by Scholes and Mitchell (1970a, b). The apparatus was the same as that used by Walter *et al.* (1978) except that the pH electrode employed was a fast responding combination electrode with a flat pH sensing tip (Activon Scientific Products, N.S.W., Australia, Model 9210). The response of the equipment was limited by the injection and fluid mixing time which was about 1.5s. Typically, 40 mg wet wt. (9.3 mg protein) of cells in 2 ml of 150 mM KCl (pH 7.0) was supplemented with carbonic anhydrase (30 µg/ml) and the salt of a permanent ion (e.g. valinomycin) at sufficient concentration

(20 µg/ml) to collapse the membrane potential and allow proton ejection to be observed. This mixture was placed in the electrode chamber and allowed to equilibrate under argon for 10 min at 25°C, at which time the pH was adjusted as required by use of 10 mM HCl or NaOH sparged with argon. Once pH had stabilized, a small volume (1 to 10 µl) of argon saturated solution (in 150 mM KCl) of either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ was injected to initiate respiration and the subsequent proton response was recorded in an omniscribe recorder (Houston Instruments, Tx, U.S.A.) at 1 mV with a full scale deflection of 0.38 pH units. The response was calibrated by injecting an appropriate amount of 5 mM HCl (in 150 mM KCl, pH 7.0) sparged with argon, the concentration of which was determined titrimetrically with reference to accurately weighed Tris-base.

Light, from a 150w Tungsten lamp (incandescent) filtered through a flat bottle filled with distilled water, was used for all light reactions. Aluminium foil was wrapped around the reaction vessel when experiments were conducted in the dark. Cells were pre-incubated with valinomycin (20 µg/ml) for 1h either in light or in the dark. When BVH was used as the electron donor, the cells were pre-incubated with 1 mM NEM and 0.5 mM HOQNO for 10 min to inhibit respiration linked $H^+$ extrusion. When DIECA, or $NaN_3$ were used, the cells were pre-incubated with either inhibitor for 10 min and in case of CCCP for 2 min, prior to pulse experiments.

A positive response to either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ by bacteria involved a rapid acidification of the medium, followed immediately by a slow ($t_{\frac{1}{2}}$ = approximately 1 min) passive diffusion of protons back across the cell membrane. The latter process tended to diminish the amplitude of the initial rapid pH transient by about 5 to 20%, depending on respiration rates and the permeability of the membrane to protons. To correct for this effect the decay curve for passive $H^+$ diffusion was extrapolated

back to a time, approximately 1 to 2s after oxidant injection, at which time the initial transient had reached half its final amplitude as shown by Scholes and Mitchell (1970b). A negative response to the oxidants ($NO_3^-$, $NO_2^-$ and $N_2O$) was indicated by a rapid alkalinization of the external medium which was corrected in the same way. The overall reactions of the various electron acceptors are given in equations 1-7.

$$O + 2e^- + 2H^+ \longrightarrow H_2O \quad (1)$$

$$NO_3^- + 5e^- + 6H^+ \longrightarrow 0.5N_2 + 3H_2O \quad (2)$$

$$NO_3^- + 4e^- + 5H^+ \longrightarrow 0.5N_2O + 2.5H_2O \quad (3)$$

$$NO_3^- + 2e^- + 2H^+ \longrightarrow NO_2^- + H_2O \quad (4)$$

$$NO_2^- + 3e^- + 4H^+ \longrightarrow 0.5N_2 + 2H_2O \quad (5)$$

$$NO_2^- + 2e^- + 3H^+ \longrightarrow 0.5N_2O + 1.5H_2O \quad (6)$$

$$NO_2^- + 2e^- + 2H^+ \longrightarrow N_2 + H_2O \quad (7)$$

2.2.12.2 Estimation of stoichiometric protons

The reactions considered in equations 1-7 (Section 2.2.12.1) involve the stoichiometric production of protons. The $^{\rightarrow}H^+$/oxidant ratios were calculated as described in Section 2.2.12.1 and then $^{\rightarrow}H^+/2e^-$ ratios were also determined. The $^{\rightarrow}H^+/2e^-$ ratio for the reduction of $NO_3^-$ and $NO_2^-$ to $N_2O$ and $N_2$ was calculated by the method of Boogerd *et al.* (1981). From equations 1-7 (Section 2.2.12.1) the following relations can be derived:

$$^{\rightarrow}H^+/2e^- \ (NO_3^- \rightarrow 0.5\ N_2) = [^{\rightarrow}H^+/NO_3^- \ (\rightarrow NO_2^-) + {}^{\rightarrow}H^+/NO_2^- \ (\rightarrow 0.5\ N_2O) + 0.5\,{}^{\rightarrow}H^+/N_2O(\rightarrow N_2) + \text{number of protons consumed}]\ [2.5]^{-1} \quad (1)$$

$$^{\rightarrow}H^+/2e^-(NO_3^- \rightarrow 0.5N_2O) = [^{\rightarrow}H^+/NO_3^-(\rightarrow NO_2^-) + {}^{\rightarrow}H^+/NO_2^-(\rightarrow 0.5N_2O) + \text{numbers of protons consumed}\ [2]^{-1} \quad (2)$$

$$^{\rightarrow}H^+/2e^-(NO_2^- \rightarrow 0.5N_2\ ) = [^{\rightarrow}H^+/NO_2^-(\rightarrow 0.5N_2O) + 0.5H^+/N_2O(\rightarrow N_2) + \text{number of protons consumed}\ [1.5]^{-1} \quad (3)$$

### 2.2.13 Determination of membrane potential ($\Delta\Psi$) and transmembrane pH gradient ($\Delta pH$) in washed cells

#### 2.2.13.1 EDTA treatment

Cells (0.5g wet wt.) were suspended in 50 mM sodium phosphate buffer (25 ml) containing 10 mM EDTA (pH 7.0) in a 50 ml Erlenmeyer flask closed with a subaseal. After evacuating for 5 min *via* a needle inserted into the subaseal, the flask was filled with argon and the reaction mixture incubated at 30°C for 10 min. The cells, collected by centrifugation at 10,000 x g for 10 min, were washed once in the phosphate buffer and then resuspended (1 to 1.5 mg dry wt./ml) in the same buffer at various pH values (6.0 to 8.0). The EDTA-treated cells were used immediately.

#### 2.2.13.2 Intracellular water space

This was determined in washed cells (not treated with EDTA) by using [$^3H_2O$] (for total pellet water) and [$^{14}C$] inulin (for extracellular water) according to the methods of Maloney *et al.* (1975) and Stock *et al.* (1977). Thus for *R. sphaeroides* f. sp. *denitrificans* the intracellular space was 1.47 ± 0.10 µl/mg dry wt.

#### 2.2.13.3 Uptake of radioactive probes

Isotopically labelled compounds, either [$^3H$]-$TPP^+$ (8.4 µM) or [$^{14}C$]-benzoic acid (8.8 µM) with either $KNO_3$ (10 mM) or $KNO_2$ (5 mM), in a total volume of 0.5 ml, were dispensed into 1.5 ml Eppendorf centrifuge tubes (2 ml glass tubes for experiments in light) fitted with subaseals. The tubes, evacuated *via* needles inserted into the subaseals, were then filled with argon. Tubes were covered with black polythene sheets for the dark reaction. The reaction was started by injecting 0.4 ml of either untreated or EDTA-treated cell-suspensions (0.8 to 1.5 mg dry wt./ml) in either $Na^+$ or $K^+$ phosphate (50 mM) or Tris-HCl (100 mM) buffer at

the appropriate pH *via* gas-tight microsyringes and the incubation continued for 10 min at 25°C. When either $O_2$ or $N_2O$ was used as the terminal electron acceptor, cell suspensions were first sparged with the appropriate gas (20 ml/min) for 15 min prior to injection of the cell suspension into the reaction mixture. After the prescribed incubation period samples were centrifuged immediately in 1.5 ml Eppendorf tubes through 0.5 ml silicone oil mixture (75% v/v fluid 550 and 25% v/v fluid 510/50 cs, Dow Corning Australia Pty. Ltd., BLacktown, N.S.W.) in an Eppendorf microfuge at 13,000 x g for 30s. This short-term centrifugation separated the cells from the aqueous phase but a further centrifugation (3 min) was done to ensure that any residual cells trapped in the oil were sedimented. When the experiments were conducted in light, the cell suspensions were quickly (10-15s) transferred from glass tubes into Eppendorf tubes in the presence of tungsten lights (150w) and then centrifuged as described above. Portions of the supernatant (200 µl) were transferred into scintillation glass vials (15 ml) and the rest of the supernatant discarded. Tubes containing the pellets were swabbed dry with cotton buds to remove any adhering droplets (Kashket and Wong, 1969). The pellets were resuspended in 200 µl of distilled water. Supernatant and pellet fractions, respectively, were added to 0.8 ml of 3M perchloric acid (PCA) in 15 ml scintillation vials. After standing overnight, to dissolve cell proteins, 5 ml of a scintillation-counting fluid, phase-combining system (PCS, Amersham, Australia) was added to each vial and the contents mixed thoroughly and radioassayed in a Packard Tri-carb 460CD liquid scintillation spectrometer. The $\Delta\Psi$ values were corrected for $TPP^+$ binding in de-energised cells (treated with 50 µM CCCP). $\Delta\Psi$ was determined from [$^3$H]-$TPP^+$ uptake and $\Delta$pH from that of [$^{14}$C]-benzoic acid.

2.2.13.4 Calculations of proton motive force ($\Delta p$ or $\tilde{\mu}_{H^+}$)

The calculations of $\Delta\Psi$ and $\Delta pH$ were made by using the Nernst equation as described by Mitchell (1966) after correcting for the uptake data for non-specifically bound [$^3$H]-$TPP^+$ and that bound in de-energised cells and extracellular counts of [$^{14}$C]-benzoic acid respectively. Membrane potential was calculated from the uptake of [$^3$H]-$TPP^+$ as follows:

$$\Delta\Psi = \frac{RT}{F} \ln \frac{[TPP^+]\ in}{[TPP^+]out}$$

or $$\Delta\Psi = -2.303\ \frac{RT}{F} \log \frac{[TPP^+]\ in}{[TPP^+]out}$$

At 25°C (T = 298°K) 2.303 RT/F ≃ 59 mV

i.e., $\Delta\Psi = 59 \times \log \frac{[TPP^+]\ in}{[TPP^+]out}$

where $\frac{[TPP^+]\ in}{[TPP^+]out}$ is the ratio of intracellular [$^3$H]-$TPP^+$ to extracellular [$^3$H] $TPP^+$.

Intracellular pH ($pH_{in}$) was calculated from the distribution of [$^{14}$C]-benzoic acid;

$$pH_{in} = pK + \log [Acid_{in}/Acid_{out}\ (10^{pH_{out}-pK} + 1) - 1]$$

$\Delta pH$ was obtained from the difference of $pH_{in}$ and $pH_{out}$ ($\Delta pH = pH_{in} \sim pH_{out}$). Proton motive force ($\Delta p$) was calculated as:

$$\Delta p = \Delta\Psi - 2.303\ \frac{RT}{F}\ \Delta pH \text{ or at } 25°C,\ \Delta p = \Delta\Psi - 59 \times \Delta pH$$

2.2.14 Proline transport

2.2.14.1 Measurement of proline uptake

[$^{14}$C]-proline uptake by washed cells in light and in the dark was determined in the presence of either $NO_3^-$ (10 mM), $NO_2^-$ (5 mM), $N_2O$ (25 mM) or $O_2$ (23.5 µM) as the terminal acceptor. The reaction mixture (0.5 ml) contained [$^{14}$C]-proline (10 µM), the terminal acceptor and

0.4 ml EDTA-treated or $Na^+$-loaded anaerobic (evacuation and flushing with argon) cell-suspension (0.8 to 1.0 mg dry wt./ml) in 50 mM potassium phosphate buffer (pH 7.0). Either NaCl or KCl (20 mM) was also included in the assay mixture to investigate the effects of these compounds on proline uptake. The assay was carried out anaerobically under argon at 25°C for 10 min. The accumulation of [$^{14}$C]-proline in the cells was determined by liquid scintillation spectrometry as described in Section 2.2.13.3. The chemical potential of proline ($\Delta\mu$pro) was determined using the Nernst equation.

2.2.14.2 Sodium loading of cells

Washed cells were suspended to 10 mg wet wt. of cells/ml in 100 mM Tris-HCl buffer (pH 7.5). The cell suspension was evacuated with a water pump for 5 min and then incubated anaerobically under argon in the presence of 10 mM EDTA (pH 7.5) at 30°C for 10 min. The cells were then diluted 20 fold with 100 mM Tris-HCl (pH 7.5) to dilute out EDTA, centrifuged at 20,000 x g for 10 min, washed twice with 50 mM Tris-HCl (pH 7.5) buffer containing 50 mM NaCl and finally suspended in the same buffer to 0.8-1.0 mg dry wt./ml for uptake studies.

2.2.15 $Na^+/H^+$ exchange studies

2.2.15.1 $\Delta$pH-dependent $Na^+$ uptake

A pH gradient was created across the cytoplasmic membranes of washed cells by first pre-equilibrating the cells in MES buffer (pH 6.0) followed by dilution of the cells in HEPES buffer (pH 8.0) and *vice versa*. The second buffer in each case contained $^{22}Na^+$ (2 $\mu$Ci/ml). The uptake of this radioisotope by the cells was measured after filtration through Millipore filters (0.45 $\mu$m). The washed cells (40 mg wet wt./ml) were

soaked for 16h at 4°C in an appropriate buffer, either 50 mM MES (pH 6.0) or 50 mM HEPES buffer (pH 8.0), each containing 1mM EDTA. These cells were then centrifuged at 20,000 x gfor 30 min at 4°C and resuspended (25 mg dry wt./ml) in the respective buffers without EDTA. To initiate ΔpH-dependent $^{22}Na^{+}$ uptake, an aliquot (20 µl) of the cells pre-equilibrated in MES buffer (pH 6.0) was diluted to 1 ml at 25°C with 50 mM HEPES buffer (pH 8.0) containing 50 mM NaCl and $^{22}NaCl$ (2 µCi/ml) and *vice versa*. Aliquots (100 µl) of the diluted cells were withdrawn at intervals and filtered through Millipore filters (0.45 µm). The cells trapped on the filters, were washed twice with 2 ml buffered choline chloride (0.1M choline in 10 mM Tris-HCl, pH 7.5). Filters plus the cells were transferred into 1.5 ml Eppendorf tubes containing 1 ml toluene-based scintillation counting fluid (0.3% w/v PPO and 0.03% w/v POPOP in toluene). The tubes were then placed in 15 ml glass vials and radioassayed in a Packard Tri-Carb : 460CD liquid scintillation spectrometer. Corrections were made for the $^{22}Na^{+}$ retained by filters in the absence of cells.

2.2.15.2 $Na^{+}$-pulse experiments

These experiments were carried out in a similar way to oxidant pulse experiments (Section 2.2.12.1) using a pH electrode and a recorder. Cells, washed and resuspended (50 mg dry wt./ml) in 50 mM KCl (pH 7.0) were used for all the experiments. $Na^{+}$-pulses (50 µl 2M NaCl) were given to either aerobic or anaerobic cells (evacuated by a water pump for 5 min and flushed with argon) either in light or in the dark. A constant flow (10 ml/min) of either $O_2$ for aerobic cells or argon for anaerobic cells, was sustained throughout the experiments. Recalibration of the pH electrode was done after each experiment using 50 µl 10 mM HCl or NaOH standards.

### 2.2.16 $K^+/H^+$ exchange studies

#### 2.2.16.1 ΔpH-dependent $K^+$ uptake

A pH gradient across the cytoplasmic membranes of washed cells was created as described previously (Section 2.2.15.1). To initiate ΔpH-dependent $K^+$ uptake, an aliquot (20 µl) of cells pre-equilibrated in 50 mM MES buffer (pH 6.0) was diluted to 1 ml at 25°C with 50 mM HEPES buffer (pH 8.0) containing 50 mM KCl and *vice versa*. Samples (100 µl) of the diluted cells were withdrawn at intervals, filtered through Millipore filters (0.45 µm) and washed twice with 2 ml buffered choline chloride. The filters containing the cells were immersed in 5 ml 5% (w/v) trichloroacetic acid (TCA) in 10 ml plastic centrifuge tubes (acid washed) and left overnight. The $K^+$ content of TCA extracts were determined in a Pye Unicam SP9 atomic absorption spectrometer. The $K^+$ content was measured at 766 nm after adding 1 mg/ml $CsCl_2$ to suppress interference by ionization.

#### 2.2.16.2 $K^+$-pulse experiments

$K^+$-pulse experiments were done in a similar way to the oxidant pulse experiments using the same equipment (pH electrode, recorder, etc.) as described previously (Section.2.2.12.1). Cells washed and resuspended (50 mg dry wt./ml) in 5 mM Tris-HCl (pH 6.5), containing 1 mM dithiothreitol (DTT), were used for these experiments. $K^+$-pulses (50 µl 2M KCl) were given to either aerobic or anaerobic (evacuated by a water pump for 5 min then flushed and kept under argon) suspension of cells either in light or in the dark. The cells were sustained under a constant flow (10 ml/min) of either $O_2$ or argon at 25°C. The pH electrode was re-calibrated after each experiment using 50 µl 10 mM HCl or NaOH.

2.2.17 $Na^+$ and $K^+$ transport

2.2.17.1 $K^+$-depletion of cells

The cells were depleted of intracellular $K^+$ by treatment with diethanolamine (DEA) at pH 9.2 (Nakamura *et al.*, 1982). Freshly harvested cells (0.5g wet wt.), washed twice in 50 mM Tris-HCl buffer (pH 7.5), were suspended in 4 ml 10 mM HEPES-NaOH buffer (pH 7.5). This cell suspension was then added to 21 ml 50 mM DEA-HCl/150 mM NaCl (pH 9.2) and incubated at 30°C for 1h. The cell suspension was then centrifuged at 20,000 x g for 10 min. The cells, washed once in an appropriate buffer, contained less than 5 mM intracellular $K^+$. The DEA-treated cells are referred to as $K^+$-depleted cells.

2.2.17.2 $^{22}Na^+$ loading of $K^+$-depleted cells

$K^+$-depleted cells prepared by DEA-treatment were washed once in 50 mM Tris-HCl buffer (pH 7.5) and then suspended in the same buffer (0.5g wet wt./ml). Then 0.1 ml of this cell suspension was diluted 10 times in 100 mM Na-HEPES buffer (pH 7.5) in a glass tube (5 x 1.2 cm) containing $^{22}NaCl$ (2 µCi). The reaction mixture was incubated at 30°C for 1h in a reciprocating water bath in air in the dark.

2.2.17.3 $Na^+$, $K^+$ and $Cu^{2+}$ determinations by atomic absorption

Cell suspensions (25 mg wet wt./ml) were filtered through Millipore filters (0.22 µm or 0.45 µm) and washed twice with 2 ml buffered choline chloride (0.2M choline chloride in 10 mM Tris-HCl, pH 7.5). The filters were immersed in 5 ml 5% (w/v) trichloroacetic acid (TCA) in 10 ml plastic centrifuge tubes (acid washed) and left overnight. The $Na^+$ and $K^+$ contents of TCA extracts were determined in a Pye Unicam SP9 atomic absorption spectrometer. The $K^+$ content was determined at 766 nm after

adding 1 mg/ml $CsCl_2$ to suppress interference by ionization. Similarly the $Na^+$-content was determined at 589 nm and 2 mg/ml KCl was added to the extracts as an ionization suppressant. The equipment was calibrated with standard solutions of KCl and NaCl before and after each set of 6 determinations. Appropriate controls for $K^+$ and $Na^+$ contents (in filters, plastic tubes, TCA and double glass distilled water) were always included. All solutions used in atomic absorption studies were dispensed with double glass distilled water. Copper content of the purified nitrite reductase was also determined by atomic absorption spectrometry. 0.5 ml purified nitrite reductase (0.12 mg protein) was dried in an acid washed Pyrex conical flask and then digested for 20 min with 2.5 ml concentrated $HNO_3$. After cooling, the sample was diluted to 2.5 ml with double glass distilled water which was then injected directly into the atomic absorption spectrometer and read at 325 nm. The equipment was calibrated with 0 to 2 ppm standard solutions of copper in double glass distilled water.

2.2.18 GC/MS studies with $^{15}NO_3^-$ and $^{15}NO_2^-$

Stoichiometries for $^{15}NO_3^-$ and $^{15}NO_2^-$ reduction by washed cells *via* $N_2O$ to $N_2$ were determined by analysing the gaseous products formed during the reaction in a GC/mass spectrometer (Hewlett Packard Model 5992B). The GC/MS was fitted with a glass column (1.83 m length, 5 mm outer and 2 mm inner diameter) packed with Tenax GC (60-80 mesh). Helium flow was 2.5 ml/min with the electron multiplier at 1600v. The reaction was conducted in a glass vial (6.5 ml) contained 1.5 ml washed cells (7.5 mg dry wt.), 20 mM MV and either $K^{15}NO_3$ (7 mM) or $K^{15}NO_2$ (5.5 mM). The contents of the vials was evacuated by a water pump (*via* needles inserted into the subaseal) for 2 min and flushed with argon. The reactions

conducted anaerobically at 30°C were initiated by injecting 40 mM $Na_2S_2O_4$ (in 1% w/v $NaHCO_3$) sparged with argon. Samples (50 µl) were withdrawn from the gas phase (4.5 ml) at intervals *via* a gas-tight microsyringe and injected into the GC/MS unit. Data were obtained by Selected Ion Monitoring (SIM) programme. The degree of ionization-induced fragmentation of $N_2O^+$ into $NO^+$ and $N_2^+$ was similar to those reported previously (Cady and Bartholomew, 1960; St. John and Hollocher, 1977). Nitrate utilization was monitored by determining the $NO_3^-$ content by the *E. coli* nitrate reductase method (Section 2.2.9.1) and that of $NO_2^-$ by the method of Nicholas and Nason (1957). To determine the $NO_3^-$:$NO_2^-$ stoichiometry, 2.5 mM KCN was included in the reaction mixture in a parallel experiment to inhibit nitrite reductase. $K^{15}NO_3$ and $K^{15}NO_2$ used were enriched 81.9 and 27.5 atom % excess respectively.

$^{15}N_2O$ the product of nitrite reductase in the photodenitrifier was also identified by GC/MS using $K^{15}NO_2$ (97 atom % excess). The reaction was carried out anaerobically in a 3.5 ml test tube containing 2 5 ml reaction mixture. The reaction mixture contained 0.2 ml enzyme (1.4 µg protein), 20 mM $K^{15}NO_2$ and 40 mM MV in 0.2M potassium phosphate buffer (pH 7.0). The reaction was started by injecting 80 mM $Na_2S_2O_4$ (in 1% w/v $NaHCO_3$ in argon). The reaction was conducted at 30°C under argon. Samples (from the gas phase) withdrawn at intervals were analysed by the GC/MS as described above.

### 2.2.19 General techniques

#### 2.2.19.1 Liquid scintillation spectrometry

Radioactivity in aqueous samples ($^{14}C$ or $^{3}H$) was measured by counting aliquots in 'PCS' scintillation fluid in Packard glass vials.

The ratio of sample volume to scintillation fluid volume was 1:5 according to the recommendations of the manufacturer (Amersham, England).

Radioactivity on Millipore filters ($^{22}Na^{+}$) was measured in toluene based scintillation fluor (0.3% w/v PPO and 0.03% w/v POPOP in toluene) in Packard glass vials. The vials were assayed in a Packard Tri-Carb liquid scintillation spectrometer (Model 460CD).

2.2.19.2 Preparation of chromatographic columns

Gel filtration columns (Sephadex G100) and ion exchange column (DEAE Sephadex A50) were prepared according to the instructions given by the manufacturers (Pharmacia Fine Chemicals, Uppsala, Sweden). The columns were equilibrated with appropriate buffers and when not in use they were stored at 2°C in the appropriate buffer in the presence of 0.1% (w/v) sodium azide.

# 3. RESULTS

# 3. RESULTS

## 3.1 GROWTH AND DENITRIFICATION IN *R. SPHAEROIDES* F. SP. *DENITRIFICANS*

### 3.1.1 Aerobic and anaerobic cultures in light and in the dark

The cells grew well photosynthetically both under aerobic and anaerobic conditions ($A_{660\ nm}$ at 30h stage; aerobic, 1.3; anaerobic 1.35) in a culture medium containing 21.4 mM Na-malate as the carbon source (composition described in Section 2.2.2). Cells grown aerobically in light were brown-red in colour whereas those of photodenitrifying cultures grown anaerobically with 20 mM $KNO_3$ as the terminal electron acceptor, were yellowish-brown. The growth rates of these two cultures were similar ($A_{660\ nm}$ = 0.60-0.67) up to mid-exponential phase (12h) and then the photodenitrifying cells reached stationary stage after 20h growth whereas those grown under aerobic conditions grew more slowly reaching the stationary phase after 30h (Fig. 3). Bacterial growth was less in air in the dark ($A_{660\ nm}$ at 30h stage = 0.87) i.e. about 70% of the phototrophic (aerobic or anaerobic) growth at 30h stage (Fig. 3). Under dark denitrifying conditions (anaerobic with 20 mM $KNO_3$) the bacteria grew slowly (Fig. 4) and mid-exponential stage was reached after 50h and a stationary phase after 144h ($A_{660\ nm}$ = 0.55).

### 3.1.2 Nitrate utilization and nitrite production

When grown under denitrifying conditions in light or in the dark, $NO_2^-$ accumulated in the culture medium. The cells (1L culture) rapidly utilized $NO_3^-$ when grown in the dark. Thus after 72h growth these cells utilized almost all the $NO_3^-$ in the culture solution (5.73 out of 5.80 mmol) and about 5.0 mmol $NO_2^-$ was produced (Fig. 4). The $NO_2^-$ was subsequently taken up when all the $NO_2^-$ had been utilized (3.1 mmol $NO_2^-$ was utilized between 7 and 96h growth). In denitrifying cultures (120 ml), grown in light,

FIG. 3: GROWTH OF *R. SPHAEROIDES* F. SP. *DENITRIFICANS*.

Cells were grown at 30°C in culture solutions containing DL-malate (Na salt, 21.4 mM) and $(NH_4)_2HPO_4$ (12 mM) as carbon and nitrogen sources, respectively. Denitrifying medium was supplemented with $KNO_3$ (20 mM). Phototrophic cultures were illuminated with 4 Phillips Philinea tubes (60w, 250v, ca 5000 lux). Denitrifying cultures were grown anaerobically at 30°C in 120 ml screw-cap bottles completely filled with sterile culture solution and closed with rubber septa through which sterile needles were inserted. In aerobically grown cultures (2L) sterile $O_2$ (250 ml/min) was sparged through culture solutions. When cells were grown in the dark, the flasks were completely covered with black polythene sheets.

△ Light anaerobic;
○ Light aerobic;
● Dark aerobic.

FIG. 4: CELLS GROWN WITH NITRATE IN THE DARK.

Cells were grown anaerobically in the dark in 300 ml screw-cap bottles completely filled with a culture solution containing 20 mM $KNO_3$ and closed with rubber septa. A sterile needle was inserted through the septum just above the liquid surface. Other growth conditions are given in Fig.3. Nitrate concentrations in the culture solution were determined by *E. coli* nitrate reductase method (Section 2.2.9.1). Nitrite concentrations were determined spectrophotometrically at 540 nm (Section 2.2.8.2).

● Growth;
△ Residual $NO_3^-$; ○ $NO_2^-$ produced.

FIG.3

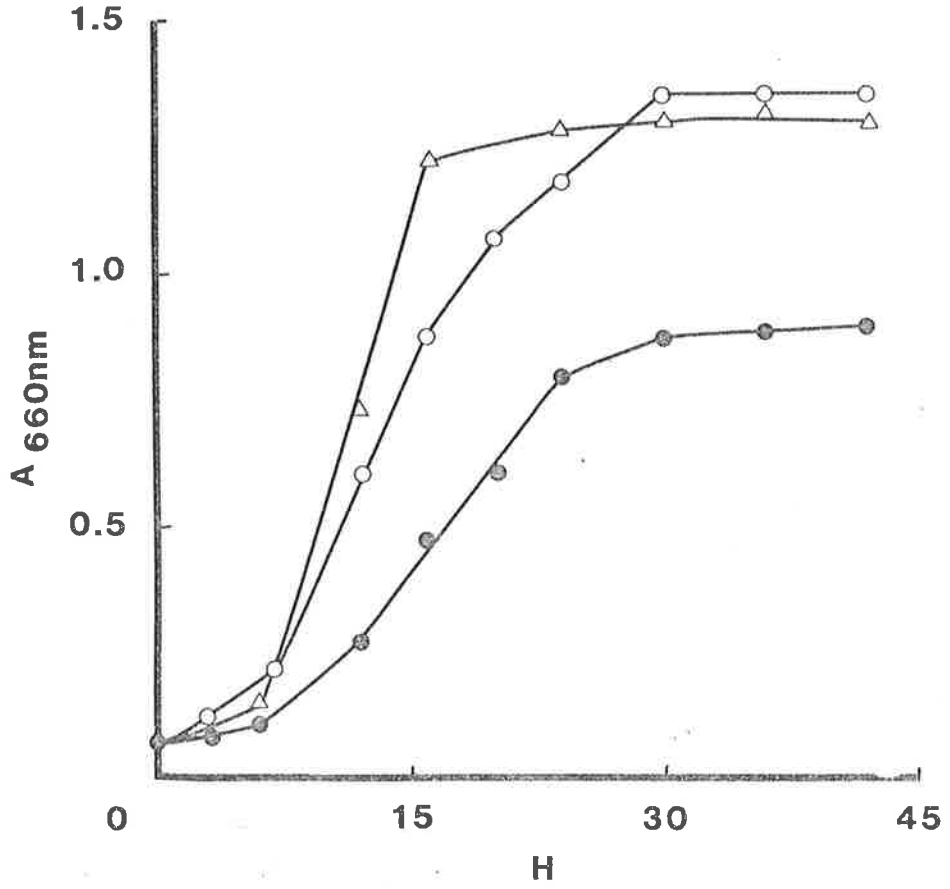
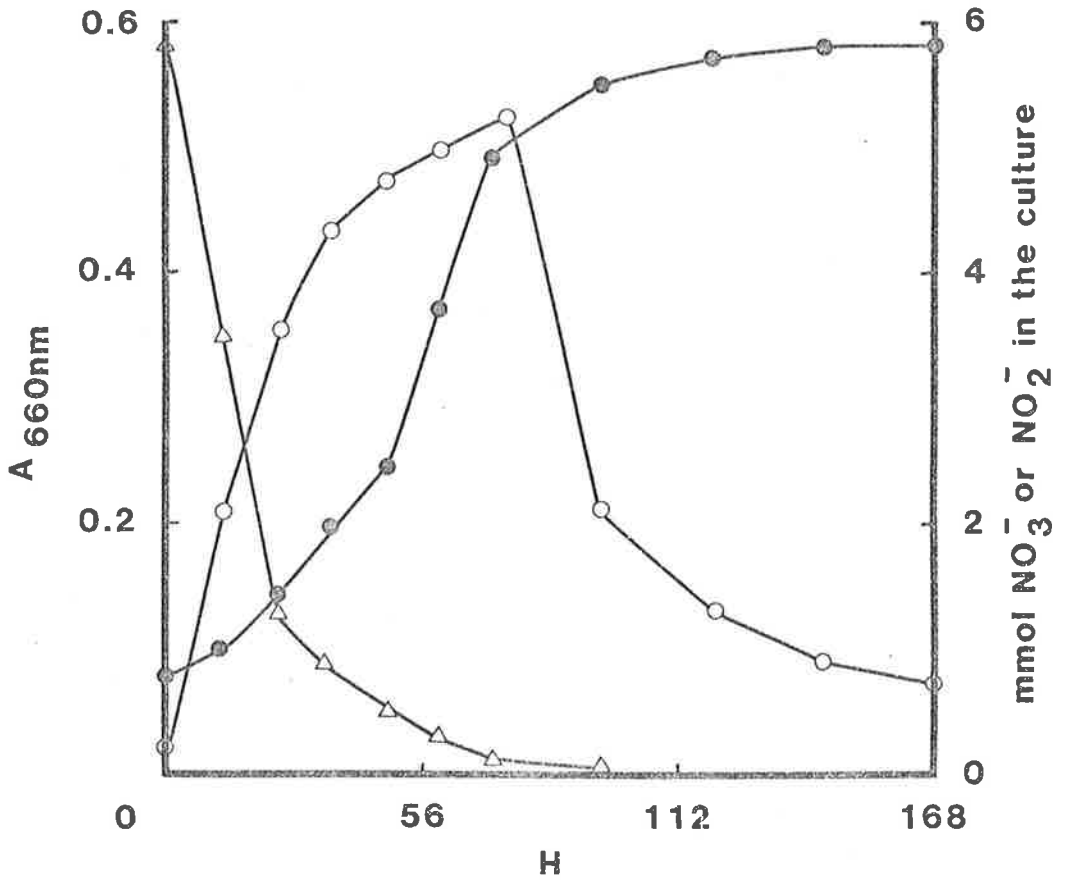

1.5
1.0
0.5
A 660nm
0
15
30
45
H

FIG.4


0.6
0.4
0.2
A 660nm
0
56
112
168
H
6
4
2
0
mmol NO3- or NO2- in the culture

$NO_3^-$ uptake was slow initially (0.07 mmol $NO_3^-$ utilized after 6h growth) followed by a steady increase in the uptake *viz.* 0.9 mmol after 20h (Fig. 5). Nitrite was detected in these cultures after 6h growth, increasing to 5.0 mM after 30h.

### 3.1.3 Nitrate, nitrite and nitrous oxide reductase activities of washed cells

Nitrate, nitrite and nitrous oxide reductase activities of washed cells were determined at various stages of growth. In cells grown with $NO_3^-$ anaerobically in the dark, the nitrate reductase activity increased linearly to 96 nmol $NO_2^-$ produced/30min/mg dry wt. at the 72h stage and then declined to 60 nmol/30 min/mg dry wt. (Fig. 6) at the stationary phase (168h). Both nitrite reductase (25 nmol $NO_2^-$ utilized/30 min/mg dry wt.) and nitrous oxide reductase activities (0.17 µmol $N_2O$ utilized/3h/mg dry wt.) remained low up to 72h, then there was a sharp increase in activities of both enzymes. The maximum activity of nitrite reductase (234 nmol $NO_2^-$ utilized/30 min/mg dry wt.) was recorded after 120h growth and then it decreased to 170 nmol after 168h. The nitrous oxide reductase activity reached a maximum after 144h growth.

In washed cells, grown anaerobically under photodenitrifying conditions, there was a steady increase in nitrate reductase activity up to 12h (130 nmol $NO_2^-$ formed/30 min/mg dry wt.) followed by a slow decrease to 45 nmol (Fig. 7) after 30h. Nitrite reductase was more active than nitrate reductase during first 20h growth period. A maximum of 432 nmol of $NO_2^-$ was utilized/30 min/mg dry wt. cells at the 12h stage of growth followed by a sharp decrease in activity. Maximum $N_2O$ reductase activity (2.55 µmol $N_2O$ utilized/3h/mg dry wt.) was recorded in 24h grown cells and there was no appreciable decrease in activity after 30h. Nitrite reductase was completely inhibited by 2.5 mM KCN so that it was possible

FIG. 5: CELLS GROWN ANAEROBICALLY WITH $NO_3^-$ IN LIGHT.

Cells were grown in light with $NO_3^-$ as the terminal acceptor as described in Fig.3. Nitrate and $NO_2^-$ concentrations in the culture solutions were determined as in Fig.4.

● Growth;
△ Residual $NO_3^-$; ○ $NO_2^-$ produced.

FIG. 6: NITRATE, NITRITE AND NITROUS OXIDE REDUCTASE ACTIVITIES AT VARIOUS STAGES OF GROWTH IN WASHED CELLS GROWN UNDER DARK DENITRIFYING CONDITIONS.

The cells were harvested at intervals by centrifuging at 20,000 x g for 20 min at 4°C in a RC5B centrifuge (SS34 rotor, Sorvall, Norwalk, USA). Cells were washed twice in 50 mM potassium phosphate buffer (pH 7.0) and resuspended (4-5 mg dry wt./ml) in the same buffer. Sodium malate was used as the electron donor (20 mM for cells assayed for nitrate and nitrite reductases, 125 mM for cells assayed for nitrous oxide reductase). Nitrate reductase was assayed anaerobically at 30°C in the presence of 2.5 mM KCN by measuring the $NO_2^-$ produced (Section 2.2.8.2) from $NO_3^-$ and nitrite reductase activity was determined by following $NO_2^-$ utilization (Section 2.2.9.3) at 30°C under anaerobic conditions. Both enzymes were assayed for 30 min. Nitrite concentrations were measured spectrophotometrically at 540 nm (Section 2.2.8.2). Nitrate and nitrite reductases were assayed in a reaction mixture (1 ml) containing 0.8 ml cells (4 mg dry wt.) in 50 mM potassium phosphate buffer (pH 7.0), 50 µl of either 20 mM $KNO_3$ or 20 mM $KNO_2$ (final conc. 1 mM) and 50 µl Na-malate (final conc. 20 mM). Nitrous oxide reductase activity was assayed (Section 2.2.9.4) by measuring $N_2O$ utilization in a GC/mass spectrometer (Hewlett Packard Model 5992B). The assay mixture (2 ml) contained 1.5 ml cells in 50 mM potassium phosphate buffer, pH 7.0, (7.5 mg dry wt.) and Na-malate (125 mM). The gas phase (4.5 ml) consisted of 20% (v/v) $N_2O$ and 80% (v/v) argon. The assay was carried out for 3h at 30°C. Experiments were conducted in triplicate and errors were within 5%.

● Growth;
△ $NO_3^-$-reductase (nmol $NO_2^-$ produced/30 min/mg dry wt.);
▲ $NO_2^-$-reductase (nmol $NO_2^-$ utilized/30 min/mg dry wt.);
○ $N_2O$-reductase (µmol $N_2O$ utilized/3h/mg dry wt.).

FIG.5

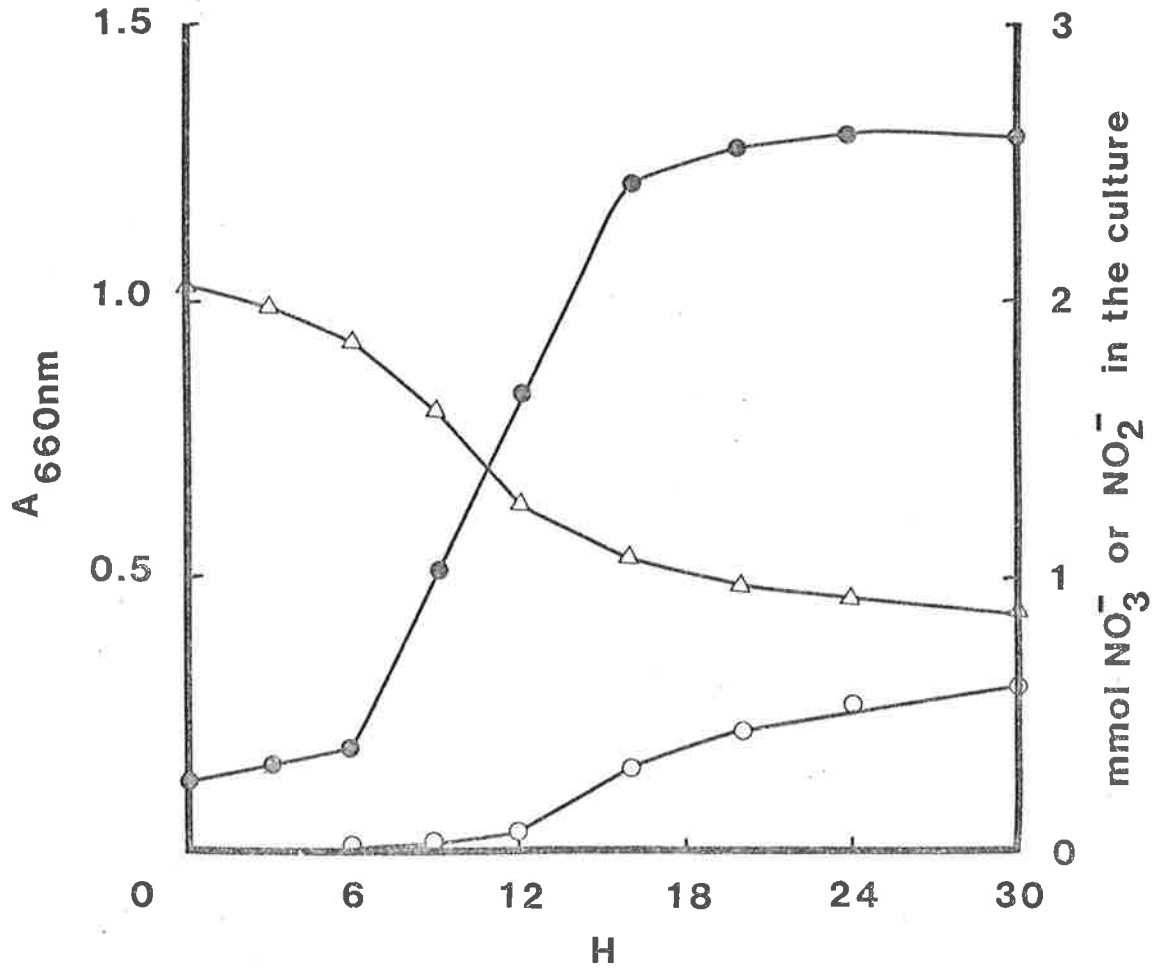
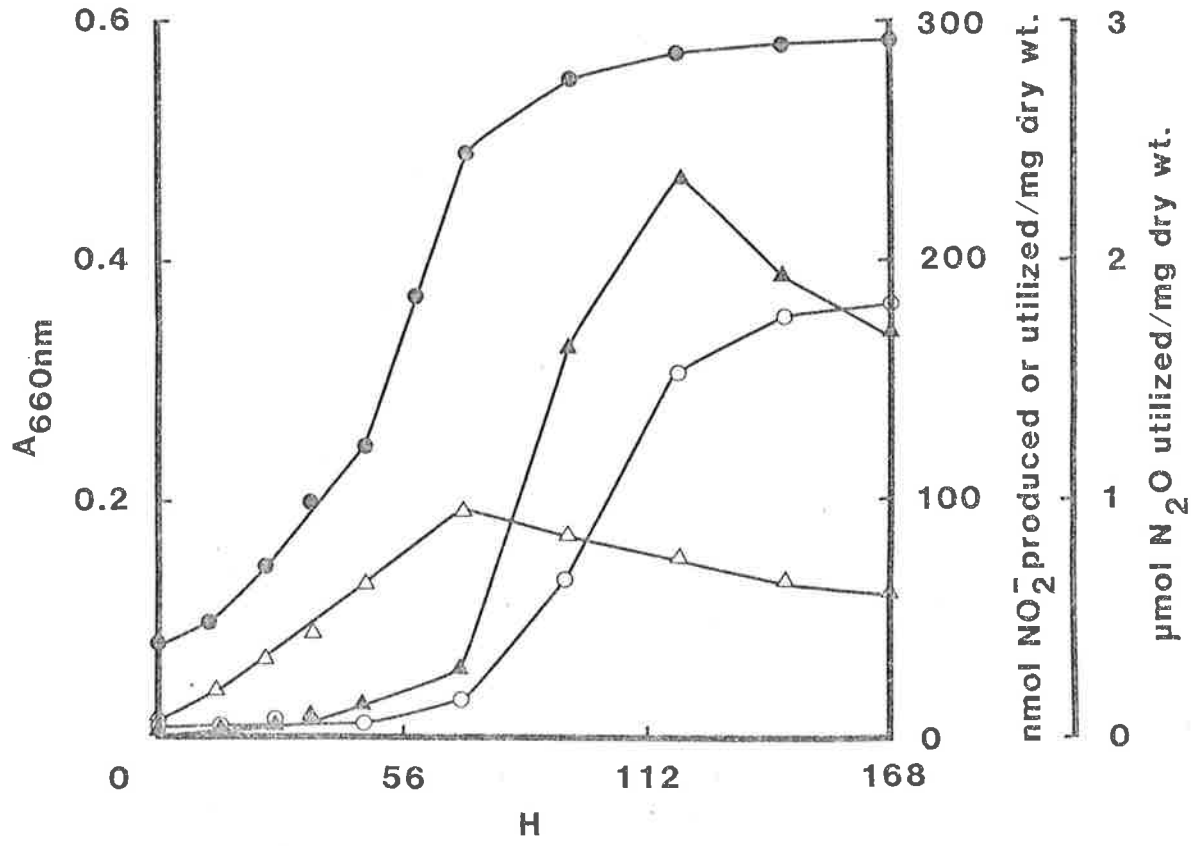

1.5
1.0
0.5
0
$A_{660nm}$
3
2
1
0
mmol $NO_3^-$ or $NO_2^-$ in the culture
0
6
12
18
24
30
H

FIG.6


0.6
0.4
0.2
0
$A_{660nm}$
300
200
100
0
nmol $NO_2^-$ produced or utilized/mg dry wt.
3
2
1
0
μmol $N_2O$ utilized/mg dry wt.
0
56
112
168
H

to determine nitrate reductase in the presence of nitrite reductase (Fig. 8). Nitrate reductase activity was very low (25 nmol/30 min/mg dry wt.) without KCN but in its presence the activity increased 5-fold because nitrite reductase was inhibited. A nitrite reductase activity of 305 nmol/30 min/mg dry wt. was recorded in the absence of KCN.

## 3.2 DENITRIFICATION OF NITRATE IN WASHED CELLS *VIA* NITRITE AND NITROUS OXIDE TO $N_2$

### 3.2.1 Effects of various electron donors

Sodium salts of a number of organic acids were utilized by washed cells to reduce $NO_3^-$, $NO_2^-$ and $N_2O$ respectively to $N_2$ gas (Table 1). Succinate, formate, citrate, fumarate and lactate were found to be the most effective donors for $NO_3^-$ reduction. Higher activities of nitrite and $N_2O$ reductases were obtained with either fumarate, citrate, lactate, malate or succinate. Malate was relatively ineffective for nitrate reductase, but it was readily utilized by nitrite and $N_2O$ reductases. Glucose was comparatively ineffective for the reduction of the three nitrogenous substrates. Reduced viologen dyes (BVH and MVH) were about 8 times more effective than other electron donors for the reduction of $NO_3^-$, $NO_2^-$ and $N_2O$ to $N_2$ gas.

### 3.2.2 Stoichiometries for $NO_3^-$, $NO_2^-$ and $N_2O$ reduction to $N_2$ gas

The results in Fig. 9 indicate that washed cells from photodenitrifying cultures produced $^{15}N_2O$ and $^{15}N_2$ from $K^{15}NO_3$ (82 atom % excess) when 20 mM MVH was the electron donor. About 80% of $^{15}NO_3^-$ was utilized within 2h when 8 µmol of $^{15}NO_3^-$ was converted into $^{15}N_2O$ and $^{15}N_2$ in the gas phase and 0.5 µmol of $NO_2^-$ accumulated in the reaction mixture. After a 3h incubation, 6 µmol of the following gases $^{15}N_2O$, $^{15}N_2$, $^{14}N_2O$

FIG. 7: NITRATE, NITRITE AND NITROUS OXIDE REDUCTASE ACTIVITIES OF WASHED CELLS GROWN UNDER PHOTODENITRIFYING CONDITIONS.

Enzyme activities were assayed anaerobically in washed cells (4-5 mg dry wt./ml) at various stages of growth as described in Fig. 6. Experiments were conducted in triplicate and errors were $\leqslant$ 5%.

- ● Growth;
- △ $NO_3^-$-reductase (nmol $NO_2^-$ produced/30 min/mg dry wt.);
- ▲ $NO_2^-$-reductase (nmol $NO_2^-$ utilized/30 min/mg dry wt.);
- ○ $N_2O$-reductase (µmol $N_2O$ utilized/3h/mg dry wt.).

FIG. 8: EFFECTS OF KCN ON NITRATE AND NITRITE REDUCTASE ACTIVITIES IN WASHED CELLS GROWN UNDER PHOTODENITRIFYING CONDITIONS.

Washed cells, grown for 16h under photodenitrifying conditions were used for these experiemnts. Assay procedures as described in Fig. 6. Experiments were done in triplicate and errors were within 5%.

- ● $NO_3^-$;
- ○ $NO_3^-$; + 2.5 mM KCN;
- △ $NO_2^-$;
- ▲ $NO_2^-$; + 2.5 mM KCN.

FIG.7

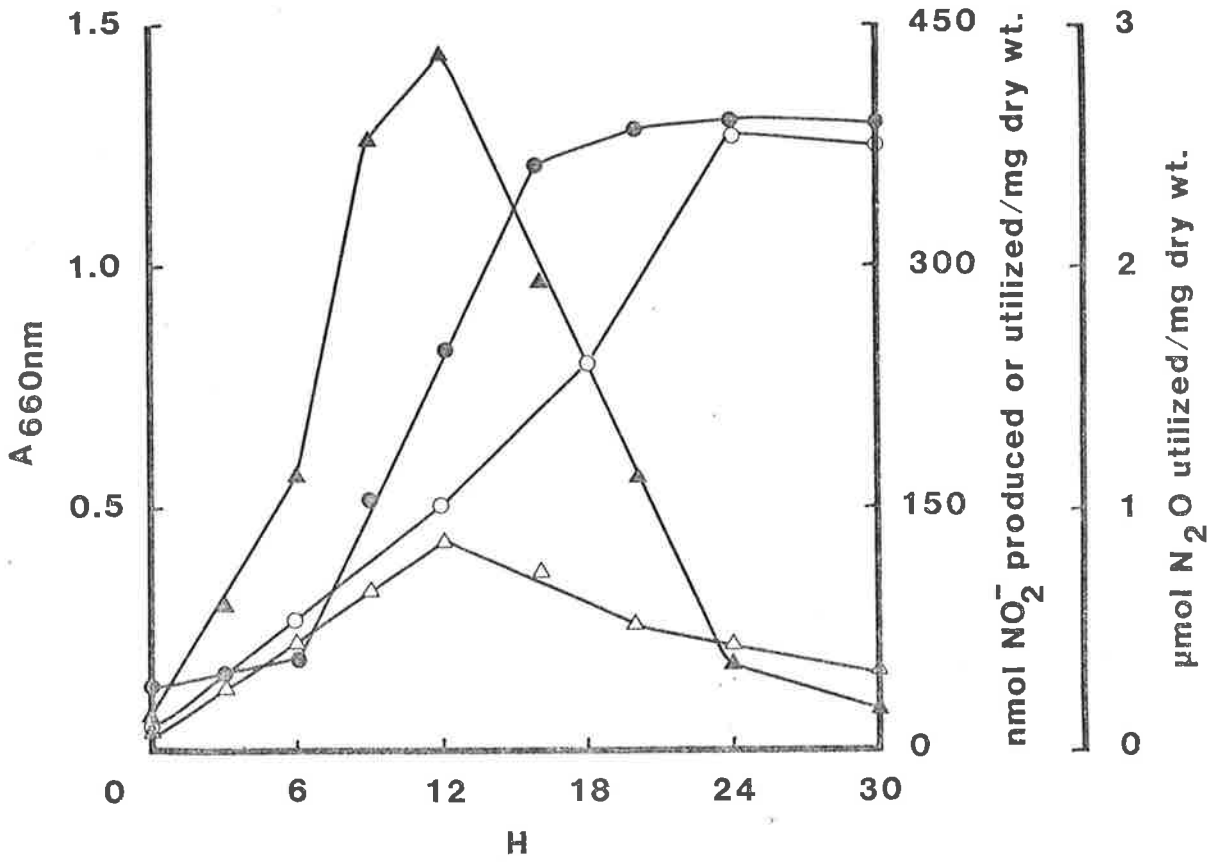

1.5
1.0
0.5
0
$A_{660nm}$
450
300
150
0
nmol $NO_2^-$ produced or utilized/mg dry wt.
3
2
1
0
µmol $N_2O$ utilized/mg dry wt.
0
6
12
18
24
30
H

FIG.8

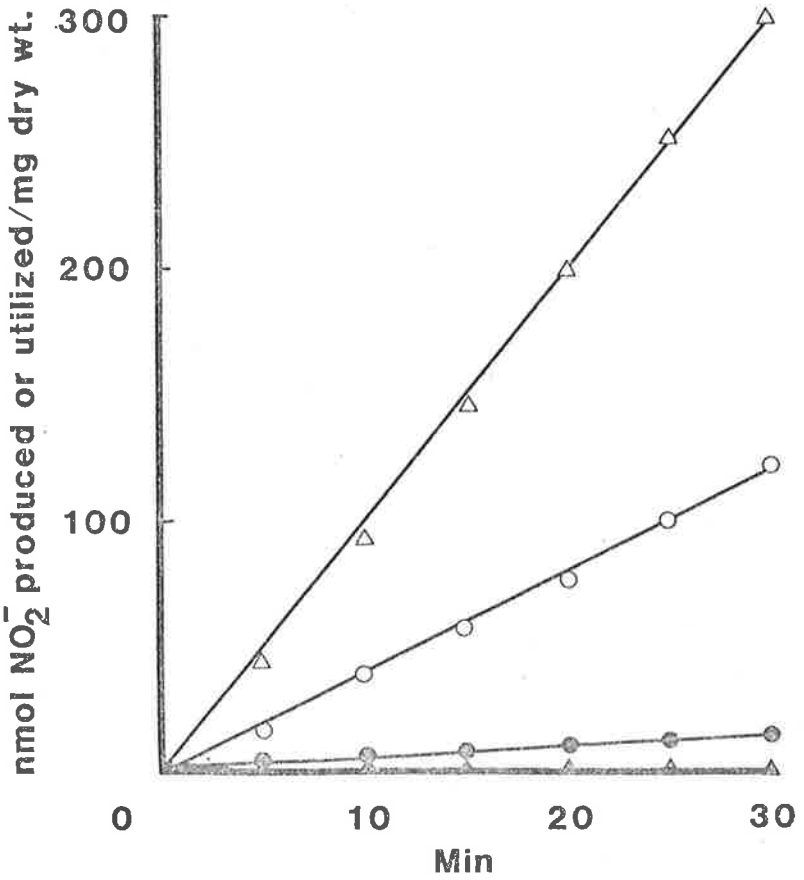

300
200
100
0
nmol $NO_2^-$ produced or utilized/mg dry wt.
10
20
30
Min

TABLE 1: UTILIZATION OF VARIOUS ELECTRON DONORS FOR $NO_3^-$, $NO_2^-$ AND $N_2O$ REDUCTION BY WASHED CELLS FROM PHOTODENITRIFYING CULTURES.

Cells were harvested at 18h growth stage, washed and resuspended (5 mg dry wt/ml) in 50 mM potassium phosphate buffer (pH 7.0) as described in Fig.6. Nitrate and nitrite reductases were assayed anaerobically at 30°C for 30 min but when either BVH or MVH (2 mM) was the electron donor, cells were incubated for 10 min. Final concentration of other electron donors was 20 mM. Nitrate reductase was assayed in the presence of 2.5 mM KCN. Nitrous oxide reductase was assayed anaerobically over a period of 3h at 30°C by following $N_2O$ utilization in a Hewlett Packard GC/mass spectrometer Model 5992B. Final concentrations of electron donors were 20 mM for BVH and MVH and 125 mM for others. Assay procedures are as described in Fig.6. Experiments were done in triplicate and errors were within 5%.

| Electron donor | $NO_3^-$-reductase (nmol $NO_2^-$ formed /30min/mg dry wt.) | $NO_2^-$-reductase (nmol $NO_2^-$ utilized /30min/mg dry wt.) | $N_2O$-reductase (nmol $N_2O$ utilized /3h/mg dry wt.) |
|---|---|---|---|
| Na-malate | 124 | 410 | 660 |
| Na-succinate | 250 | 309 | 510 |
| Na-lactate | 189 | 480 | 685 |
| Na-fumarate | 225 | 495 | 723 |
| Na-citrate | 239 | 451 | 698 |
| Na-pyruvate | 115 | 160 | 224 |
| Na-ascorbate | 163 | 95 | 87 |
| Na-formate | 239 | 123 | 150 |
| Glucose | 158 | 145 | 174 |
| BVH | 390[a] | 1367[b] | 5022 |
| MVH | 420[a] | 1418[b] | 5121 |

[a] nmol $NO_2^-$ produced/10 min/mg dry wt.

[b] nmol $NO_2^-$ utilized/10 min/mg dry wt.

FIG. 9: REDUCTION OF $^{15}NO_3^-$ *VIA* $^{15}NO_2^-$ TO $^{15}N_2O$ AND $^{15}N_2$ BY WASHED CELLS FROM PHOTODENITRIFYING CULTURES.

Cells were grown anaerobically under photodenitrifying conditions for 16h as described in Fig. 3. Assays were carried out anaerobically at 30°C with 20 mM MVH as the electron donor. Gaseous products were analysed in a GC/mass spectrometer (Hewlett Packard Model 5992B) as described in Section 2.2.18. Experiments were done in triplicate and errors were $\leqslant$ 5%. Stoichimetries for $NO_3^-$ : $NO_2^-$ $\equiv$ 1:1; $NO_3^-$ : $N_2$ $\equiv$ 2:1.

● Residual $NO_3^-$ in the culture solution;
○ $NO_2^-$ produced in the medium : Gases produced;
▲ $^{15}N_2O$;
△ $^{15}N_2$.

FIG. 10: REDUCTION OF $^{15}NO_2^-$ TO $^{15}N_2O$ AND $^{15}N_2$ BY WASHED CELLS FROM PHOTODENITRIFYING CULTURES.

Cells were grown anaerobically under photodenitrifying conditions for 16h as described in Fig. 3. Assays were carried out anaerobically at 30°C with 20 mM MVH as the electron donor. Gaseous products were analysed in a GC/mass spectrometer (Hewlett Packard Model 5992B). Assay procedures are described in Section 2.2.18. Experiments were conducted in triplicate and errors were $\leqslant$ 5%. Stoichiometry for $NO_2^-$ : $N_2$ = 2:1.

○ Residual $NO_2^-$ in the culture medium.
Gases produced;
● $^{15}N_2O$;
△ $^{14}N_2O$;
□ $^{15}N_2$;
▲ $^{14}N_2$.

FIG.9

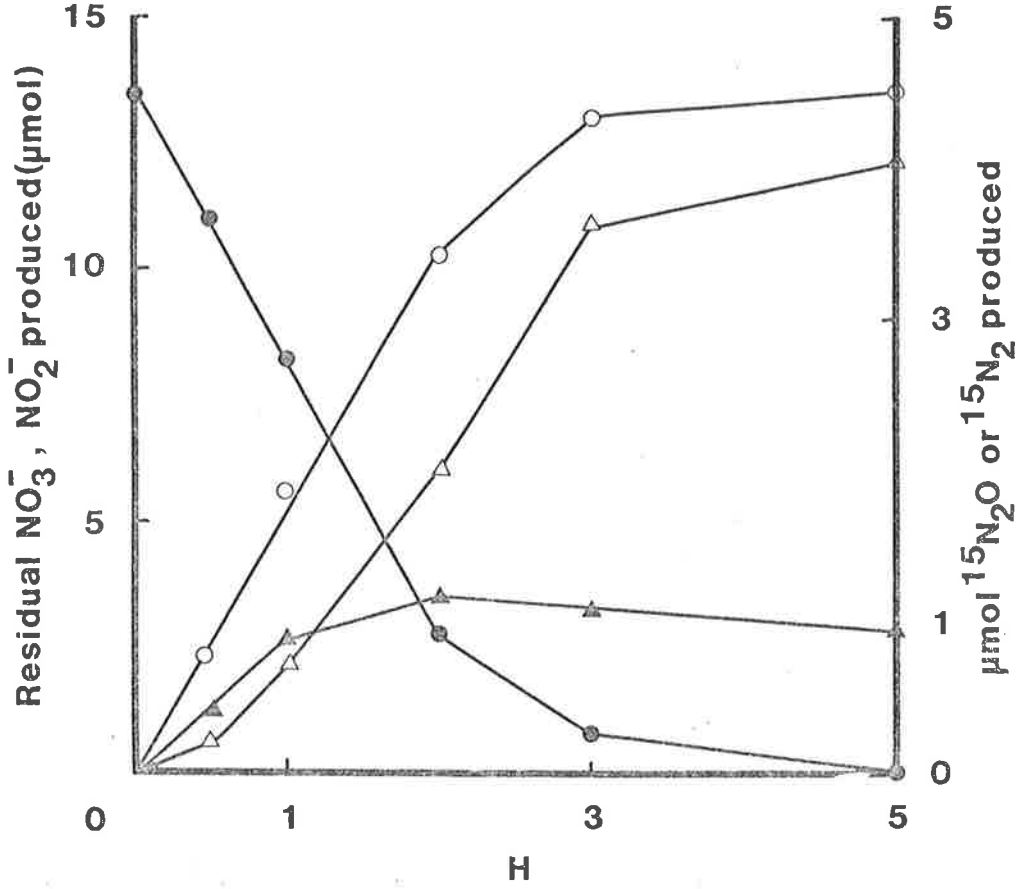
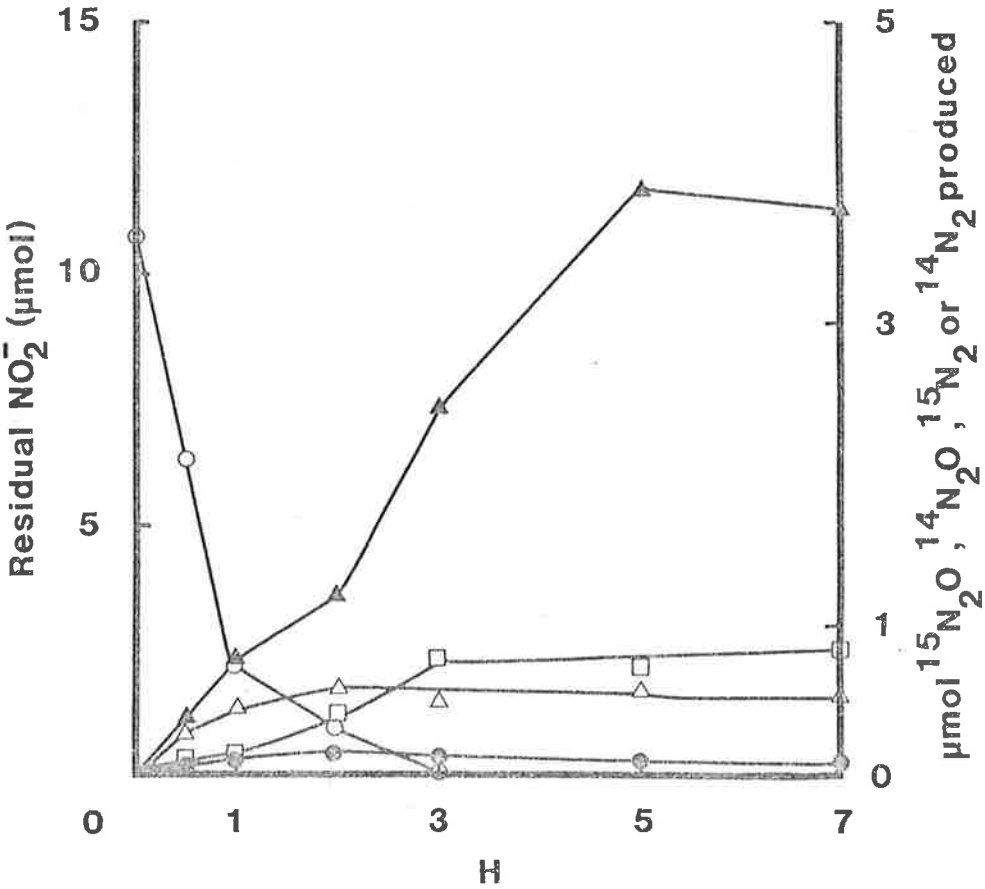

15
10
5
0
1
3
5
H
Residual $NO_3^-$, $NO_2^-$ produced(μmol)
μmol $^{15}N_2O$ or $^{15}N_2$ produced
5
3
1
0

FIG.10


15
10
5
0
1
3
5
7
H
Residual $NO_2^-$ (μmol)
μmol $^{15}N_2O$, $^{14}N_2O$, $^{15}N_2$ or $^{14}N_2$ produced
5
3
1
0

and $^{14}N_2$ were detected in the gas phase when 12.6 µmol of $NO_3^-$ was utilized resulting in a $2NO_3^-:1N_2O:1N_2$ stoichiometry. In a parallel experiment with washed cells 10.6 µmol of $NO_3^-$ was utilized and 10.2 µmol $NO_2^-$ was produced after a 2h incubation with 20 mM MVH as the electron donor in the presence of 2.5 mM KCN (to inhibit nitrite reductase). Thus, $NO_3^-:NO_2^-$ stoichiometry was 1:1.

Washed cells from photodenitrifying cultures, incubated with $K^{15}NO_2$ and MVH, also produced $^{15}N_2O$ and $^{15}N_2$ (Fig. 10). A 2:1 stoichiometry was observed for $NO_2^-$ utilization (10.7 µmol) and for $N_2O$ or $N_2$ produced (5.25 µmol) after a 3h incubation.

Washed cells from photodenitrifying cultures utilized $N_2O$ and produced $N_2$ as the sole product (Fig. 11). Both the utilization of $N_2O$ and production of $N_2$ was linear over a 3h incubation period with 20 mM MVH as the electron donor. Nitrous oxide was reduced stoichiometrically to $N_2$ (1:1) by these cells. The overall stoichiometry for nitrate reduction in the photodenitrifier was as follows: $2NO_3^-:2NO_2^-:1N_2O:1N_2$.

### 3.2.3 Effects of inhibitors

Nitrate reductase activity in washed cells was inhibited by amytal (63%) and KCNS (47%) whereas HOQNO, $NaN_3$, antimycin A, CCCP and oligomycin were relatively ineffective (Table 2). Thiourea, DIECA, O-phenanthroline and 8-hydroxyquinoline had no effect on enzyme activity. Nitrite reductase activity was completely inhibited by 2.5 mM KCN (Table 3) and it was also markedly reduced by DIECA (75%) and HOQNO (63%), whereas antimycin A, oligomycin and CCCP inhibited to a lesser extent (15 to 30%). Utilization of $N_2O$ was strongly inhibited (60 to 80%) by DIECA, $NaN_3$, $C_2H_2$, KCNS and HOQNO whereas KCN reduced the activity by about 30% (Table 4).

FIG. 11: NITROUS OXIDE REDUCTASE ACTIVITY IN WASHED CELLS FROM PHOTODENITRIFYING CULTURES.

Washed cells were prepared from photodenitrifying cultures grown for 16h as described in Fig. 3. $N_2O$-reductase was assayed anaerobically at 30°C with 20 mM MVH as the electron donor as described in Fig. 6. The quantity of $Na_2S_2O_4$ used to reduce MVH was titrated previously to make the solution just blue in order to avoid inactivation of $N_2OR$ by excess of it. Experiments were conducted in triplicate and errors were $\leqslant$ 5%. Stoichiometry for $N_2O$ : $N_2$ = 1:1.

○ Residual $N_2O$;

● $N_2$ formed.

FIG. 11

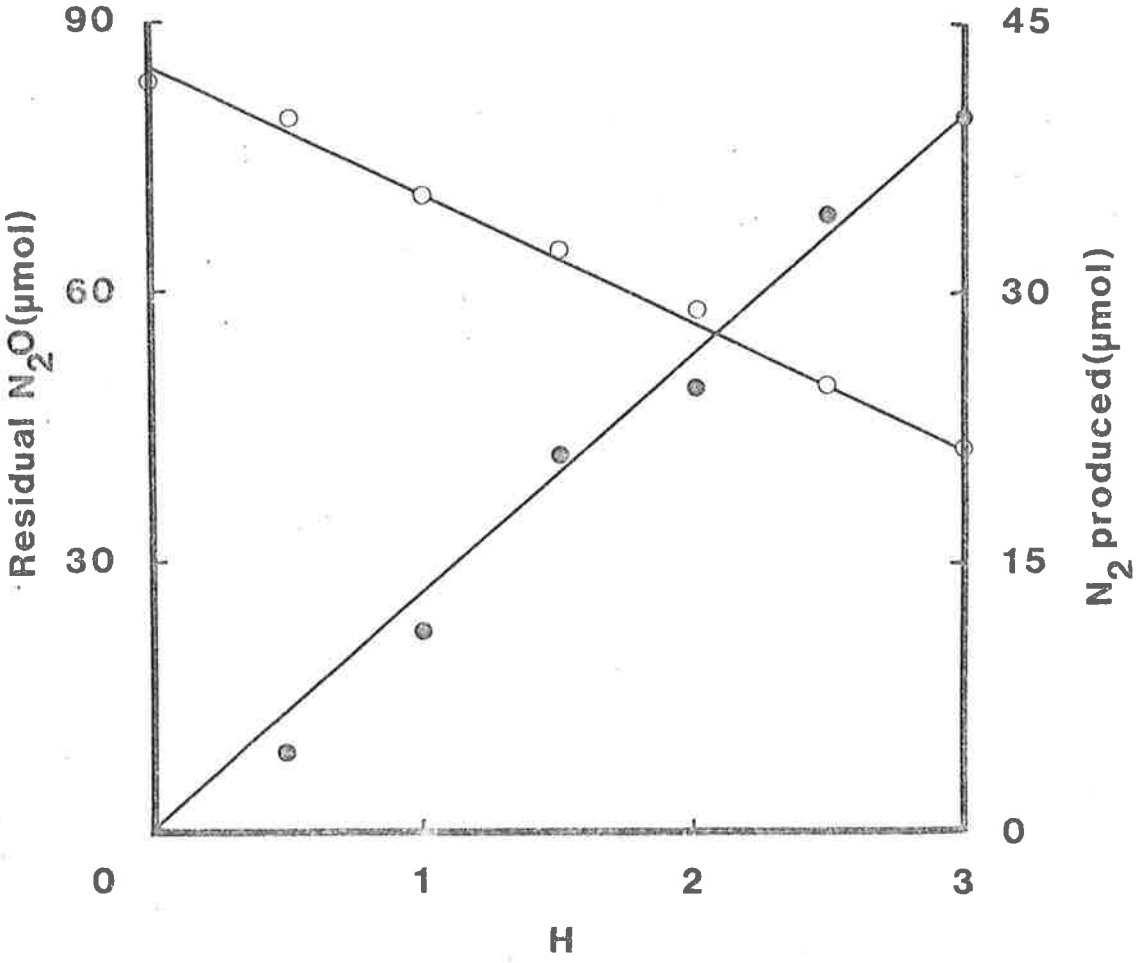


TABLE 2: INHIBITION OF NITRATE REDUCTASE ACTIVITY IN WASHED CELLS.

Washed cells (5 mg dry wt./ml) from photodenitrifying cultures (18h), were pre-incubated with 2.5 mM KCN for 10 min and then for a further 10 min with the inhibitor. Na-malate (20 mM) was used as the electron donor. Other assay conditions as in Table 1. $NaN_3$ and KCNS were dissolved in distilled water and other compounds in 95% v/v $C_2H_5OH$. Appropriate controls were done to check for any effects of alcohol and the inhibitors on the enzyme assay. Experiments were done in triplicate and errors were $\leqslant 5\%$.

| Inhibitor | Final Conc. (mM) | Activity ($nmol\ NO_2^-$ produced/ mg dry wt./30 min) | % Inhibition |
|---|---|---|---|
| - | - | 126 | 0 |
| $NaN_3$ | 1.0 | 110 | 13 |
| KCNS | 10.0 | 67 | 47 |
| Alcohol control | - | 115 | 0 |
| Amytal | 5.0 | 42 | 63 |
| Antimycin A | 0.10 | 98 | 15 |
| HOQNO | 0.10 | 75 | 35 |
| CCCP | 0.05 | 89 | 23 |
| Oligomycin | 250 µg/ml | 100 | 13 |

TABLE 3: INHIBITION OF NITRITE REDUCTASE ACTIVITY IN WASHED CELLS.

Washed cells (5 mg dry wt./ml) from photodenitrifying cultures (16h), were pre-incubated with the inhibitors for 10 min and then assayed as described in Table 1. KCN, $NaN_3$, KCNS, DIECA and thiourea were dissolved in distilled water, others in 95% (v/v) $C_2H_5OH$. Appropriate controls were done to check for any effects of $C_2H_5OH$ or inhibitors on the enzyme assay. Experiments were conducted in triplicate and errors were $\leqslant 5\%$.

| Inhibitor | Final conc. (mM) | Activity (nmol $NO_2^-$ utilized/ 30 min/mg dry wt.) | % Inhibition |
|---|---|---|---|
| - | - | 425 | 0 |
| KCN | 2.5 | 0 | 100 |
| $NaN_3$ | 1.0 | 410 | 4 |
| KCNS | 10.0 | 360 | 15 |
| DIECA | 1.0 | 102 | 75 |
| Thiourea | 2.0 | 285 | 33 |
| Amylal | 5.0 | 128 | 70 |
| Antimycin A | 0.1 | 336 | 21 |
| HOQNO | 0.1 | 157 | 63 |
| CCCP | 0.05 | 361 | 15 |
| Oligomycin | 250 µg/ml | 323 | 24 |

TABLE 4: EFFECTS OF INHIBITORS ON NITROUS OXIDE REDUCTASE ACTIVITY IN WASHED CELLS.

Washed cells (5 mg dry wt./ml) from photodenitrifying cultures (16h), were pre-incubated with the inhibitor for 10 min and then assayed anaerobically at 30°C for 3h using 20 mM MVH as the electron donor as described in Table 1. Appropriate controls were done to check for any effects of alcohol on $N_2O$ utilization by washed cells. Experiments were conducted in triplicate and errors were within 5%.

| Inhibitor | Final conc. (mM) | Activity (umol $N_2O$ utilized/ mg dry wt./3h) | % Inhibition |
|---|---|---|---|
| - | - | 5.95 | 0 |
| $NaN_3$ | 1 | 1.20 | 80 |
| KCN | 2 | 4.08 | 32 |
| KCNS | 4 | 2.15 | 64 |
| $C_2H_2$ | 80% (v/v gas phase) | 1.23 | 78 |
| DIECA | 2 | 1.10 | 82 |
| HOQNO | 0.1 | 2.45 | 59 |
| Amytal | 5.0 | 2.10 | 65 |

## 3.3 NITRATE, NITRITE AND NITROUS OXIDE REDUCTASES IN WASHED CELLS GROWN AEROBICALLY AND ANAEROBICALLY IN LIGHT AND IN THE DARK

### 3.3.1 Effects of nitrate on the production of nitrate and nitrite reductases in aerobically grown cells

The synthesis of nitrate and nitrite reductases was enhanced anaerobically at 30°C by adding $KNO_3$ to washed cells which had been grown aerobically in light. When these cells were incubated anaerobically in light with $NO_3^-$ as the terminal acceptor, the synthesis of nitrate and nitrite reductases occurred after a lag period of about 30 min (Fig. 12). About 4 µmol $NO_3^-$ was utilized and 1 µmol $NO_2^-$ accumulated in the medium after 30 min whereas after a 2h incubation, 72 µmol $NO_2^-$ was produced and 160 µmol $NO_3^-$ was utilized. Nitrate and nitrite reductase activities were first detected after a 30 min incubation when enzyme activities were very low e.g. 9 nmol $NO_3^-$ utilized/10 min/mg protein (nitrate reductase); 4 nmol $NO_2^-$ utilized/10 min/mg protein (nitrite reductase). However, activities of both enzymes increased steadily as incubation progressed. Thus after a 2h incubation, nitrate reductase activity was 544 nmol $NO_3^-$ utilized/10 min/mg protein and that of nitrite reductase was 474 nmol $NO_2^-$ utilized/10 min/mg protein.

When washed cells which had been grown aerobically in the dark, were incubated anaerobically in the dark in the presence of $NO_3^-$ as the terminal acceptor, nitrate and nitrite reductase activities were detected after about 30 min (Fig. 13). The utilization of $NO_3^-$ (3 µmol) and production of $NO_2^-$ (1 µmol) was very low during the 30 min period. The activities of nitrate and nitrite reductases increased rapidly as the incubation continued and after 2h the enzyme activities were 379 nmol $NO_3^-$ utilized/10 min/mg protein and 311 nmol $NO_2^-$ utilized/10 min/mg protein respectively.

FIG. 12: PRODUCTION OF NITRATE AND NITRITE REDUCTASES IN WASHED CELLS WHICH HAD BEEN GROWN AEROBICALLY IN LIGHT.

Cells from aerobic phototrophic cultures were harvested by centrifugation (Sorvall RC5B centrifuge, SS34 rotor) at 20,000 x g for 20 min at 4°C then washed once in 50 mM potassium phosphate buffer (pH 7.5) and resuspended (20 mg wet wt./ml) in the same buffer. These cell suspensions (20 ml) together with 20 mM Na-malate and 10 mM $KNO_3$ were dispensed into 100 ml Erlenmeyer flasks fitted with subaseals and then evacuated for 5 min by a water suction pump followed by a flushing with argon for 5 min. The production of nitrate and nitrite reductases were carried out by incubating these cell suspensions anaerobically at 30°C under argon in light. Samples of the cell suspension were analysed for $NO_3^-$ utilization and $NO_2^-$ production. The bulk of the cells were centrifuged at 20,000 x g for 20 min, washed once in 50 mM potassium phosphate buffer (pH 7.5) and resuspended (20 mg wet wt./ml) in the same buffer. Nitrate and nitrite reductase activities were assayed in these washed cell suspensions. Nitrate utilization and $NO_2^-$ production were determined at 410 nm and 540 nm, respectively, in a Hitachi Perkin Elmer spectrophotometer (Sections 2.2.8.1 and 2.2.8.2). Nitrate and nitrite reductases were assayed anaerobically at 30°C for 10 min using BVH (2 mM) as the electron donor. Assay procedures are as described in Fig.6. Experiments were done in triplicate and errors were within 5%.

- □ $NO_3^-$ utilization;
- ● $NO_2^-$ production;
- ○ $NO_3^-$-reductase (nmol $NO_3^-$ utilized/10 min/mg protein);
- △ $NO_2^-$-reductase (nmol $NO_2^-$ utilized/10 min/mg protein).

FIG. 13: PRODUCTION OF NITRATE AND NITRITE REDUCTASES IN WASHED CELLS PREVIOUSLY GROWN AEROBICALLY IN THE DARK.

Anaerobic suspension of washed cells, grown aerobically in the dark for 24h, were prepared as described in Fig. 12. Cell suspensions were incubated anaerobically at 30°C under argon (after first evacuating for 5 min by a water suction pump) in the dark with 20 mM Na-malate and 10 mM $KNO_3$. Determination of $NO_3^-$ utilization, $NO_2^-$ production as well as nitrate and nitrite reductase activities were done as described in Fig.12. Experiments were conducted in triplicate and errors were $\leq$ 5%.

- □ $NO_3^-$ utilization;
- ● $NO_2^-$ production ;
- ○ $NO_3^-$-reductase (nmol $NO_3^-$ utilized/10 min/mg protein);
- △ $NO_2^-$-reductase (nmol $NO_2^-$ utilized/10 min/mg protein).

FIG. 12

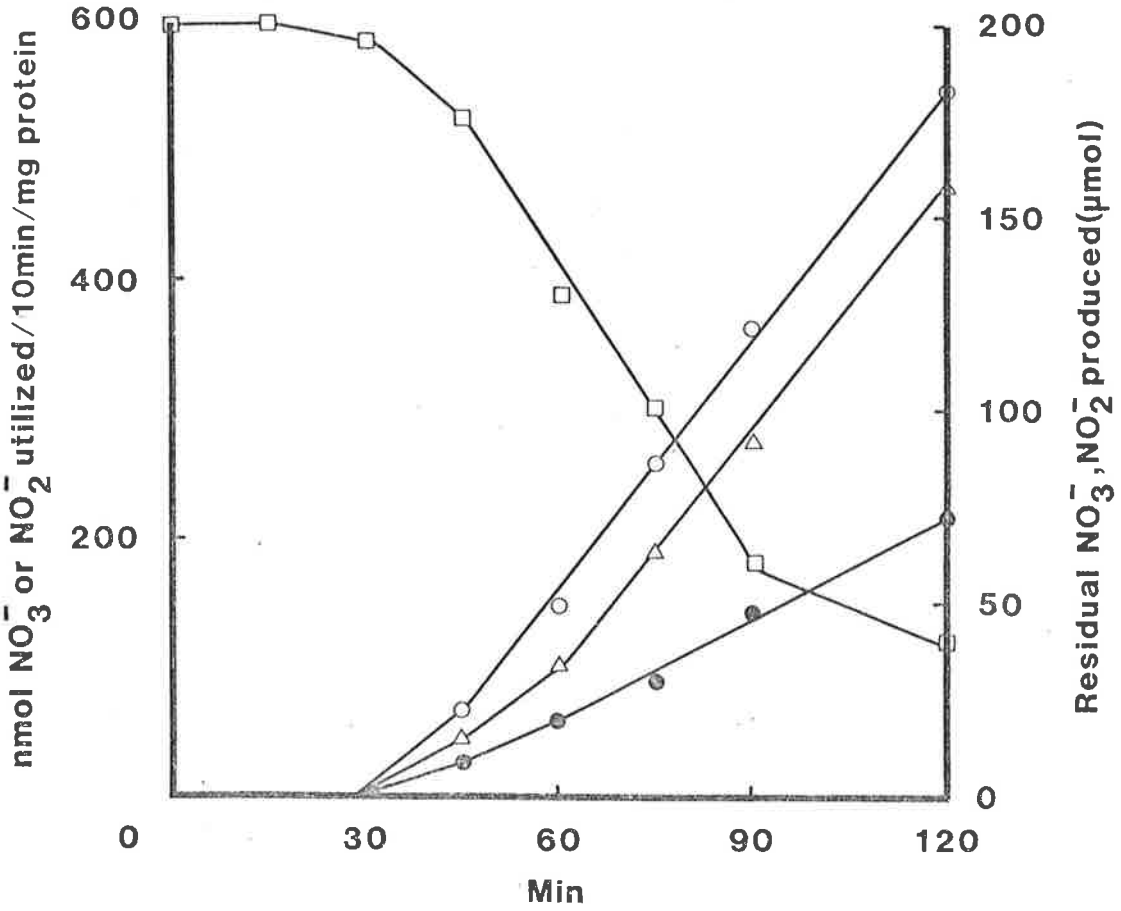
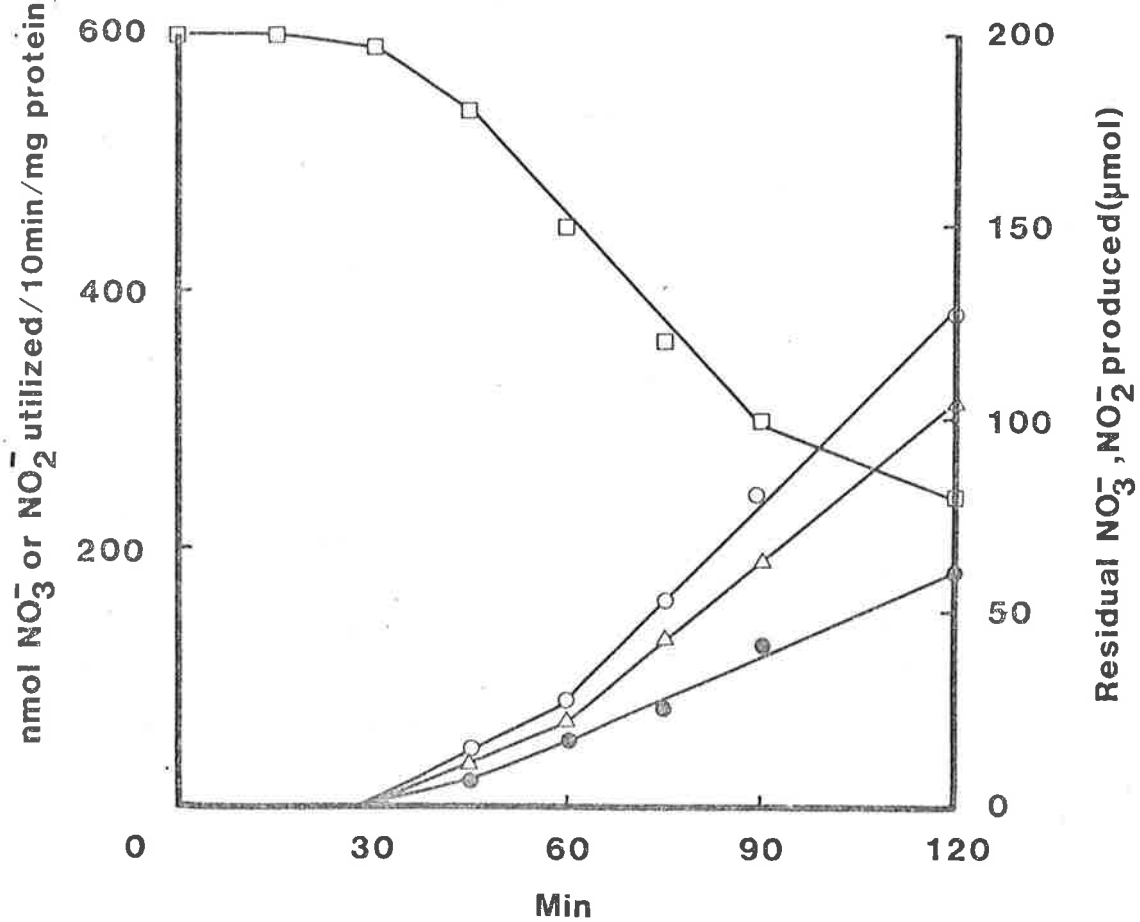

600
400
200
0
30
60
90
120
Min
200
150
100
50
0
nmol $NO_3^-$ or $NO_2^-$ utilized/10min/mg protein
Residual $NO_3^-$, $NO_2^-$ produced(μmol)

FIG. 13


600
400
200
0
30
60
90
120
Min
200
150
100
50
0
nmol $NO_3^-$ or $NO_2^-$ utilized/10min/mg protein
Residual $NO_3^-$, $NO_2^-$ produced(μmol)

The time courses for the production of nitrate and nitrite reductases in washed cells which had been grown aerobically in light are given in Figs. 14A and B. Nitrate and nitrite reductase activities were detected in these washed cells after a 30 min anaerobic incubation in light with $NO_3^-$ whereas enzyme activities were not detected when cells were incubated aerobically with $NO_3^-$ in light. After 30 min aerobic incubation with $NO_3^-$ in light, when anaerobic conditions were created by evacuating and flushing with argon, nitrate and nitrite reductases were produced in these cells after a further incubation of 30 min. On the other hand, if aerobic conditions were sustained or either 0.2 mg/ml of either chloramphenicol or rifampicin was added before the onset of anaerobic conditions in the presence of nitrate then neither enzyme was detected.

### 3.3.2 Activities of denitrifying enzymes

Nitrate, nitrite and nitrous oxide reductases were determined in washed cells which had been grown under various conditions. In washed cells grown aerobically either in light or in the dark, these enzymes were produced by incubating them anaerobically for 2h in the presence of $NO_3^-$. After 2h induction period the washed cells grown aerobically in light had the following activities of 44.8, 36.6 and 29.1 nmol $NO_3^-$, $NO_2^-$ or $N_2O$ utilized/min/mg protein respectively for nitrate, nitrite and nitrous oxide reductases (Table 5). The washed cells from photodenitrifying cultures had the following activities for the three enzymes: 52.7, 42.9 and 35.6 nmol $NO_3^-$, $NO_2^-$ or $N_2O$ utilized/min/mg protein respectively. The activities of these denitrifying enzymes in washed cells from dark denitrifying cultures were about 60-90% of those of cells from photodenitrifying cultures. Washed cells which had been grown aerobically in the dark, after 2h anaerobic incubation with $NO_3^-$, had similar activities for nitrate, nitrite and nitrous oxide reductases as for cells from dark denitrifying cultures.

FIG. 14: TIME COURSE FOR APPEARANCE OF NITRATE AND NITRITE REDUCTASES IN WASHED CELLS GROWN AEROBICALLY IN LIGHT.

Anaerobic suspension of washed cells (grown aerobically in light for 24h) were prepared as described in Fig.12. These cell suspensions were incubated anaerobically with 20 mM Na-malate in light at 30°C. The following treatments were included:

- ○ anaerobic incubation with 10 mM $KNO_3$ added at zero time;
- ◔ aerobic incubation ($O_2$ flushing) with 10 mM $KNO_3$ for 30 min then anaerobically;
- △ aerobic incubation with 10 mM $KNO_3$;
- □ anaerobic incubation with 10 mM $KNO_3$ and 0.2 mg/ml chloramphenicol added at zero time;
- ▩ anaerobic incubation with 10 mM $KNO_3$ and 0.2 mg/ml rifampicin added at zero time.

Nitrate and nitrite reductases were assayed as described in Fig.12. Experiments were conducted in triplicate and errors were within 5%.

A, $NO_3^-$-reductase (nmol $NO_3^-$ utilized/10 min/mg protein);
B, $NO_2^-$-reductase (nmol $NO_2^-$ utilized/10 min/mg protein).

FIG. 14A

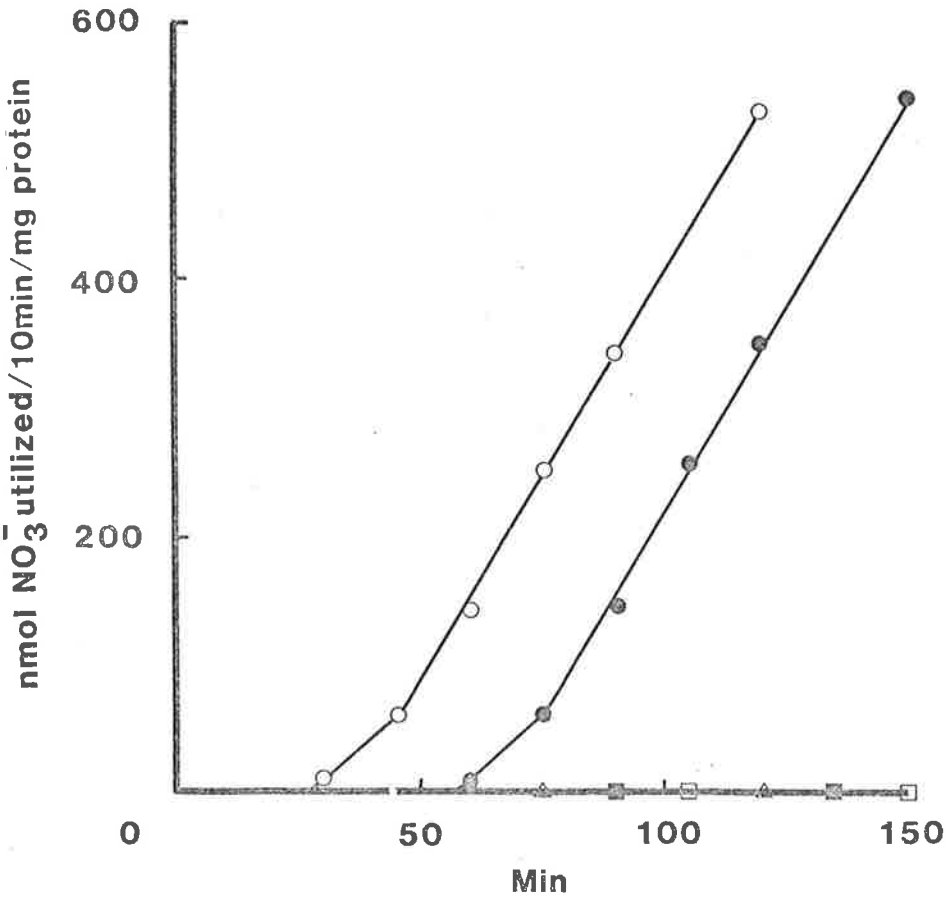



FIG. 14B

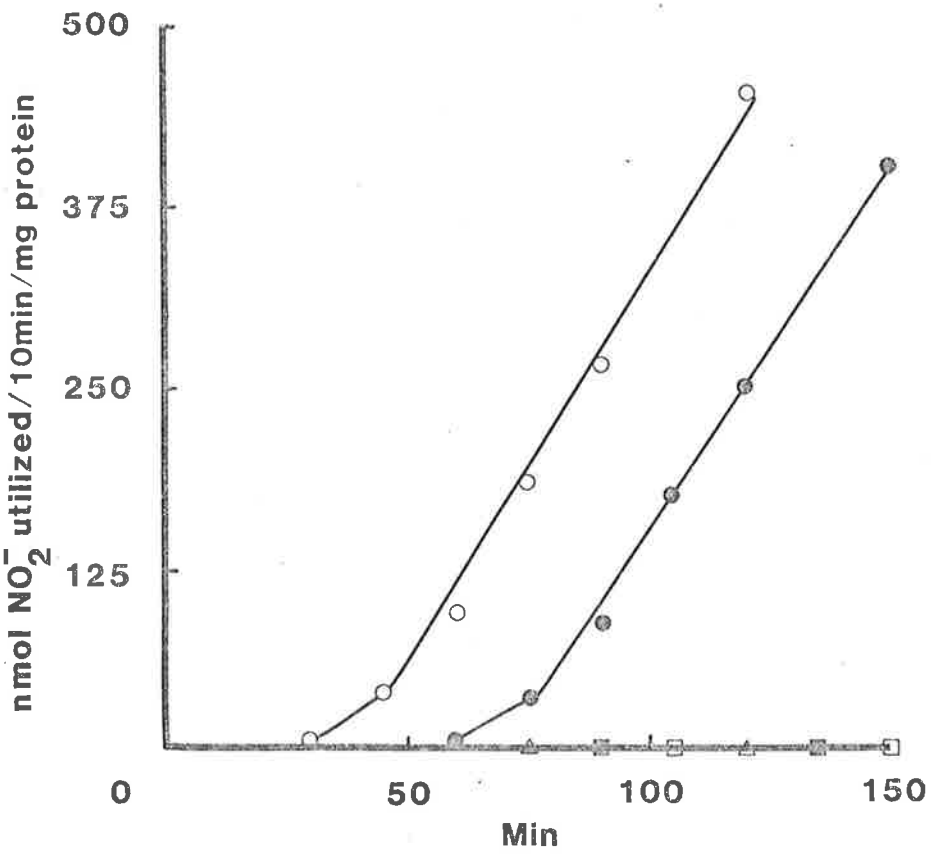


TABLE 5: A COMPARISON OF THE ACTIVITIES OF DENITRIFYING ENZYMES PRODUCED BY $NO_3^-$ IN WASHED CELLS PREVIOUSLY GROWN AEROBICALLY OR ANAEROBICALLY IN LIGHT AND IN THE DARK.

Anaerobic suspensions of washed cells were prepared as described in Fig.10. Experiments were carried out anaerobically at 30°C either in light or in the dark with 10 mM Na-malate and 10 mM $KNO_3$ as indicated. Samples were analysed for nitrate and nitrite reductase activities as described in Fig.12. Nitrous oxide reductase was assayed as described in Section 2.2.9.4. Experiments were done in triplicate and results were within 5% error.

| Growth conditions | Conditions for production of enzymes | Activity (nmol $NO_3^-$, $NO_2^-$ or $N_2O$ utilized/min/mg protein) | | |
|---|---|---|---|---|
| | | $NO_3^-$-reductase | $NO_2^-$-reductase | $N_2O$-reductase |
| 1. Light, aerobic, omit $NO_3^-$, 24h | Washed cells, light, anaerobic, $NO_3^-$, 2h incubation | 44.8 | 36.6 | 29.1 |
| 2. Light, anaerobic, 20 mM $KNO_3$, 24h | Washed cells, no treatment | 52.7 | 42.9 | 35.6 |
| 3. Dark, aerobic, omit $NO_3^-$, 24h | Washed cells, dark, anaerobic, $NO_3^-$, 2h incubation | 30.5 | 35.0 | 25.9 |
| 4. Dark, anaerobic 20 mM $KNO_3$, 5 days | Washed cells, no treatment | 32.5 | 37.5 | 27.5 |

### 3.3.3 Effects of chloramphenicol, rifampicin and $O_2$ on the production of nitrate and nitrite reductases in aerobically grown cells.

Results in Figs. 15 and 16 show that low levels of nitrate, nitrite and nitrous oxide reductases were produced (30-38 nmol $NO_3^-$ $NO_2^-$ or $N_2O$ utilized/10 min/mg protein) in washed cells previously grown aerobically either in light or in the dark, by incubating these cells under anaerobic conditions only. However, the activities of these two enzymes were about 16 times greater when $NO_3^-$ was included. When either $O_2$ was sparged through the reaction mixture or chloramphenicol added after 1h of adding $NO_3^-$, the production of both nitrate and nitrite reductases was inhibited. On the other hand when rifampicen was added (after 1h) the synthesis of these two enzymes continued at a slow rate for about 15 min.

### 3.3.4 Effects of $O_2$ tension on nitrate, nitrite and nitrous oxide reductase activities

There was no production of nitrate reductase in washed cells from aerobic phototrophic cultures when 1% v/v $O_2$ was included in the gas phase in the presence of $NO_3^-$ (Table 6). However, these cells incubated anaerobically under argon, produced after 2h, a nitrate reductase with an activity of 44.8 nmol $NO_3^-$ utilized/min/mg protein. Washed cells from photodenitrifying cultures had a similar enzyme activity (49.5 nmol $NO_3^-$ utilized/min/mg protein). This activity was decreased by about 25% and 50% respectively, in the presence of 5% and 10% v/v $O_2$ and was completely inhibited in the presence of 30% (v/v) $O_2$. Nitrite and nitous oxide reductase activities were similarly inhibited by $O_2$ (Table 6).

## 3.4 PURIFICATION AND PROPERTIES OF NITRATE AND NITRITE REDUCTASES

### 3.4.1 Subcellular distribution of the enzymes

Activities of nitrate, nitrite and nitrous oxide reductases were

FIG. 15: EFFECTS OF CHLORAMPHENICOL, RIFAMPICIN AND $O_2$ ON THE SYNTHESIS OF NITRATE AND NITRITE REDUCTASES IN WASHED CELLS FROM AEROBIC PHOTOTROPHIC CULTURES.

Anaerobic suspension of washed cells, grown aerobically in light for 24h, were prepared as described in Fig.12. Cells were incubated anaerobically in light at 30°C with 20 mM Na-malate and 10 mM $KNO_3$. In the control experiment, cells were incubated anaerobically without $NO_3^-$. After a 1h incubation 0.2 mg/ml of either chloramphenicol or rifampicin were added or $O_2$ was sparged through the cell suspension. Samples were centrifuged, washed once and resuspended (20 mg wet wt./ml) in 50 mM potassium phosphate buffer (pH 7.5). Nitrate and nitrite reductase activities were determined in these cells as described in Fig.12. Experiments were done in triplicate and errors were within 5%.

A, $NO_3^-$-reductase (nmol $NO_3^-$ utilized/10 min/mg protein);
B, $NO_2^-$-reductase (nmol $NO_2^-$ utilized/10 min/mg protein).

▲ - $NO_3^-$;
○ + $NO_3^-$;
□ chloramphenicol;
● rifampicin;
△ $O_2$.

FIG. 15A

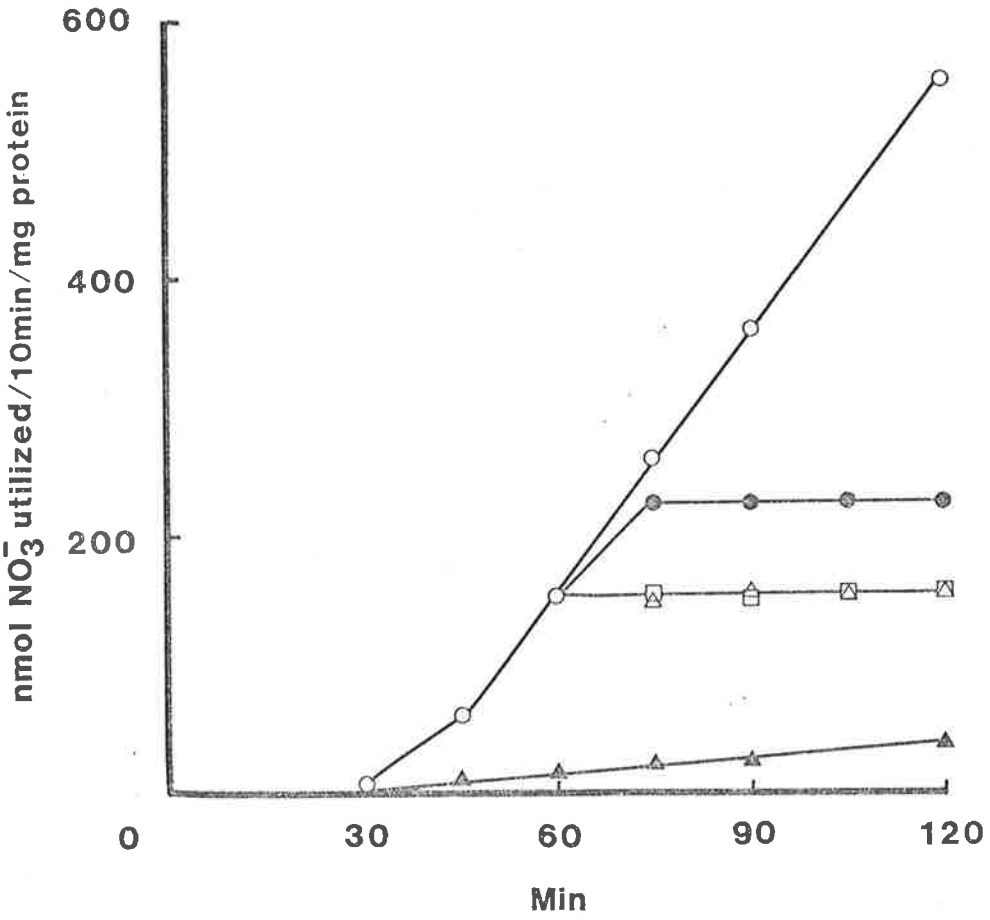



FIG. 15B

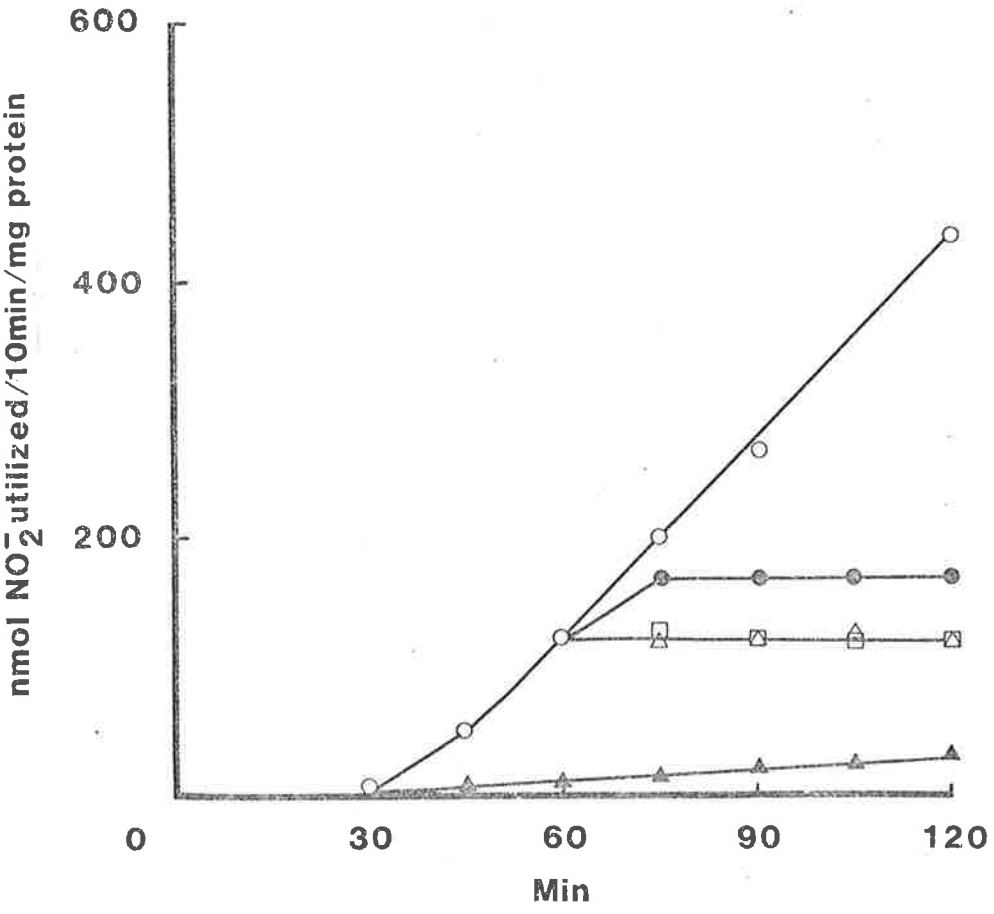


FIG. 16: EFFECTS OF CHLORAMPHENICOL, RIFAMPICIN AND $O_2$ ON THE PRODUCTION OF NITRATE AND NITRITE REDUCTASES IN WASHED CELLS GROWN AEROBICALLY IN THE DARK.

Anaerobic suspension of washed cells (20 mg wet wt./ml) in 50 mM potassium phosphate buffer (pH 7.5) were prepared from dark aerobic cultures (24h). Production of nitrate and nitrite reductases were followed in the dark as described in Fig. 15. Nitrate and nitrite reductase activities were determined as described in Fig. 12. Experiments were done in triplicate and errors were $\leqslant 5\%$.

A, Nitrate reductase (nmol $NO_3^-$ utilized/10min/mg protein).
B, Nitrite reductase (nmol $NO_2^-$ utilized/10min/mg protein).

▲ - $NO_3^-$;
○ + $NO_3^-$;
□ Chloramphenicol;
● Rifampicin;
△ $O_2$.

FIG. 16A

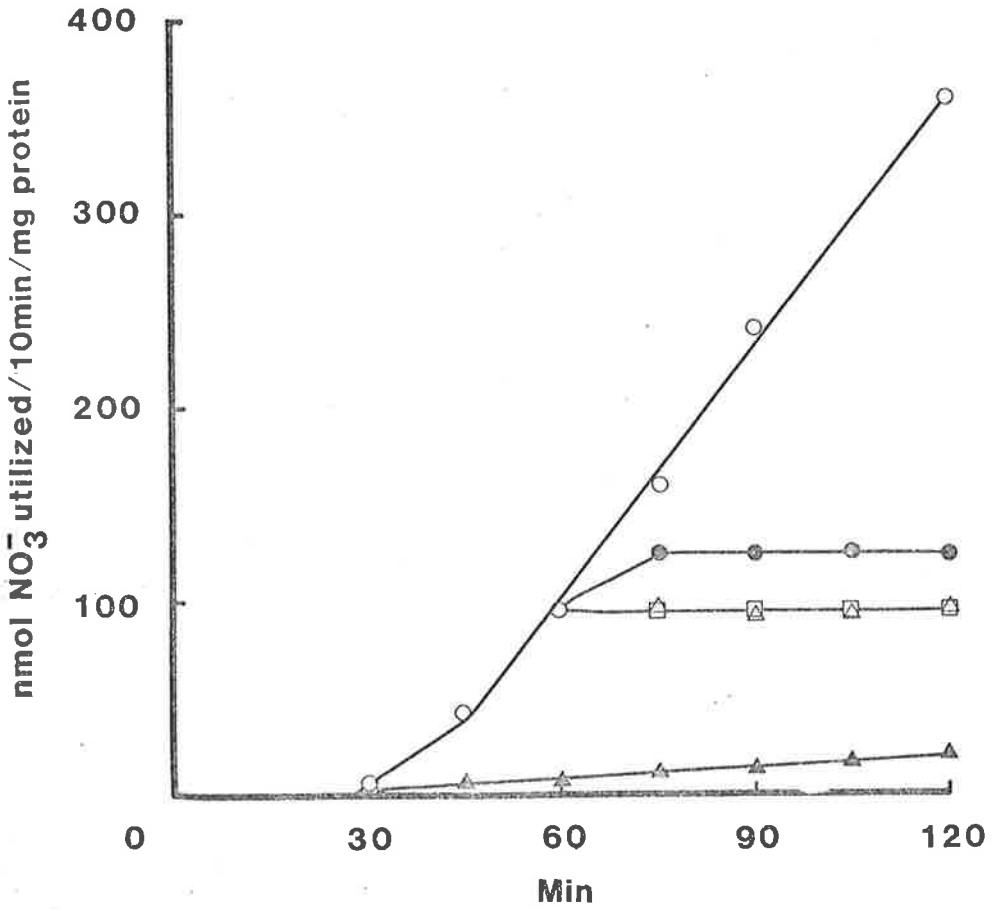



FIG. 16B

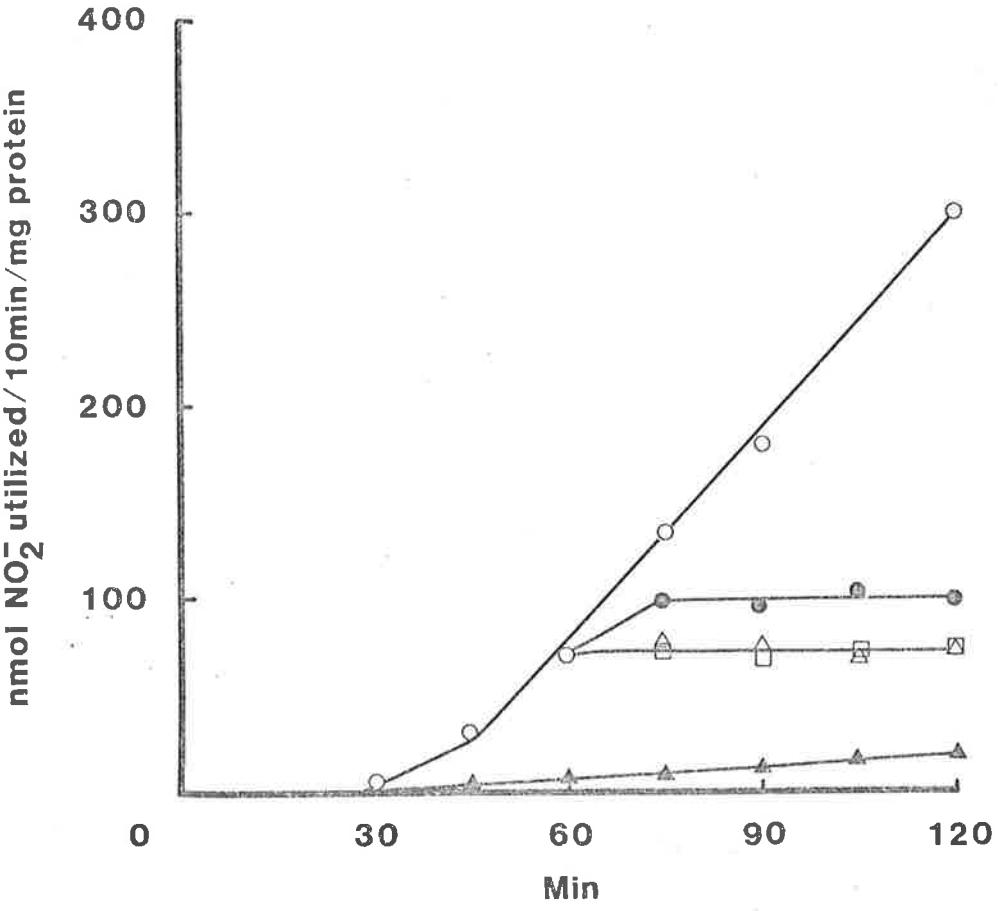


TABLE 6: EFFECTS OF $O_2$ ON NITRATE, NITRITE AND NITROUS OXIDE REDUCTASE ACTIVITIES IN WASHED CELLS FROM AEROBIC PHOTOTROPHIC AND PHOTODENITRIFYING CULTURES.

Cells were grown aerobically (without $NO_3^-$) or anaerobically (with $NO_3^-$) in light as described in Section 2.2.3. Preparation of anaerobic suspension of washed cells; production of nitrate, nitrite and nitrous oxide reductases in light and measurement of these enzyme activities in cell suspensions were determined as described in Fig.12 and Sections 2.2.9.2, 2.2.9.3 and 2.2.9.4. Experiments were done in triplicate and errors were ≤5%.

| Growth conditions | Conditions for production of enzymes | Assay conditions | Enzyme activities (nmol $NO_3^-$, $NO_2^-$ or $N_2O$ utilized/min/mg protein) | | |
|---|---|---|---|---|---|
| | | | $NO_3^-$-reductase | $NO_2^-$-reductase | $N_2O$-reductase |
| 1. Light aerobic, omit $NO_3^-$ (24h) | Washed cells, light, $NO_3^-$, 1% v/v $O_2$, 2h incubation | Anaerobic | 0 | 0 | 0 |
| | Washed cells, light, $NO_3^-$, 2h anaerobic incubation | Anaerobic | 44 | 35 | 29 |
| 2. Light anaerobic, + $NO_3^-$ (24h) | Washed cells, no treatment | Anaerobic | 50 | 39 | 34 |
| | | 5% $O_2$,95% Ar | 38 | 31 | 27 |
| | | 10% $O_2$,90% Ar | 25 | 24 | 18 |
| | | 30% $O_2$,70% Ar | 0 | 0 | 0 |

determined in washed cells from photodenitrifying cultures as well as in spheroplasts and cellular fractions (Table 7). Spheroplasts were prepared by lysozyme treatment and sphaeroplast membranes by either sonication or osmotic shock treatment as described in Section 2.2.7. Glucose-6-phosphate dehydrogenase was assayed since it is a typical cytoplasmic protein marker (Conard and Schlegel, 1978). Results in Table 7 show that more than 80% of nitrate, nitrite and nitrous oxide reductases were retained in the spheroplast supernatant fraction whereas glucose-6-phosphate dehydrogenase was detected in spheroplast lysates.

### 3.4.2 Purification of the enzymes

#### 3.4.2.1 Nitrate reductase

Extracts of washed cells previously grown under photodenitrifying conditions, were prepared by the French Pressure cell method (Section 2.2.6) and centrifuged at 20,000 x g for 20 min. The supernatant ($S_{20}$) contained 584 mg protein and 310 units of activity (µmol $NO_2^-$ produced/min) in total. The $S_{20}$ fraction was then centrifuged at 240,000 x g for 1h at 4°C. The pellet fraction ($P_{240}$) contained 710 units (total) of nitrate reductase (Table 8). Nitrite reductase activity in this fraction was about 10% of that of nitrate reductase. Nitrate reductase was solubilized from the pellet by heat treatment at 50°C for 20 min. Ammonium sulphate fractionation of the solubilized enzyme ($Ht_{50}$ $S_{240}$) between 20-60% saturation resulted in a 12-fold purification. After dialysing this fraction against 0.1M potassium phosphate buffer (pH 7.0) for 16h, it was loaded on to a DEAE-Sephacel column (1.6 x 16 cm), pre-equilibrated with the same buffer. The enzyme was eluted with a NaCl gradient between 0.25 to 0.31M in the same buffer (Fig. 17). The enzyme was further purified by gel filtration on a Sephadex-G100 column (1.6 x 93 cm) resulting in 130-fold purification with an 18% recovery (Fig. 18).

TABLE 7: SUBCELLULAR DISTRIBUTION OF NITRATE, NITRITE AND NITROUS OXIDE REDUCTASES.

Spheroplasts were prepared from washed cells (grown anaerobically in light with $NO_3^-$) by lysozyme-EDTA treatment as described in Section 2.2.7. These spheroplasts were centrifuged at 5,000 x g at 4°C, ($P_5$) washed twice in 0.5M potassium phosphate buffer, pH 7.5. From the washed spheroplasts ($P_5$), membrane fractions were prepared by either osmotic shock (OS) or sonication (SON, Section 2.2.7). Nitrate reductase was assayed by nitrite production in the presence of 2.5 mM KCN and nitrite reductase by $NO_2^-$ utilization using BVH as the electron donor (Sections 2.2.9.2 and 2.2.9.3). Nitrous oxide reductase was also assayed with BVH by measuring $N_2O$ utilization from the gas phase with a GC/mass spectrometer. Glucose-6-phosphate dehydrogenase activity was measured by oxidation of NADH at 340 nm (Section 2.2.9.5). Assays were done in triplicate and the results were within 5% error.

| Preparations | Total Protein (mg) | Activity (µmol/min/mg protein) | | | |
|---|---|---|---|---|---|
| | | $NO_3^-$-reductase | $NO_2^-$-reductase | $N_2O$-reductase | G-6-P-dehydrogenase |
| 1. Cells | 42.0 | 0.07 | 0.24 | 0.27 | 0.02 |
| 2. Supernatant left after separating spheroplasts ($S_5$) | 8.9 | 0.25 | 1.13 | 0.82 | 0 |
| 3. Spheroplasts ($P_5$) | 29.2 | 0.05 | 0.20 | 0.21 | 0.03 |
| 4. Spheroplast lysate(O.S.$S_{240}$) | 15.1 | 0.03 | 0.11 | 0.12 | 0.03 |
| 5. Spheroplast membranes (O.S.$P_{240}$) | 11.0 | 0.02 | 0.06 | 0.06 | 0 |
| 6. Spheroplast lysate (SON $S_{240}$) | 14.9 | 0.02 | 0.08 | 0.09 | 0.03 |
| 7. Spheroplast membranes (SON $P_{240}$) | 8.2 | 0.03 | 0.06 | 0.06 | 0 |

TABLE 8: PURIFICATION OF NITRATE REDUCTASE FROM CELLS GROWN ANAEROBICALLY IN LIGHT WITH NITRATE.

All purification steps were performed as described in Section 3.4.2.1. Enzyme activity was determined by following the production of $NO_2^-$ from $NO_3^-$ using BVH as the electron donor as described in Section 2.2.9.6. One unit is defined as µmole $NO_2^-$ produced/min. Specific activity is defined as the number of units/mg protein. Assays were conducted in triplicate and errors were $\leqslant 5\%$.

| Extract | Total protein (mg) | Total units | Specific activity | Fold purification | % Recovery |
|---|---|---|---|---|---|
| 1. $S_{20}$ (crude extract centrifuged at 20,000 x g for 20 min, supernatant) | 584 | 310 | 0.5 | - | 100 |
| 2. $P_{240}$ ($S_{20}$ centrifuged at 240,000 x g for 1h, pellet) | 369 | 710 | 1.9 | 1 | 229 |
| 3. [2] Resuspended in 0.1M Tris-HCl (pH 9.0), heat treatment at 50°C for 20min, centrifuged at 240,000 x g, supernatant ($Ht_{50}$ $S_{240}$) | 33 | 575 | 17.4 | 9 | 185 |
| 4. $(NH_4)_2SO_4$ fractionation of [3]; pellet between 20-60% dissolved in buffer, dialysed for 16h against 0.1M K-($PO_4$), buffer pH 7.0 | 20.6 | 461 | 22.4 | 12 | 149 |
| 5. [4]-Loaded onto Sephacel ion exchange column (1.6 x16cm), eluted at 0.28-0.32M NaCl gradient | 2.97 | 250 | 84.2 | 44 | 81 |
| 6. [5] Concentrated by Amicon ultrafiltration through PM10; dialysed for 16h against 0.1M K-($PO_4$) buffer, pH 7.0, and then loaded on to Sephadex G-100 column; fractions 30-36, pooled and concentrated by ultrafiltration | 0.20 | 55 | 275.0 | 143 | 18 |

FIG. 17: ACTIVITY PROFILE OF NITRATE REDUCTASE IN FRACTIONS FROM A SEPHACEL ION EXCHANGE COLUMN.

The column (1.6 x 16 cm) was pre-equilibrated with 0.1M potassium phosphate buffer (pH 7.0). After loading the dialysed sample (fraction 4, Table 8) the column was then washed with the same buffer (30 ml). The enzyme was eluted with 0-0.5M NaCl gradient in 0.1M potassium phosphate buffer (pH 7.0). Enzyme activity was determined by measuring $NO_2^-$ production from $NO_3^-$ as described in Section 2.2.9.6.

— % $T_{280nm}$;
○ Nitrate reductase activity (µmol $NO_2^-$ produced/min/ml fraction).

FIG. 18: ACTIVITY PROFILE OF NITRATE REDUCTASE IN FRACTIONS FROM SEPHADEX G-100 COLUMN.

The column (1.6 x 93 cm) was pre-equilibrated with 0.1M potassium phosphate buffer (pH 7.0). Active samples from ion exchange chromatography (fractions 34-42, Fig.17) were pooled, concentrated to 2 ml by ultrafiltration (Amicon) through PM-10 membrane and then loaded on to the Sephadex G-100 column. Enzyme was eluted from this column by 0.1M potassium phosphate buffer (pH 7.0). Enzyme activity was determined as described in Section 2.2.9.6.

— % $T_{280nm}$;
○ Nitrate reductase activity (µmol $NO_2^-$ produced/min/ml fraction).

FIG. 17

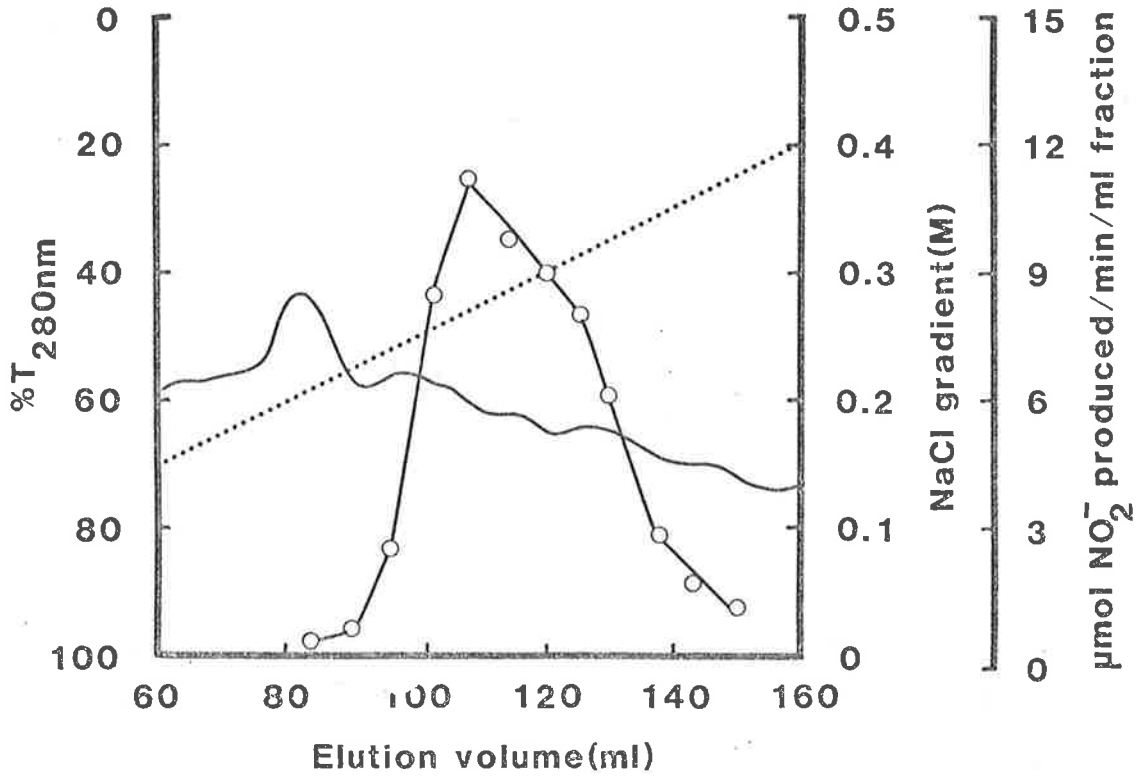
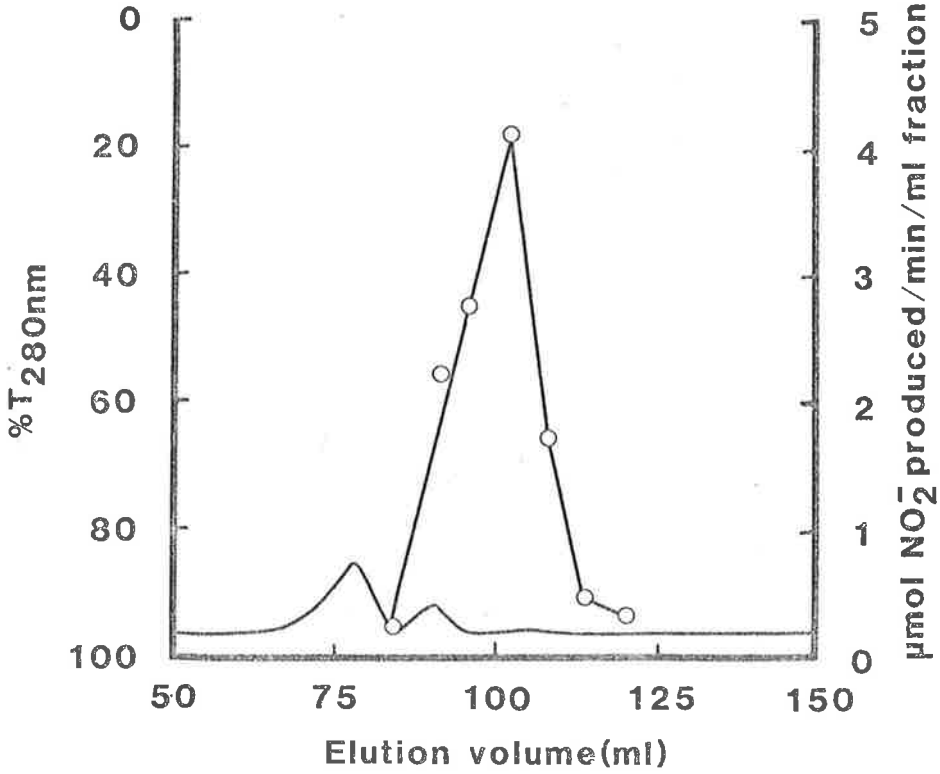

0
20
40
60
80
100
%T 280nm
0.5
0.4
0.3
0.2
0.1
0
NaCl gradient(M)
15
12
9
6
3
0
μmol NO2- produced/min/ml fraction
60
80
100
120
140
160
Elution volume(ml)

FIG. 18


0
20
40
60
80
100
%T 280nm
5
4
3
2
1
0
μmol NO2- produced/min/ml fraction
50
75
100
125
150
Elution volume(ml)

3.4.2.2 Nitrite reductase

A 168-fold purified nitrite reductase was prepared from washed cells grown under photodenitrifying conditions with an 18% recovery as shown in Table 9. When the crude extract ($S_{20}$), prepared by French Pressure cell method (Section 2.2.6), was centrifuged at 240,000 x g for 1h at 4°C, 82% of the enzyme activity was recovered in the supernatant fraction ($S_{240}$). This fraction was heat treated at 70°C for 10 min and then cooled in ice and centrifuged at 240,000 x g for 1h, resulting in a 4-fold purification. Ammonium sulphate fractionation of fraction 3, produced a 12-fold purification. After dialysis against 0.1M Tris-HCl buffer containing 0.2M NaCl (pH 7.5) for 16h, this fraction was loaded on to a DEAE-Sephadex-A50 column (4 x 10 cm) pre-equilibrated with the same buffer. The enzyme was eluted with a NaCl gradient between 0.26 to 0.29M in the same buffer (Fig. 19). The enzyme was finally purified by gel-filtration on a Sephadex G-100 column (15 x 150 cm) as shown in Fig. 20.

3.4.3 Properties of the enzymes

3.4.3.1 Nitrate reductase

The molecular weight of this enzyme was variable. Three different aggregated forms were obtained by gel filtration and also by polyacrylamide gel electrophoresis (PAGE) in the presence of 1M urea. Molecular weight of these aggregated forms were 112 Kd, 100 Kd and 70 Kd.

Absorption spectra of the partially purified enzyme (fraction 5, Table 8) reduced with sodium dithionite revealed peaks at 418,521 and 551 nm indicating the presence of a cytochrome *c*. The purified enzyme (fraction 6, Table 8) however did not contain cytochrome *c*.

When Na-molybdate was replaced with Na-tungstate (1 mM) in the growth medium the synthesis of nitrate reductase was markedly inhibited since little or no enzyme activity was detected in extracts of these cells.

TABLE 9: PURIFICATION SCHEME FOR NITRITE REDUCTASE FROM CELLS GROWN UNDER PHOTODENITRIFYING CONDITIONS.

All purification steps were performed as described in Section 3.4.2.2. Enzyme activity was determined by following the utilization of $NO_2^-$ using BVH as the electron donor (Section 2.2.9.7). One enzyme unit is defined as µmole $NO_2^-$ utilized/min and specific activity as number of units/mg protein. Assays were done in triplicate and errors were ⩽5%.

| Extract | Total protein (mg) | Total units | Specific activity | Fold purification | % Recovery |
|---|---|---|---|---|---|
| 1. $S_{20}$ (crude extract centrifuged at 20,000 x g for 20min, supernatant) | 861 | 441 | 0.5 | 1 | 100 |
| 2. $S_{240}$ ($S_{20}$ centrifuged at 240,000 x g for 1hr supernatant) | 341 | 363 | 1.0 | 2 | 82 |
| 3. [2]-Heat treated at 70°C for 10min, centrifuged at 240,000 x g for 1h, supernatant ($Ht_{70}$ $S_{240}$) | 157 | 298 | 1.9 | 4 | 68 |
| 4. $(NH_4)_2SO_4$-fractionation of [3]; $P_{30-50\%}$ dissolved in buffer, dialysed for 16h against 0.1M Tris-HCl containing 0.2M NaCl (pH 7.5) | 37 | 225 | 6.1 | 12 | 51 |
| 5. [4]-Loaded onto DEAE-Sephadex A50 column (3x10cm) pre-equilibrated with 0.1M Tris-HCl containing 0.2M NaCl (pH 7.5), enzyme eluted at 0.26-0.29M NaCl gradient | 4 | 140 | 35.0 | 70 | 32 |
| 6. [5]-Concentrated to 3ml by Amicon ultrafiltration through PM10; dialysed for 16h against 0.1M Tris-HCl buffer (pH 7.5) and loaded onto a Sephadex G100 column (1.5x150cm) pre-equilibrated and then eluted with the same buffer. | 0.95 | 80 | 84.2 | 168 | 18 |

FIG. 19: ELUTION PROFILE OF NITRITE REDUCTASE FROM DEAE-SEPHADEX A50 COLUMN.

After ammonium sulphate fractionation, the sample (fraction 4, Table 9) was dialysed for 16h against 0.1M Tris-HCl containing 0.2M NaCl (pH 7.5) and then loaded onto a DEAE-Sephadex $A_{50}$ column (3 x 10 cm) pre-equilibrated with the same buffer. The column was then washed with 0.1M Tris-HCl buffer containing 0.2M NaCl (150 ml), pH 7.5. Then the enzyme was eluted with 0.2-0.5M NaCl in 0.1M Tris-HCl (pH 7.5). Nitrite reductase activity was determined by following the utilization of $NO_2^-$ as described in Section 2.2.9.7.

— % $T_{280nm}$;
○ Activity (µmol $NO_2^-$ utilized/min/ml fraction).

FIG. 20: SEPARATION OF NITRITE REDUCTASE ON SEPHADEX G-100 COLUMN.

Active samples from the DEAE Sephadex $A_{50}$ column (fractions 20-30, Fig. 19) were pooled and concentrated to 3 ml by Amicon ultrafiltration using a PM-10 membrane. The concentrated sample was loaded onto the Sephadex G100 column (1.5 x 150 cm) pre-equilibrated with 0.1M Tris-HCl (pH 7.5). The enzyme was eluted from this column with the same buffer. Enzyme activity was determined as described in Section 2.2.9.7.

— % $T_{280nm}$;
○ Enzyme activity (µmol $NO_2^-$ utilized/min/ml fraction).

FIG.19

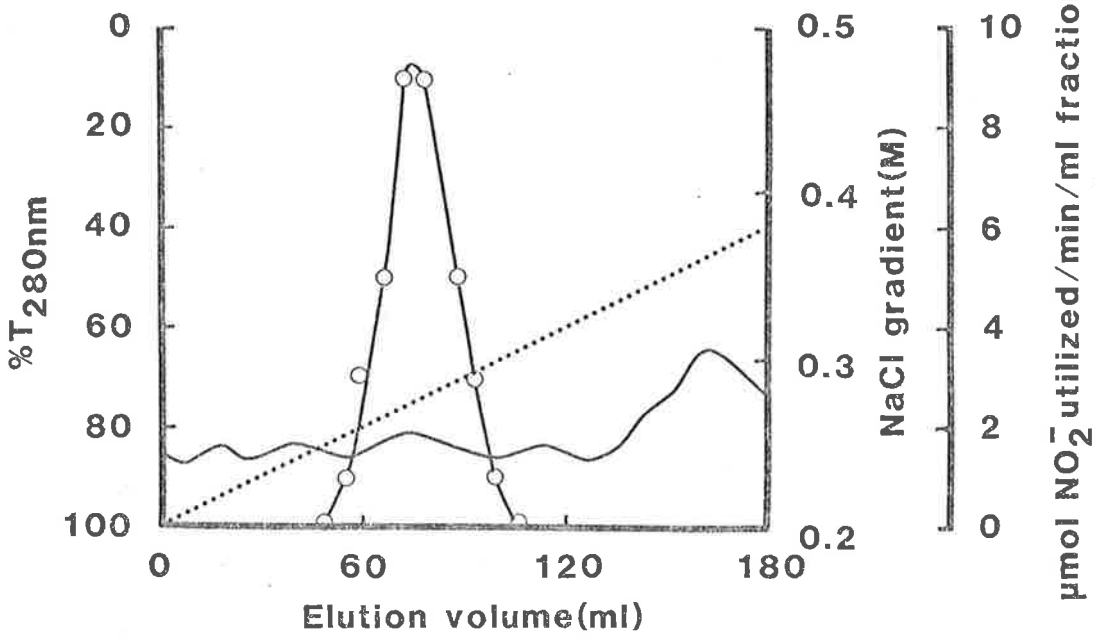
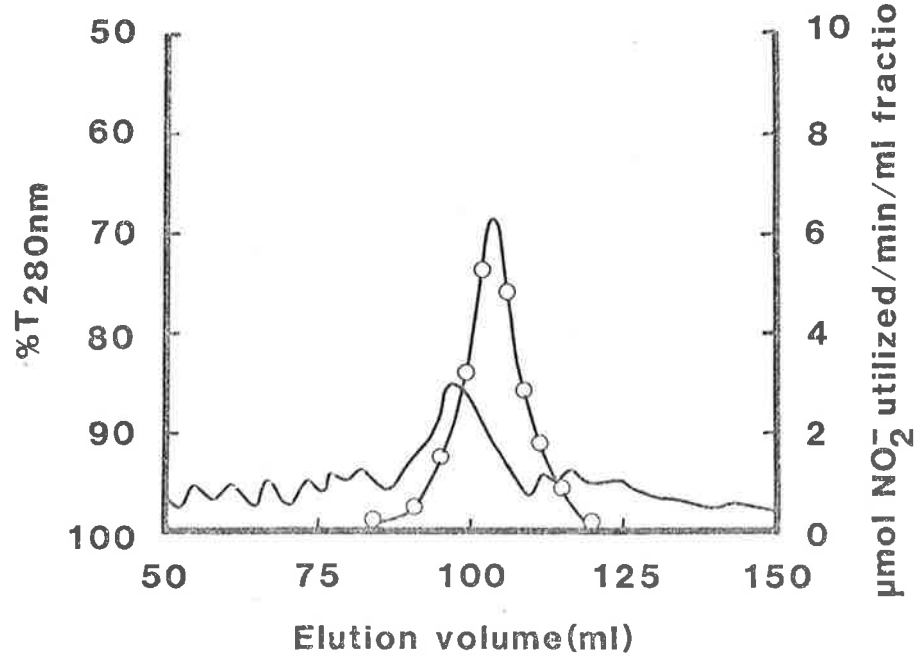

0
20
40
60
80
100
%T280nm
0
60
120
180
Elution volume(ml)
0.5
0.4
0.3
0.2
NaCl gradient(M)
10
8
6
4
2
0
μmol NO2- utilized/min/ml fraction

FIG.20


50
60
70
80
90
100
%T280nm
50
75
100
125
150
Elution volume(ml)
10
8
6
4
2
0
μmol NO2- utilized/min/ml fraction

The effect of pH on nitrate reductase activity was investigated by the dithionite-BV method (Section 2.2.9.6). The pH optimum was about 6.5. The apparent $K_m$ for $NO_3^-$ at pH 6.5 was 1.6 mM.

When BVH in the assay mixture was replaced by various other electron donors, no enzyme activity was observed in the presence of either NADH, NADPH, $FMNH_2$, $FADH_2$ or reduced forms of either cytochrome $b_2$, ubiquinone-10 or cytochrome $c_2$.

Bathophenanthroline inhibited nitrate reductase activity by 75% (Table 10). The enzyme activity was markedly reduced (70%) by KCNS at 5 mM whereas O-phenanthroline, α,α-dipyridyl and 8-hydroxyquinoline restricted enzyme activity by 20-35%. *p*-CMB reduced the activity by 40% and this effect was partially reversed by adding cysteine.

3.4.3.2 Nitrite reductase

The molecular weight of the purified enzyme (fraction 6, Table 9), determined by gel-filtration using a Sephadex G-100 column (Section 2.2.10) was 82 Kd. Bovine serum albumin (dimer, 135 Kd; monomer, 67 Kd), ovalbumin (43 Kd), chymotrypsinogen A (25 Kd) and ribonuclease A (13.7 Kd) were used as marker proteins (Fig. 21). Polyacrylamide disc gel electrophoresis of fraction 6 (Table 9) produced a single protein band. When the gel was immersed in a solution of $KNO_2$ containing BVH under argon (Section 2.2.11) the protein band on the gel was decolourized (Fig. 22). The molecular weight of the two identical subunits, determined by SDS polyacrylamide gel electrophoresis was 40 Kd (Figs. 23 and 24).

The effects of various electron donors on nitrite reduction by the purified enzyme (fraction 6, Table 9) are given in Table 11. In addition to BVH and MVH, the enzyme utilized reduced forms of 2,6-dichlorophenol-indophenol and phenazine methosulfate whereas $FMNH_2$, NADH, NADPH, baker's

TABLE 10: EFFECTS OF INHIBITORS ON BVH-LINKED NITRATE REDUCTASE ACTIVITY.

The purified enzyme (fraction 6, Table 8) was assayed anaerobically with BVH as the electron donor as described in Section 2.2.9.6. Enzyme activity (70 nmol $NO_2^-$ produced/min) without inhibitor was considered 100%. All the assays were conducted in triplicate and errors were $\leqslant$ 5%. Appropriate controls were done to check for any effects of alcohol and inhibitors on enzyme assay.

| Inhibitor | Final conc. (mM) | % Inhibition |
|---|---|---|
| O-phenanthroline | 5 | 20 |
| Bathophenanthroline | 1 | 75 |
| α,α-dipyridyl | 5 | 20 |
| KCNS | 5 | 70 |
| 8-Hydroxyquinoline | 5 | 35 |
| *p*-CMB | 0.5 | 40 |
| *p*-CMB + cysteine | 0.5 + 2 | 5 |

FIG. 21: MOLECULAR WEIGHT DETERMINATION OF PURIFIED NITRITE REDUCTASE USING A SEPHADEX G-100 COLUMN.

The column (1.5 x 150 cm) was pre-equilibrated with 0.1M Tris-HCl, buffer (pH 7.5). The marker proteins (2 mg/ml) and the enzyme (fraction 6, Table 9) were loaded onto the column and eluted in turn with the same buffer (Section 2.2.10). The elution volumes (Ve) for each protein were recorded and $Ve/_{Vo}$ ratios (Vo = void volume) were plotted against molecular weights of the respective proteins.

- ● BSA dimer (135 Kd);
- ○ Nitrite reductase (82 Kd);
- □ BSA monomer (67 Kd);
- △ Ovalbumin (43 Kd);
- ▲ Chymotrypsinogen A (25 Kd);
- ▪ Ribonuclease A (13.7 Kd).

FIG.21

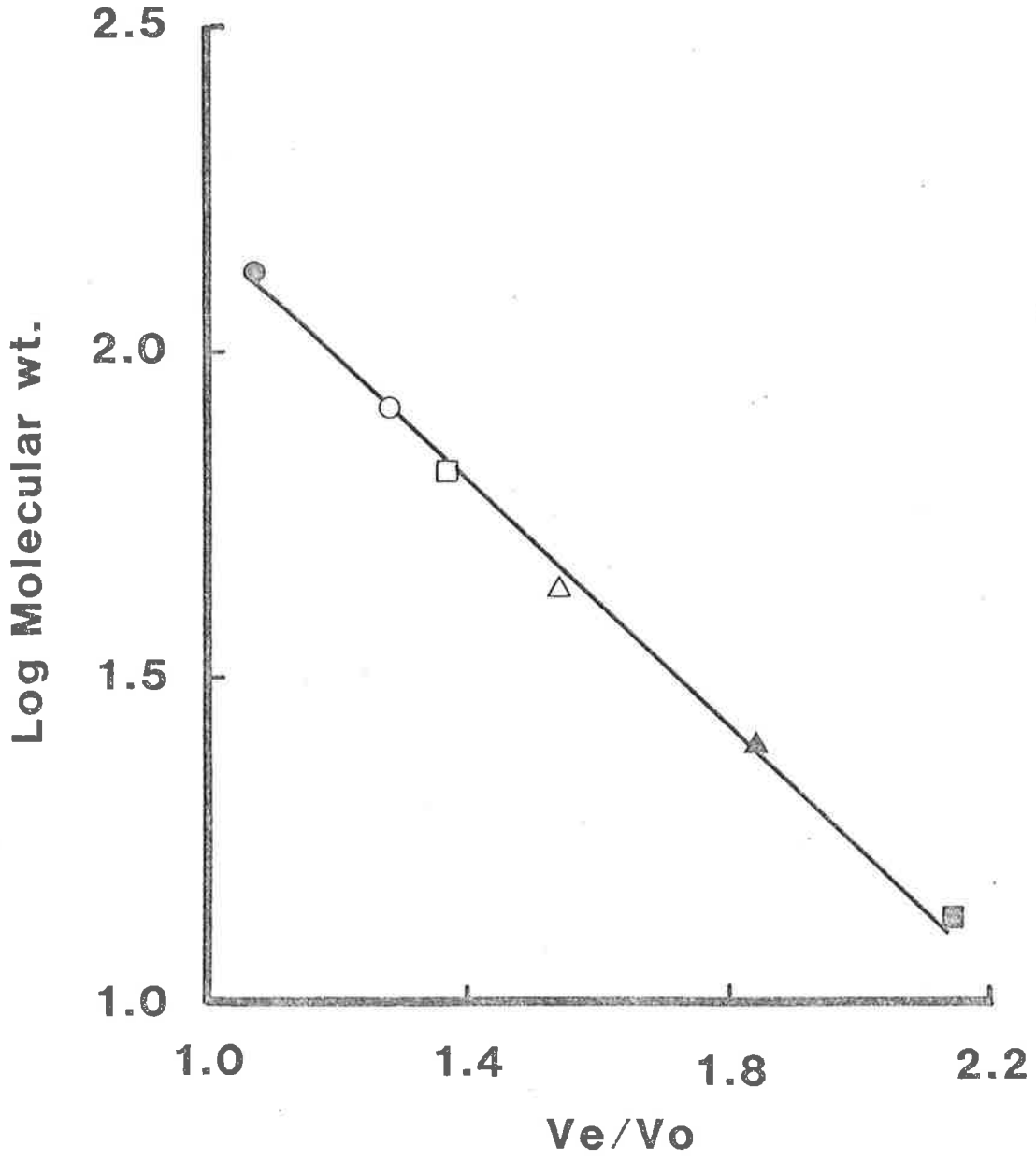


FIG. 22: POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) OF PURIFIED NITRITE REDUCTASE.

PAGE of nitrite reductase (fraction 6, Table 9) was carried out in 7.5% w/v polyacrylamide gels as described in Section 2.2.11. Gel A was stained with Coomassie blue (Section 2.2.11) and Gel B was used to determine enzyme activity as described in Section 2.2.11. B.P.B. = Bromophenol blue.

FIG.22

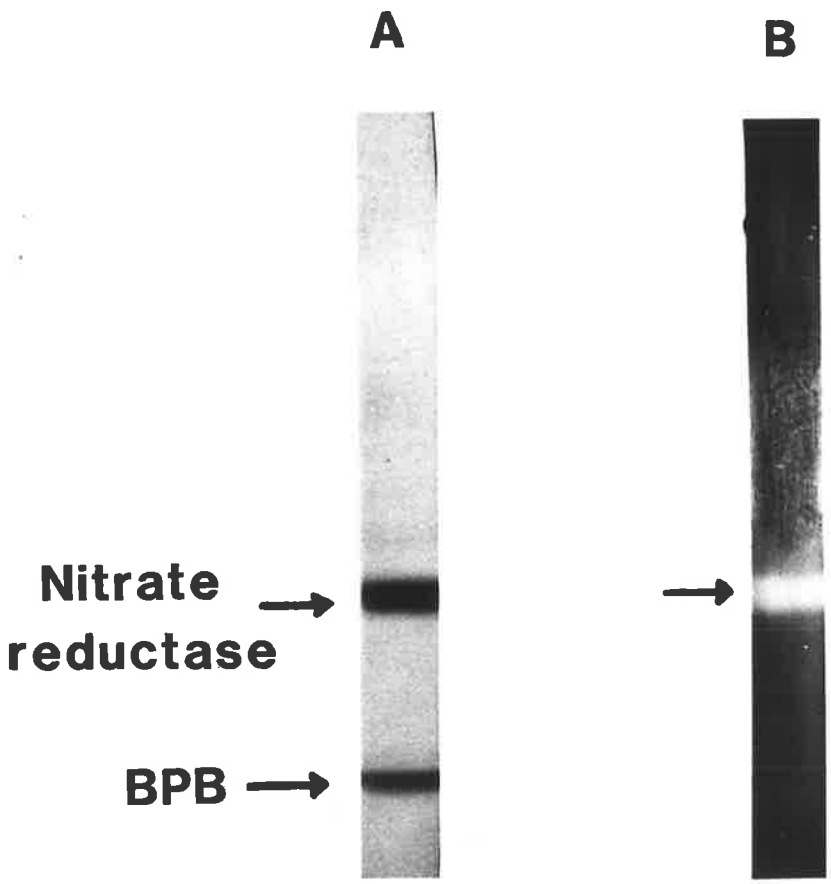


FIG. 23: SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) OF NITRITE REDUCTASE PREPARATIONS.

SDS-PAGE was carried out on a 12% (w/v) polyacrylamide gel slab in the presence of 0.1% (w/v) SDS (Section 2.2.11). The gel was stained with Coomassie blue (Section 2.2.11).

Lane A, molecular weight markers:
1 - phosphorylase *b* (94Kd);
2 - Albumin (67 Kd);
3 - Ovalbumin (43 Kd);
4 - Carbonic anhydrase (30 Kd), 5-trypsin; inhibitor (21.1 Kd);

Lane B, DEAE-Sephadex $A_{50}$ extract (20 µg protein);

Lane C, Gel filtration extract (2 µg protein);

Lane D, Gel filtration extract (1 µg protein).

FIG.23

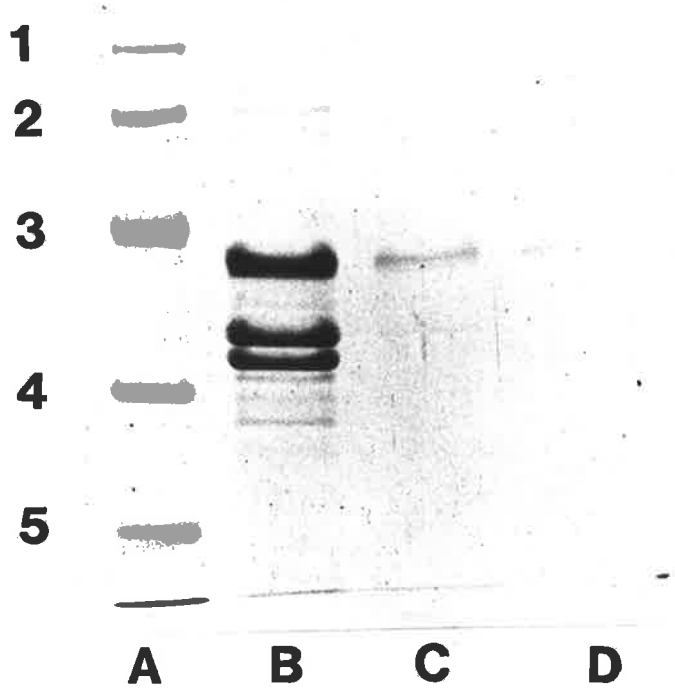


FIG. 24: MOLECULAR WEIGHT DETERMINATION OF THE SUBUNIT OF PURIFIED NITRITE REDUCTASE BY SDS-PAGE.

SDS-PAGE of nitrite reductase (fraction 6, Table 9), was carried out as described in Fig. 23. Mobility of each protein was determined relative to that of bromophenol blue.

- ● Phosphorylase b (94 Kd);
- ○ Albumin (67 Kd);
- △ Ovalbumin (43 Kd);
- ▩ Nitrite reductase (40 Kd);
- ▲ Carbonic anhydrase (30 Kd);
- □ Trypsin inhibitor (21.1 Kd).

FIG. 24

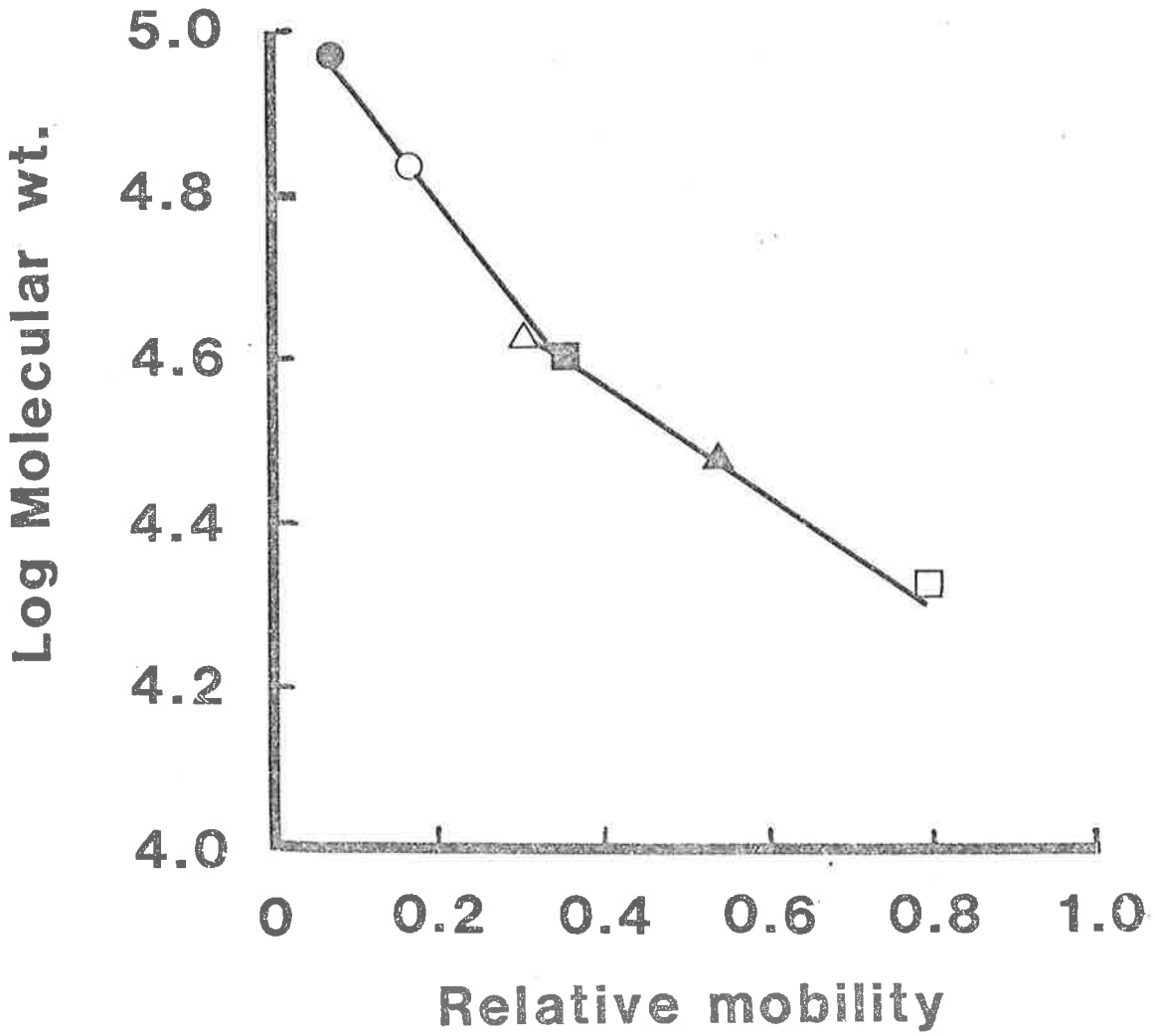

5.0
4.8
4.6
4.4
4.2
4.0
Log Molecular wt.
0
0.2
0.4
0.6
0.8
1.0
Relative mobility

TABLE 11: UTILIZATION OF VARIOUS ELECTRON DONORS BY PURIFIED NITRITE REDUCTASE.

Enzyme (fraction 6, Table 9) was assayed under anaerobic conditions as described in Section 2.2.9.7. BV, MV, DCPIP, PMS, FMN and cytochrome *c* respectively were reduced in the reaction mixture with 100 µl $Na_2S_2O_4$ (6 mg/ml, dissolved in 1% w/v $NaHCO_3$) to start the reaction. NADH and NADPH were injected into the reaction mixture. Final concentration of each electron donor was 100 µM. Enzyme activity (65 nmol $NO_2^-$ utilized/min) with BVH as the electron donor is regarded as 100%. Assays were done in triplicate and errors were within 5%.

| Electron donor | % Activity compared to BVH |
|---|---|
| BVH | 100 |
| MVH | 108 |
| $DCPIP_{red}$ | 100 |
| $PMS_{red}$ | 92 |
| $FMNH_2$ | 5 |
| Horse heart cytochrome $c_{red}$ | 2 |
| Lactate + Lactate dehydrogenase (Cyt $b_2$) | 3 |
| NADH | 0 |
| NADPH | 0 |

yeast lactate/lactic dehydrogenase (cytochrome $b_2$) and reduced forms of horse heart cytochrome *c* and cytochrome $c_2$ from this bacterium were ineffective.

The effects of inhibitors on the purified enzyme presented in Table 12 indicate that KCN, CO and DIECA markedly inhibited enzyme activity. The CO inhibition in the dark was not reversed when the inhibited enzyme was exposed to light. Inhibition (100%) of enzyme activity by DIECA and its reversal by Cu indicate that Cu may be required for enzyme activity. Among other inhibitors, O-phenanthroline and 8-hydroxyquinoline restricted enzyme activity by about 20 to 30%, whereas $NaN_3$, Na-EDTA, α,α-dipyridyl, *p*-CMB, rotenone, amytal, antimycin A and 2-n-heptyl-4-hydroxyquinoline-N-oxide were without effect.

The copper content of the enzyme was determined by atomic absorption spectrometry (Section 2.2.17.3). The purified enzyme contained 2 mol of Cu per mol protein, i.e. 1 mol Cu per subunit.

The enzyme activity was assayed over the pH range of 5 to 9 with reduced benzyl viologen as the electron donor. Appropriate controls were done at each pH value to correct for any chemical reduction of nitrite by the donor system. The optimum pH for enzyme activity was 7.0 (Fig. 25).

About 80% of enzyme activity was retained on heating either crude or purified enzyme at 70°C for 10 min (Fig. 26).

The $K_m$ value for nitrite (fraction 6) was 46.6 ± 2.8 µM determined by Lineweaver-Burk plots (Fig. 27).

The gaseous product of the purified enzyme supplied with $K^{15}NO_2$ was found to be $^{15}N_2O$ (Section 2.2.18). When fragmentation of $^{15}N_2O$ in to $^{15}N_2$ and $^{15}NO$ ($2^{15}N_2O \rightarrow {}^{15}N_2 + 2^{15}NO$) in the mass spectrometer were taken into account the stoichiometry for $^{15}NO_2^-$:$^{15}N_2O$ was 2:1 (Fig. 28).

TABLE 12: EFFECTS OF INHIBITORS ON PURIFIED NITRITE REDUCTASE.

The purified enzyme (fraction 6, Table 9) was assayed anaerobically with reduced benzyl viologen as the electron donor as described in Section 2.2.9.7. Enzyme activity (65 nmol $NO_2^-$ utilized/min) without an inhibitor is regarded as 100%. Assays were done in triplicate and errors were $\leqslant$ 5%. Appropriate controls were taken to check any effects of alcohol and the inhibitors on the enzyme assay.

| Inhibitor | Final conc. (mM) | % inhibition |
|---|---|---|
| KCN | 0.1 | 50 |
| | 0.2 | 76 |
| | 0.5 | 100 |
| CO (Dark) | Sparged for 5 min | 90 |
| CO (Light) | Sparged for 5 min | 88 |
| DIECA | 0.1 | 55 |
| | 0.2 | 85 |
| | 0.3 | 100 |
| *p*-CMB | 1.0 | 4 |
| O-Phenanthroline | 1.0 | 18 |
| 8-Hydroxyquinoline | 1.0 | 30 |

FIG. 25: EFFECTS OF pH ON PURIFIED NITRITE REDUCTASE ACTIVITY.

Nitrite reductase (0.79 µg protein, fraction 6, Table 9) was assayed anaerobically with BVH as the electron donor as described in Section 2.2.9.7. Appropriate controls were conducted at various pH values to correct for any chemical reduction of nitrite by BVH. Experiments were conducted in triplicate and restuls were within 5% error.

FIG. 26: EFFECTS OF TEMPERATURE ON THE ACTIVITY OF PURIFIED NITRITE REDUCTASE.

Nitrite reductase (0.79 µg protein, fraction 6, Table 9) was assayed (in triplicate) anaerobically with BVH as the electron donor as described in Section 2.2.9.7. Experimental errors were $\leqslant$ 5%.

FIG.25

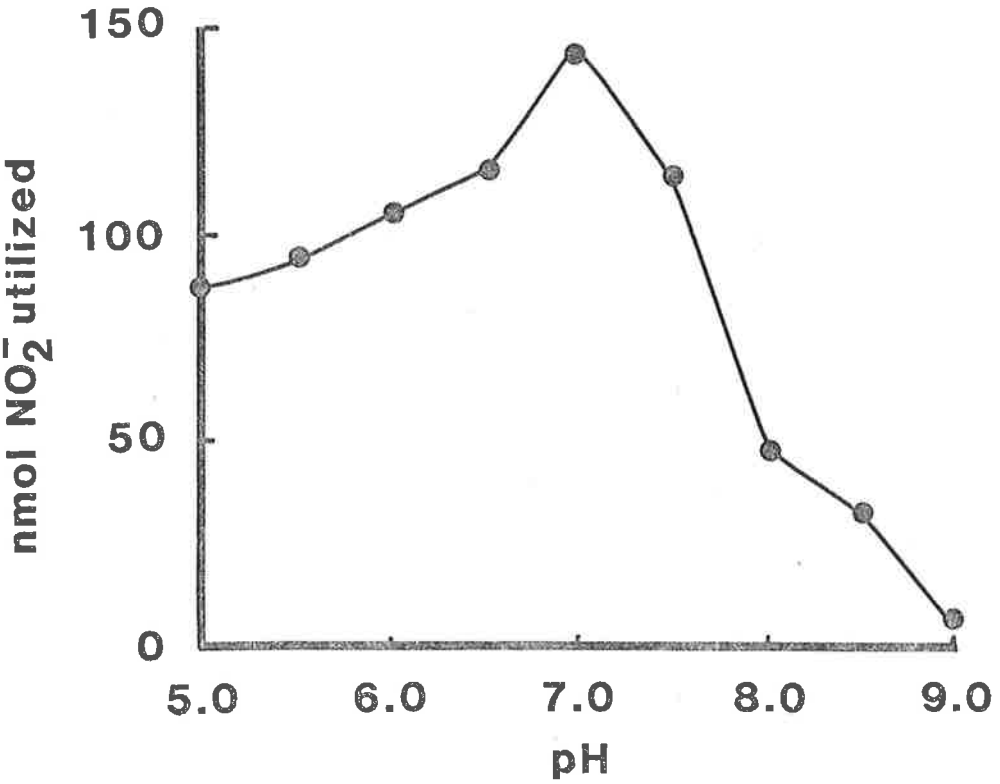

150
100
50
0
nmol $NO_2^-$ utilized
5.0
6.0
7.0
8.0
9.0
pH

FIG.26

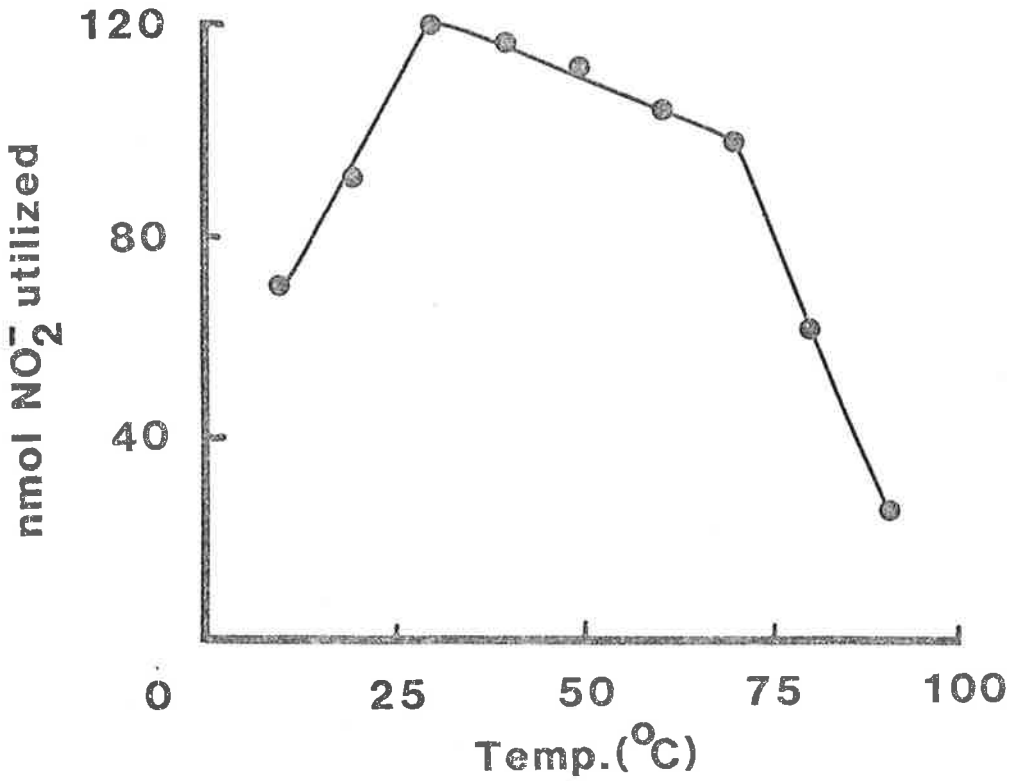

120
80
40
nmol $NO_2^-$ utilized
0
25
50
75
100
Temp.(°C)

FIG. 27: LINEWEAVER-BURK PLOTS OF PURIFIED NITRITE REDUCTASE.

Nitrite reductase (0.79 µg protein, fraction 6, Table 9) was assayed anaerobically with BVH as the electron donor (Section 2.2.9.7). Nitrite was varied from 0-300 µM in the assay mixtures.

FIG. 28: TIME COURSE FOR $^{15}N_2O$ PRODUCTION FROM $^{15}NO_2^-$ BY PURIFIED NITRITE REDUCTASE.

The enzyme (fraction 6, Table 9) activity was assayed (in triplicate) by a GC-mass spectrometer (Hewlett Packard HP5992B) by measuring the $N_2O$ produced (Section 2.2.18. ). $NO_2^-$ utilization by the enzyme was assayed as described in Section 2.2.8.2. Experimental errors were $\leqslant$ 5%.

● $^{15}N_2O$ production;

○ $^{15}NO_2^-$ utilization;

stoichiometry $NO_2^-$ : $N_2O \equiv 2:1$.

FIG.27

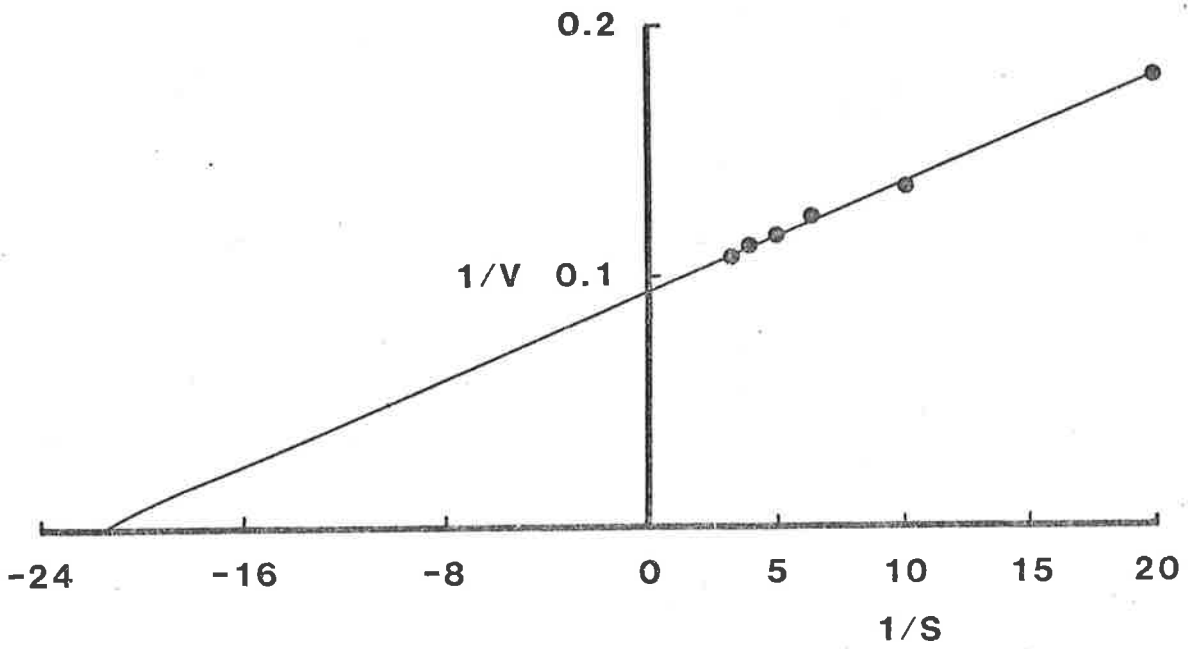
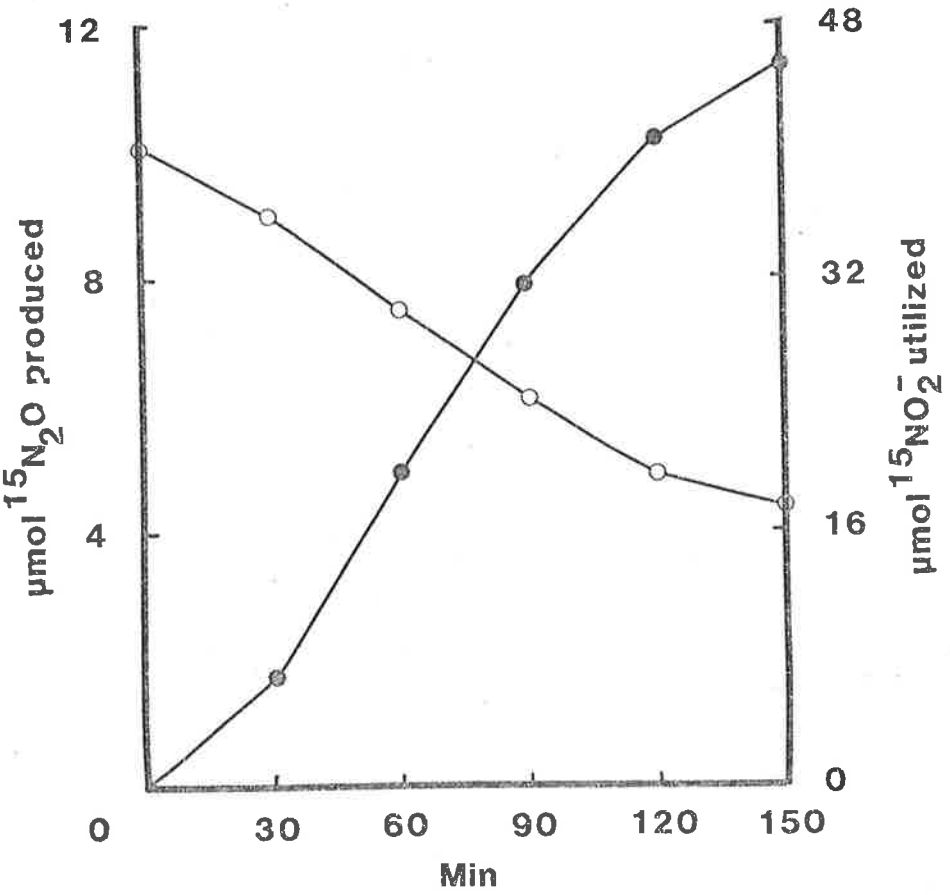

0.2
1/V 0.1
-24
-16
-8
0
5
10
15
20
1/S

FIG.28


12
8
4
0
μmol $^{15}N_2O$ produced
48
32
16
0
μmol $^{15}NO_2^-$ utilized
0
30
60
90
120
150
Min

## 3.5 ENERGY CONSERVATION DURING DENITRIFICATION IN WASHED CELLS

### 3.5.1 Proton translocation and oxidant pulse experiments

The proton current generated by the respiratory chain can not be determined directly under steady-state conditions as there is no mechanism for detecting the flux of protons when the rate of $H^+$-efflux exactly balances that of re-entry. It is, however, possible to measure the initial ejection of protons which accompanies the onset of respiration before proton re-entry occurs. Oxidant pulse experiments have been used to measure changes in proton concentration in the external medium of dense cell suspensions in response to a brief burst (pulse) of respiratory activity (Scholes and Mitchell, 1970a,b). Proton translocation in washed cells of *R. sphaeroides* f. sp. *denitrificans* with $NO_3^-$, $NO_2^-$, $N_2O$ and $O_2$ respectively, as the terminal acceptor in light and in the dark have been studied in this way.

#### 3.5.1.1 Oxidant pulse response in absence of a permeant ion

Addition of either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ to illuminated washed cells previously grown under photodenitrifying conditions, resulted in a rapid respiration-dependent proton translocation (Figs. 29 and 30; A,C,E,G). The illuminated cells had a faster initial decay phase (acidification) than those in the dark and a passive back flow to alkalinity in light (Fig. 29; A,C,E,G) which was more pronounced with $NO_2^-$ (Fig. 30; A,C,E,G). The addition of $NO_3^-$ to washed cells in the dark produced primarily an acidification whereas with $NO_2^-$ there was first a sharp alkaline transient followed by acidification. The alkaline effect was not detected with either $N_2O$ or $O_2$ (Fig. 30; E,G). The total $H^+$ extrusions associated with the reduction of $NO_3^-$, $NO_2^-$ or $N_2O$ to $N_2$ are presented in Figs. 29 and 30. Carbonyl-cyanide-m-chlorophenyl hydrazone (CCCP) inhibited the ex-

FIG. 29: OXIDANT PULSE RESPONSE OF WASHED CELLS IN LIGHT.

Cells grown for 16h under photodenitrifying conditions were washed twice in 150 mM KCl (pH 7.0) and resuspended in the same solution (4.65 mg protein/ml). Either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ was injected into an anaerobic suspension of washed cells (9.3 mg protein) in 150 mM KCl (pH 7.0) containing 30 µg/ml carbonic anhydrase in a 7.5 ml glass vessel in light as indicated ↓. The bar corresponds to 10 ng atom $H^+$. Acidification resulted in a downward deflection (see Section 2.2.12.1 for details).

A,B, 13 nmol $NO_3^-$; C,D, 20 nmol $NO_2^-$; E,F, 30 ng atom $N_2O$; G,H, 12 ng atom O; B,D,F,H with 20 µM CCCP and either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$.

FIG. 30: OXIDANT PULSE RESPONSE OF WASHED CELLS IN THE DARK.

Washed cells, from photodenitrifying cultures (16h) were prepared as decribed in Fig. 29. Either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ was injected into an anaerobic suspension of washed cells (9.3 mg protein) in 150 mM KCl (pH 7.0) containing 30 µg/ml carbonic anhydrase in a 7.5 ml glass vessel in the dark as indicated ↓. The bar corresponds to 10 ng atom $H^+$. Acidification resulted in a downward deflection.

A,B, 13 nmol $NO_3^-$; C,D, 20 nmol $NO_2^-$; E,F, 30 ng atom $N_2O$; G,H, 12 ng atom $O_2$. B,D,F,H, with 20 µM CCCP and $NO_3^-$, $NO_2^-$, $N_2O$ and $O_2$ respectively.

FIG.29

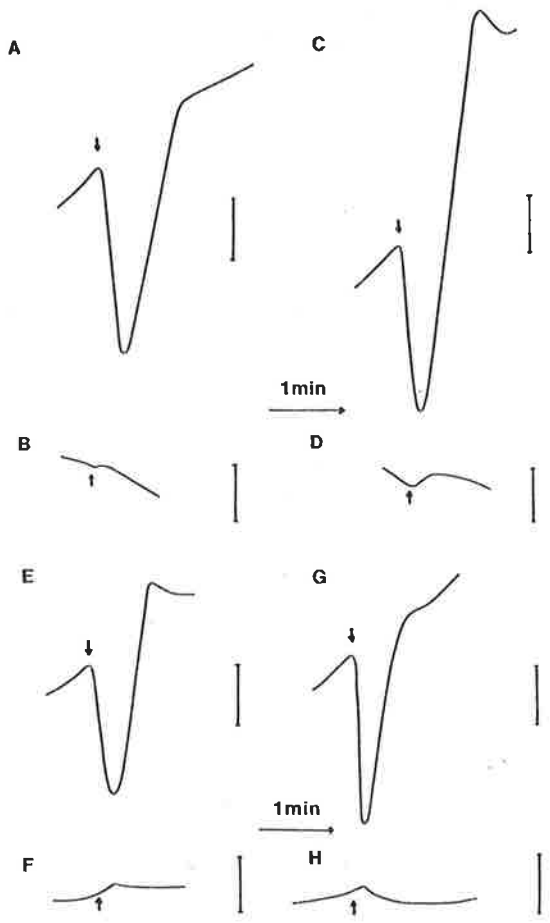
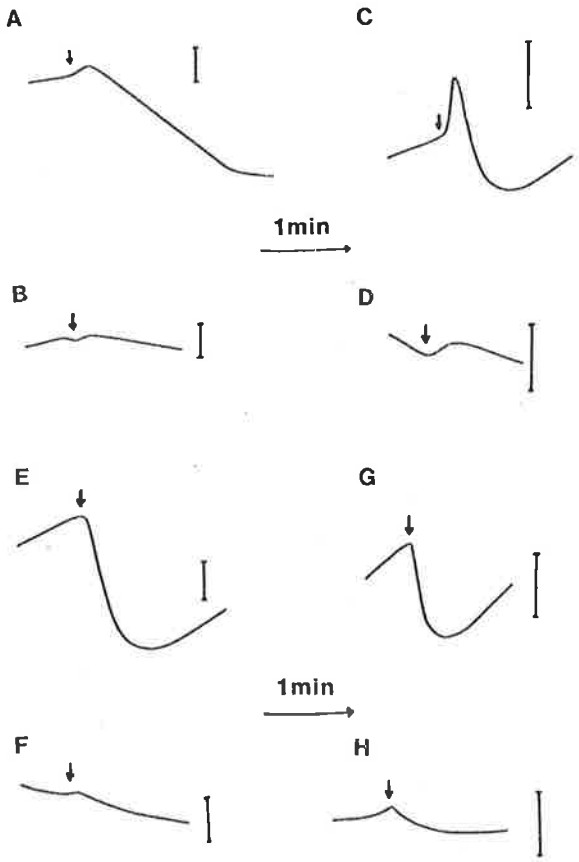



FIG.30



trusion of protons either in light or in the dark (Figs. 29 and 30; B,D,F,H). Illuminated cells had higher $^{\rightarrow}H^+$/oxidant ratios than those in the dark. Thus $^{\rightarrow}H^+$/oxidant ratios for $NO_3^-$, $NO_2^-$ and $O_2$ in the dark were 1.98, 1.02 and 1.37 whereas those in light were 3.24, 1.98 and 3.10. $^{\rightarrow}H^+/N_2O$ ratios were similar in light (1.11) to those in the dark (1.07).

#### 3.5.1.2 Stoichiometries of $O_2$ and $NO_3^-$ respiration dependent proton translocation in the presence of valinomycin

Valinomycin, an ionophore which catalyses the electrical uniport of $K^+$, was used to collapse the membrane potential. This collapse increased the ion permeability of the cell membranes. Maximal $^{\rightarrow}H^+$/oxidant ratios were obtained when cells were pre-incubated for 1h with 20 µg/ml valinomycin. Thus washed cells pre-incubated with valinomycin, rapidly acidified the reaction mixture in response to the addition of either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ in light and in the dark (Figs. 31 and 32; A-E,G,I). In the presence of 1 mM DIECA, $NO_3^-$ was reduced to $NO_2^-$ only while 1 mM $NaN_3$ inhibited $N_2O$ reduction, resulting in the accumulation of $N_2O$ from either $NO_3^-$ or $NO_2^-$. The stoichiometry of proton translocation, $^{\rightarrow}H^+/2e^-$ or $^{\rightarrow}H^+$/oxidant, depends on the amount of oxidant added; the limit ratio is achieved when the amount of oxidant approaches zero (Scholes and Mitchell, 1970b). $^{\rightarrow}H^+$/oxidant limit ratios obtained in washed cells of the photodenitrifier in light were: $NO_3^-$, 7.95; $NO_2^-$, 5.0; $N_2O$, 5.7 and $O_2$, 7.25 (Fig. 33). Comparable values in the dark were 4.45, 2.80, 2.55 and 4.40 (Fig. 34). The maximum $^{\rightarrow}H^+/2e^-$ ratios determined experimentally for cells reducing these nitrogenous compounds to $N_2$ were 4.82 ($NO_3^-$), 5.43 ($NO_2^-$) and 6.20 ($N_2O$) in light and the comparable values in the dark were 3.99, 4.10 and 4.17 respectively, (Tables 13 and 14). For the reduction of $NO_3^- \rightarrow NO_2^-$, $NO_3^- \rightarrow 0.5\ N_2O$ and $NO_2^- \rightarrow 0.5\ N_2O$ by these cells in light, the $^{\rightarrow}H^+/2e^-$ ratios were 4.56, 5.19 and 5.95 respectively. Comparable

FIG. 31: pH RECORDINGS PRODUCED BY INJECTING $NO_3^-$, $NO_2^-$, $N_2O$ AND $O_2$ RESPECTIVELY INTO AN ANAEROBIC SUSPENSION OF WASHED CELLS IN LIGHT.

Washed cells (9.3 mg protein), from photodenitrifying cultures (16h), were pre-incubated with valinomycin (20µg/ml) for 1h in light and the reactions were conducted in light (Section 2.2.12.1). The bar corresponds to 10 ng atom $H^+$. Acidification resulted in a downward deflection.

A,B,C, 10 nmol $NO_3^-$; D,E,F, 10 nmol $NO_2^-$; G,H, 12.7 ng atom $N_2O$; I, 4.64 ng atom O; B,E,H, with 1 mM $NaN_3$; C,F, with 1 mM DIECA.

FIG. 32: pH RECORDINGS PRODUCED BY INJECTING EITHER $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ INTO AN ANAEROBIC SUSPENSION OF WASHED CELLS IN THE DARK.

Washed cells (9.3 mg protein) from photodenitrifying cultures (16h) were pre-incubated with valinomycin (20µg/ml) for 1h in the dark and the pH recordings were conducted in the dark (Section 2.2.12.1). The bar corresponds to 10 ng atom $H^+$. Acidification produced a downward deflection.

A,B,C, 10 nmol $NO_3^-$; D,E,F, 10 nmol $NO_2^-$; G and H 10.1 ng atom $N_2O$; I, 11.6 ng atom O; B,E and H with 1 mM $NaN_3$ and either $NO_3^-$, $NO_2^-$ or $N_2O$; C and F with 1 mM DIECA and either $NO_2^-$ or $N_2O$.

FIG.31

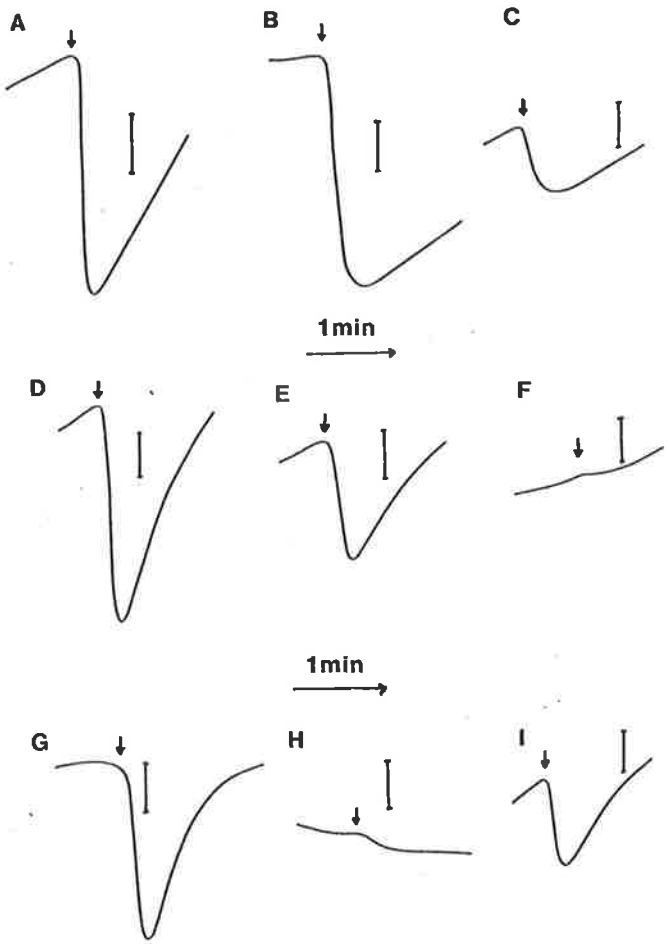



FIG.32

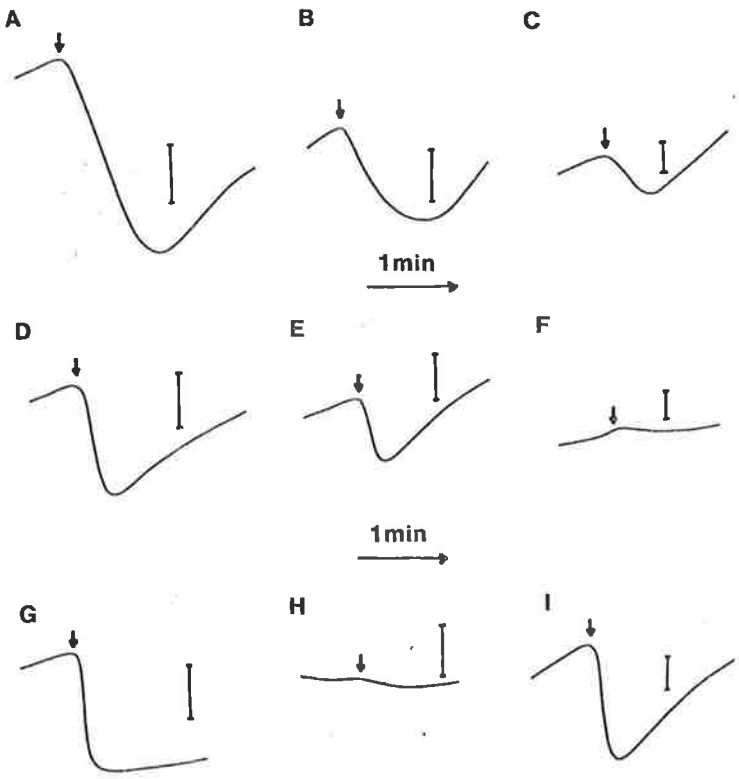


FIG. 33: RELATION BETWEEN $^{\rightarrow}H^{+}$/OXIDANT RATIO AND THE AMOUNT OF OXIDANT ADDED IN OXIDANT PULSE EXPERIMENTS WITH VALINOMYCIN TREATED CELLS IN LIGHT.

The washed cells (9.3 mg protein), from photodenitrifying cultures (16h), were treated with 20 µg/ml valinomycin for 1h in light and reactions were conducted in light as described in Section 2.2.12.1. Standard error of mean varied from ± 0.07 to ± 0.20.

● $NO_3^-$;
○ $NO_2^-$;
▲ $N_2O$;
△ $O_2$.

FIG. 34: RELATION BETWEEN $^{\rightarrow}H^{+}$/OXIDANT RATIO AND THE AMOUNT OF OXIDANT ADDED IN OXIDANT PULSE EXPERIEMNTS WITH VALINOMYCIN TREATED CELLS IN THE DARK.

Washed cells (9.3 mg protein), from photodenitrifying cultures (16h), were treated with 20 µg/ml valinomycin for 1h in the dark and reactions were conducted in the dark as described in Section 2.2.12.1. Standard error of mean varied from ± 0.1 to ± 0.3.

● $NO_3^-$;
○ $NO_2^-$;
▲ $N_2O$;
△ $O_2$.

FIG.33

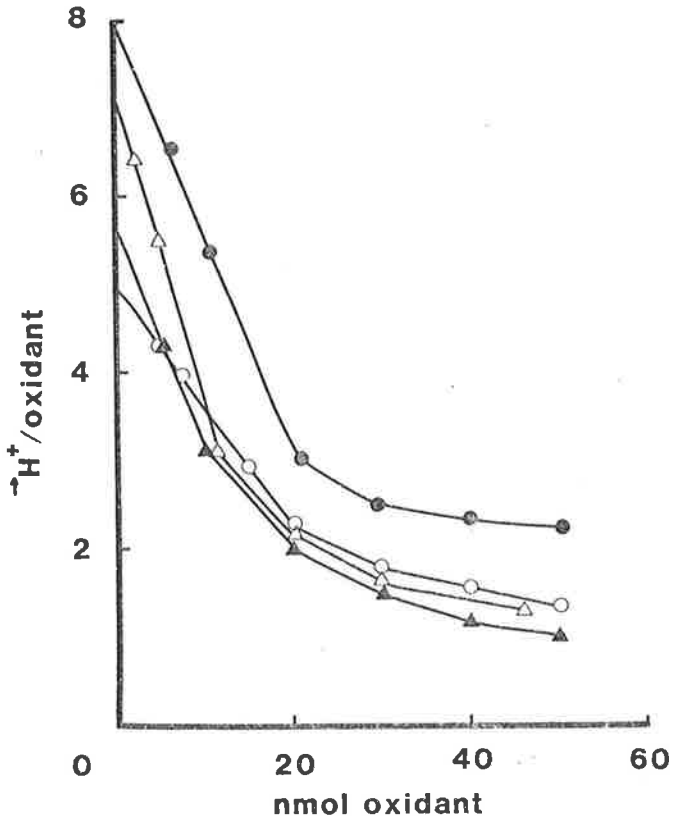
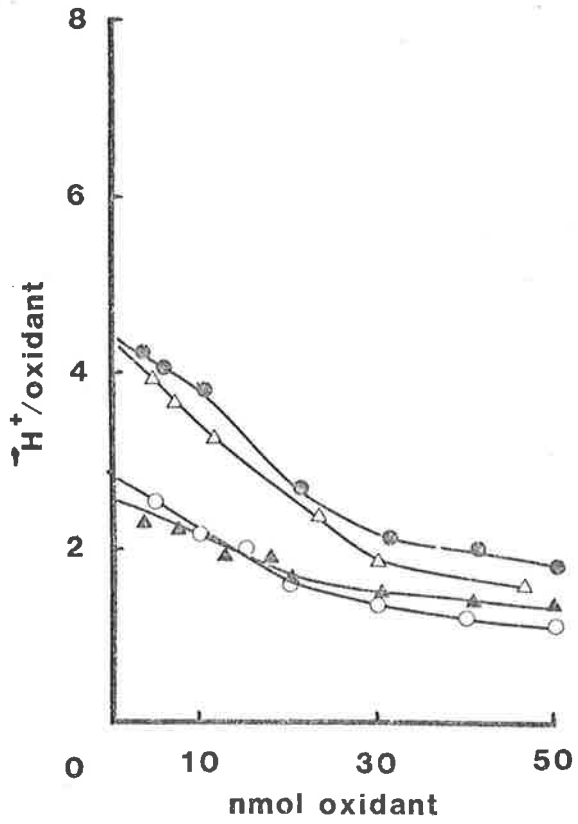

8
6
4
2
0
20
40
60
nmol oxidant
$H^+$/oxidant

FIG.34


8
6
4
2
0
10
30
50
nmol oxidant
$H^+$/oxidant

values in the dark were 3.90, 3.84 and 4.88. With $O_2$ as the terminal electron acceptor the $^{\rightarrow}H^+/2e^-$ values were 6.43 in light and 3.95 in the dark (Tables 13 and 14).

3.5.1.3 Stoichiometries for proton consumption with BVH as the electron donor

In order to obtain accurate proton consumption ratios, in the presence of an external electron donor, it is necessary to inhibit the electron flow from endogenous substrates. Injection of small amounts (up to 50 nmol) $NO_3^-$, $NO_2^-$ or $N_2O$ to washed cells in the dark preincubated with 1 mM NEM and 0.5 mM HOQNO (to inhibit proton extrusion produced by endogenous substrates) resulted in a rapid alkalinization of the extracellular medium when BVH was the electron donor (Figs. 35 and 36; A-E,G). The alkalinization produced by the addition of various amounts of either $NO_3^-$, $NO_2^-$ or $N_2O$ to washed cells in the dark with or without valinomycin was linear in each case (Figs. 37 and 38). The partial reactions (e.g. $NO_3^- \rightarrow NO_2^-$, $NO_3^- \rightarrow 0.5\ N_2O$ and $NO_2^- \rightarrow 0.5\ N_2O$) with either 1 mM DIECA (inhibitor of nitrite reductase) or 1 mM $NaN_3$ (inhibitor of nitrous oxide reductase) also resulted in a rapid alkalinization (Figs. 35 and 36; B,C,E), in response to the addition of the respective substrate. The $^{\rightarrow}H^+$/oxidant ratios for complete reduction of $NO_3^-$, $NO_2^-$ (*via* $N_2O$) and $N_2O$ to $N_2$ were -6.12, -3.46 and -2.02 without a permeant ion (Table 15) and -6.04, -3.99 and -1.87 with valinomycin (Table 16). In the reduction of $NO_3^- \rightarrow NO_2^-$, $NO_3^- \rightarrow 0.5\ N_2O$ and $NO_2^- \rightarrow 0.5\ N_2O$, $^{\rightarrow}H^+$/oxidant values of -1.95, -4.79 and -3.03 were obtained in the absence of valinomycin, and -1.93, -4.88 and -2.87 respectively, in its presence.

3.5.2 Proton electrochemical gradients

3.5.2.1 Uptake of radioactive probes

Probes ($[^3H]$-$TPP^+$ and $[^{14}C]$-benzoic acid) used to determine membrane

TABLE 13: ENDOGENOUS PROTON TRANSLOCATION IN VALINOMYCIN-TREATED CELLS IN LIGHT.

Washed cells from photodenitrifying cultures, were treated with valinomycin (20 µg/ml) for 1h in light. These cells (4.64 mg protein/ml) were then pre-incubated with the appropriate inhibitor ($NaN_3$ or DIECA) for 10 min and then assayed for proton extrusion in light as described in Section 2.2.12.1. Positive ratios indicate proton extrusion and figures in parentheses are the number of pulses in each case.

| Oxidant | Inhibitor | Conc. (mM) | Reaction | $^{\rightarrow}H^+$/oxidant | $^{\rightarrow}H^+/2e^-$ | Theoretical $^{\rightarrow}H^+/2e^-$ |
|---|---|---|---|---|---|---|
| $NO_3^-$ | None | - | $NO_3^- \rightarrow 0.5\ N_2$ | 6.04 ± 0.30 (5) | 4.82 | 4.8 |
| | $NaN_3$ | 1 | $NO_3^- \rightarrow 0.5\ N_2O$ | 5.37 ± 0.07 (3) | 5.19 | 5.0 |
| | DIECA | 1 | $NO_3^- \rightarrow NO_2^-$ | 2.56 ± 0.15 (4) | 4.56 | 4.0 |
| $NO_2^-$ | None | - | $NO_2^- \rightarrow 0.5\ N_2$ | 4.15 ± 0.13 (5) | 5.43 | 5.33 |
| | $NaN_3$ | 1 | $NO_2^- \rightarrow 0.5\ N_2O$ | 2.95 ± 0.22 (3) | 5.95 | 6.0 |
| | DIECA | 1 | - | 0 | 0 | 0 |
| $N_2O$ | None | - | $N_2O \rightarrow N_2$ | 4.20 ± 0.27 (6) | 6.20 | 6.0 |
| | $NaN_3$ | 1 | - | 0 | 0 | 0 |
| O | None | - | $O \rightarrow H_2O$ | 6.43 ± 0.27 (6) | 6.43 | 6.0 |

TABLE 14: ENDOGENOUS PROTON TRANSLOCATION IN VALINOMYCIN-TREATED CELLS IN THE DARK.

Washed cells, previously grown under photodenitrifying conditions, were treated with 20 µg/ml valinomycin for 1h in the dark. These cells (4.64 mg protein/ml) were then pre-incubated with the appropriate inhibitor ($NaN_3$ or DIECA) for 10 min and then assayed in the dark for proton extrusion as described in Section 2.2.12.1. Positive ratios indicate proton extrusion and figures in parentheses, the number of pulses in each case.

| Oxidant | Inhibitor | Conc. (mM) | Reaction | $\rightarrow H^+$/oxidant | $\rightarrow H^+/2e^-$ | Theoretical $\rightarrow H^+/2e^-$ |
|---|---|---|---|---|---|---|
| $NO_3^-$ | None | - | $NO_3^- \rightarrow 0.5\ N_2$ | 3.98 ± 0.15 (5) | 3.99 | 4.8 |
| | $NaN_3$ | 1 | $NO_3^- \rightarrow 0.5\ N_2O$ | 2.68 ± 0.07 (4) | 3.84 | 5.0 |
| | DIECA | 1 | $NO_3^- \rightarrow NO_2^-$ | 1.90 ± 0.13 (4) | 3.90 | 4.0 |
| $NO_2^-$ | None | - | $NO_2^- \rightarrow 0.5\ N_2$ | 2.15 ± 0.25 (4) | 4.10 | 5.33 |
| | $NaN_3$ | 1 | $NO_2^- \rightarrow 0.5\ N_2O$ | 1.38 ± 0.07 (3) | 4.88 | 6.0 |
| | DIECA | 1 | - | 0 | 0 | 0 |
| $N_2O$ | None | - | $N_2O \rightarrow N_2$ | 2.17 ± 0.22 (6) | 4.17 | 6.0 |
| | $NaN_3$ | 1 | - | 0 | 0 | 0 |
| O | None | - | $O \rightarrow H_2O$ | 3.95 ± 0.20 (5) | 3.95 | 6.0 |

FIG. 35: ALKALINIZATION FOLLOWING THE ADDITION OF N-OXIDES INTO AN ANAEROBIC SUSPENSION OF WASHED CELLS WITH BVH AS THE ELECTRON DONOR.

Washed cells (4.64 mg protein/ml), previously grown for 16h under photodenitrifying conditions, were pre-incubated with 1 mM NEM, 0.5 mM HOQNO and either $NaN_3$ or DIECA for 10 min prior to assay. BVH (1 mM) was the electron donor in all the experiments (see Section 2.2.12.1 for details). The bar corresponds to 50 ng atom $H^+$. Alkalinization resulted in an upward deflection.

A,B,C, addition of 42 nmol $NO_3^-$; D,E,F, 40 nmol $NO_2^-$; G,H, 40 ng atom $N_2O$; B,E,H, with 1 mM $NaN_3$; C,F, with 1 mM DIECA.

FIG. 36: ALKALINIZATION PRODUCED BY INJECTING N-OXIDES INTO AN ANAEROBIC SUSPENSION OF VALINOMYCIN TREATED CELLS.

Washed cells, from photodenitrifying cultures (16h), were treated with valinomycin (20 µg/ml) in the dark and then pre-incubated with 1 mM NEM, 0.5 mM HOQNO and either $NaN_3$ or DIECA for 10 min before adding BVH (1 mM). Details of the experiment are given in Section 2.2.12.1. The bar corresponds to 50 ng atom $H^+$. Alkalinization resulted in an upward deflection.

A,B,C, addition of 50 nmol $NO_3^-$; D,E,F, 50 nmol $NO_2^-$; G,H, 50 ng atom $N_2O$; B,E,H, with 1 mM $NaN_3$; C,F, with 1 mM DIECA.

FIG.35

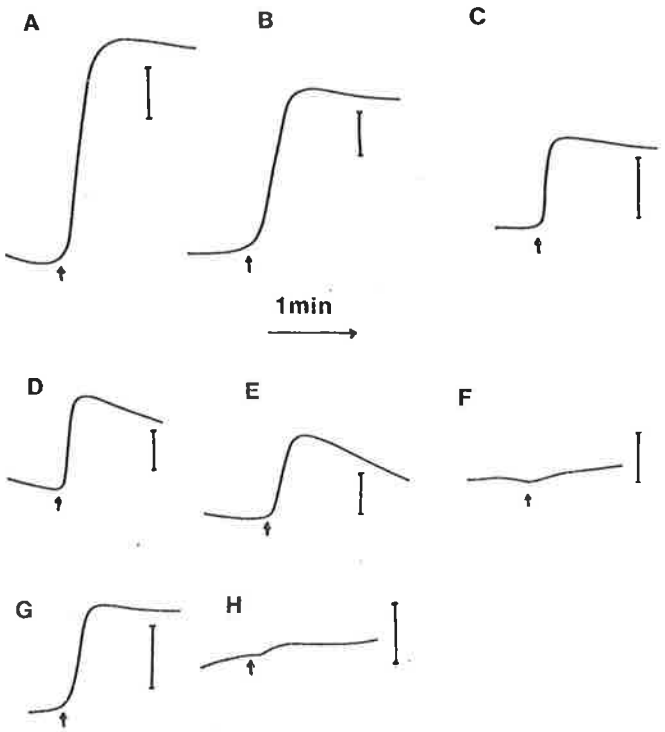



FIG.36

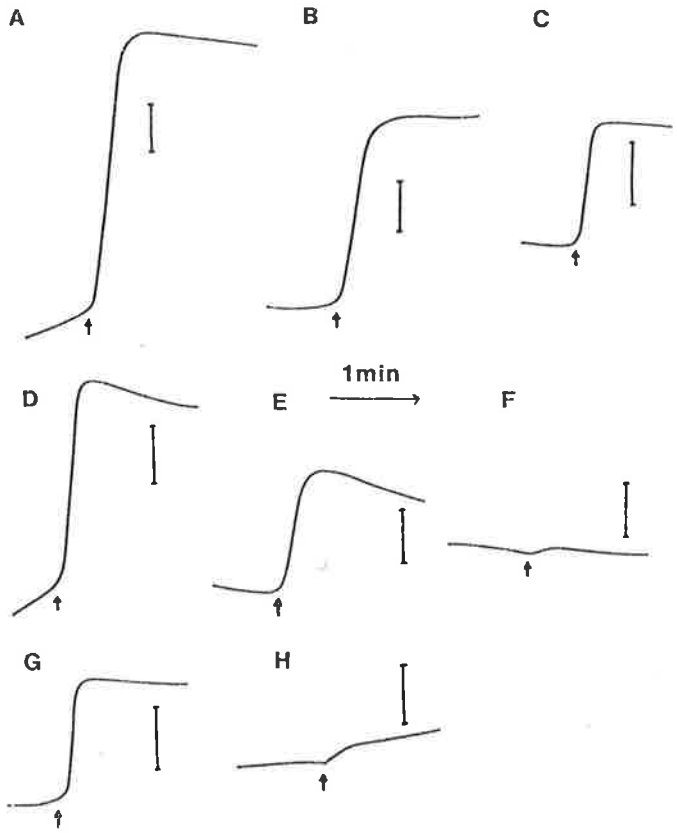


FIG. 37: RELATION BETWEEN PROTON CONSUMPTION AND THE AMOUNT OF OXIDANT ADDED TO WASHED CELLS.

Washed cells (4.64 mg protein/ml), previously grown for 16h under photodenitrifying conditions were pre-incubated with 1 mM NEM and 0.5 mM HOQNO for 10 min. BVH (1 mM) was used as the electron donor. Oxidant pulse experiments were done as described in Section 2.2.12.1. Standard error of means varied from ± 0.10 to ± 0.29.

● $NO_3^-$;
○ $NO_2^-$;
▲ $N_2O$.

FIG. 38: RELATION BETWEEN PROTON CONSUMPTION AND THE AMOUNT OF OXIDANT ADDED IN VALINOMYCIN-TREATED CELLS.

Washed cells (4.64 mg protein/ml), from photodenitrifying cultures (16h), were treated with 20 µg/ml valinomycin for 1h in the dark and then pre-incubated with 1 mM NEM and 0.5 mM HOQNO for 10 min. BVH (1 mM) was used as the electron donor. Oxidant pulse experiments were conducted as described in Section 2.2.12.1. Standard error of means varied from ± 0.09 to ± 0.21.

● $NO_3^-$;
○ $NO_2^-$;
▲ $N_2O$.

FIG.37

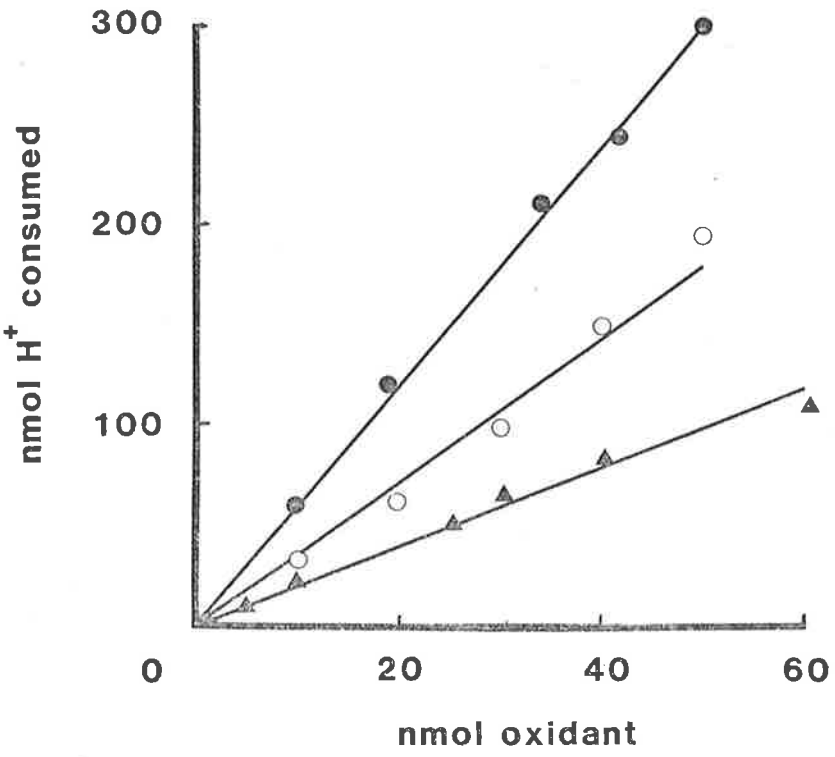

300
200
100
0
20
40
60
nmol H+ consumed
nmol oxidant

FIG.38

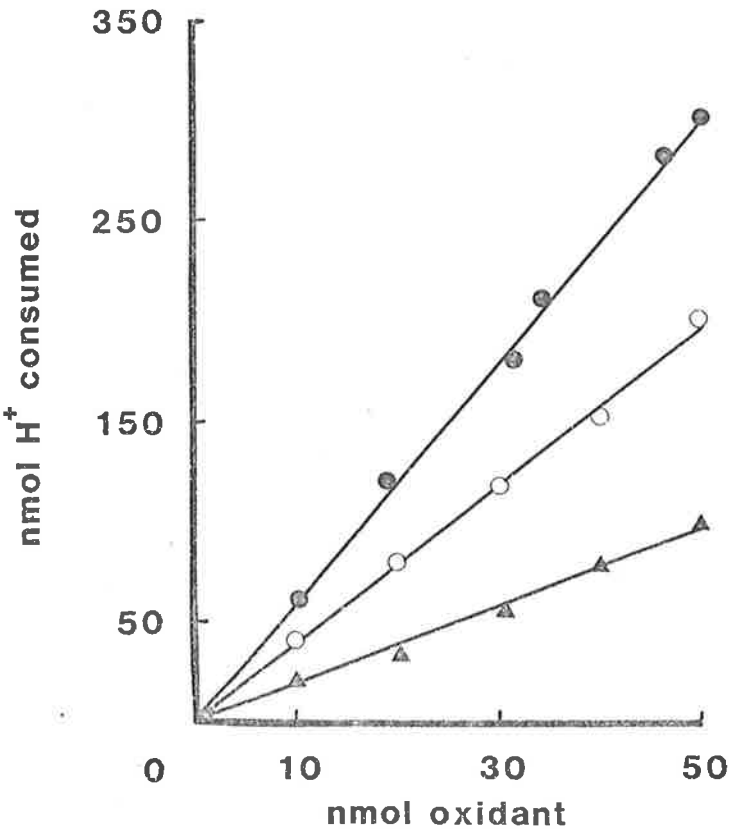

350
250
150
50
0
10
30
50
nmol H+ consumed
nmol oxidant

TABLE 15: EXOGENOUS PROTON TRANSLOCATION IN WASHED CELLS.

Washed cells (4.64 mg protein/ml), grown under photodenitrifying conditions, were pre-incubated with 1 mM NEM and 0.5 mM HOQNO for 10 min. These cells were then assayed for proton consumption using 1mM BVH as the electron donor as described in Section 2.2.12.1. Figures in parenthesis indicate the number of pulses given. Negative ratios indicate proton consumption by cells .

| Oxidant | Inhibitor | Conc. (mM) | Reaction | $^{\rightarrow}H^{+}$/oxidant | Theoretical $^{\rightarrow}H^{+}$/oxidant |
|---|---|---|---|---|---|
| $NO_3^-$ | None | - | $NO_3^- \rightarrow 0.5\ N_2$ | -6.12 ± 0.29 (7) | -6.0 |
| | $NaN_3$ | 1 | $NO_3^- \rightarrow 0.5\ N_2O$ | -4.79 ± 0.21 (4) | -5.0 |
| | DIECA | 1 | $NO_3^- \rightarrow NO_2^-$ | -1.95 ± 0.20 (3) | -2.0 |
| $NO_2^-$ | None | - | $NO_2^- \rightarrow 0.5\ N_2$ | -3.46 ± 0.37 (5) | -4.0 |
| | $NaN_3$ | 1 | $NO_2^- \rightarrow 0.5\ N_2O$ | -3.03 ± 0.10 (4) | -3.0 |
| | DIECA | 1 | - | 0 | 0 |
| $N_2O$ | None | - | $N_2O \rightarrow N_2$ | -2.02 ± 0.17 (6) | -2.0 |
| | $NaN_3$ | 1 | - | 0 | 0 |

TABLE 16: EXOGENOUS PROTON TRANSLOCATION IN VALINOMYCIN-TREATED CELLS.

Washed cells from photodenitrifying cultures were treated with 20 µg/ml valinomycin for 1h in the dark. These cells (4.64 mg protein/ml) were then pre-incubated with 1 mM NEM and 0.5 mM HOQNO and either $NaN_3$ or DIECA for 10 min and then assayed for proton consumption with BVH (1 mM) as the electron donor (Section 2.2.12.1). Negative ratios indicate proton consumption and figures in parentheses, the number of pulses.

| Oxidant | Inhibitor | Conc. (mM) | Reaction | $^{\rightarrow}H^+$/oxidant | Theoretical $^{\rightarrow}H^+$/oxidant |
|---|---|---|---|---|---|
| $NO_3^-$ | None | - | $NO_3^- \rightarrow 0.5\ N_2$ | -6.04 ± 0.21 (8) | -6.0 |
| | $NaN_3$ | 1 | $NO_3^- \rightarrow 0.5\ N_2O$ | -4.88 ± 0.23 (5) | -5.0 |
| | DIECA | 1 | $NO_3^- \rightarrow NO_2^-$ | -1.93 ± 0.18 (6) | -2.0 |
| $NO_2^-$ | None | - | $NO_2^- \rightarrow 0.5\ N_2$ | -3.99 ± 0.14 (9) | -4.0 |
| | $NaN_3$ | 1 | $NO_2^- \rightarrow 0.5\ N_2O$ | -2.87 ± 0.21 (6) | -3.0 |
| | DIECA | 1 | - | 0 | 0 |
| $N_2O$ | None | - | $N_2O \rightarrow N_2$ | -1.87 ± 0.19 (5) | -2.0 |
| | $NaN_3$ | 1 | - | 0 | 0 |

potential ($\Delta\Psi$) and transmembrane pH gradient ($\Delta$pH) were readily taken up by washed cells previously grown anaerobically with $NO_3^-$ in light. An equilibrium state was reached within 10 min. The EDTA-treatment of the cells was necessary to make them permeable to [$^3$H]-TPP$^+$ (Kashket, 1981b). In order to obtain maximal [$^3$H]-TPP$^+$ uptake various concentrations of EDTA were used and 10 mM EDTA was optimal both in light and in the dark (Figs. 39A,B). The uptake of [$^3$H]-TPP$^+$ was higher in light (equivalent to 20 mV) than that in the dark. However, the uptake of [$^{14}$C]-benzoate by cells treated with EDTA at concentrations corresponding to those used in the $\Delta\Psi$ assays ([$^3$H]-TPP$^+$ uptake) were similar to those in the absence of EDTA (Figs. 39A,B). EDTA-treated cells were metabolically active since they had similar nitrate and nitrite reductase activities to those of untreated cells.

3.5.2.2 Measurement of $\Delta$pH

The EDTA-treated cells previously grown phototrophically with $NO_3^-$ under anaerobic conditions, readily incorporated [$^{14}$C]-benzoic acid, reaching an equilibrium within 10 min at 25°C both aerobically or anaerobically either in light or in the dark. This uptake was dependent on external pH ($pH_{out}$) in the dark (Fig. 40A) and in the light (Fig. 40B). The effect of light in stimulating $\Delta$pH was observed only at $pH_{out} \leqslant 7.0$. Thus with either $NO_3^-$ or $NO_2^-$ the $\Delta$pH in light was 0.5 to 0.7 pH units higher than those in the dark at $pH_{out}$ 6.0. With $N_2O$ similar values of $\Delta$pH were obtained in the dark (1.39) and in the light (1.44), whereas with $O_2$, $\Delta$pH declined from 1.57 in the dark to 1.19 in the light when $pH_{out}$ was 6.0. The stimulatory effect of light on $\Delta$pH in the presence of either $NO_3^-$, $NO_2^-$ or $N_2O$ was less pronounced at $pH_{out} \geqslant 7.0$ than at lower pH values. The $\Delta$pH was inhibited in light in the presence of $O_2$.

FIG. 39: EFFECTS OF EDTA TREATMENT ON [$^{3}$H]-TPP$^{+}$ AND [$^{14}$C]-BENZOIC ACID UPTAKE BY WASHED CELLS IN LIGHT AND IN THE DARK.

Washed cells (0.5 g wet wt.), previously grown for 16h under photodenitrifying conditions, were suspended in 50 mM sodium phosphate buffer (25 ml), pH 7.0, containing various concentrations of Na-EDTA (pH 7.0) in separate 50 ml Erlenmeyer flasks fitted with subaseals. The flasks were evacuated (2 min) by a water pump *via* needles inserted into the subaseals, flushed with argon (5 min) then incubated for 10 min at 30°C. After this treatment, the cells were washed in 50 mM sodium phosphate buffer (pH 7.0) and resuspended in the same buffer (1 to 1.5 mg dry wt./ml). These cells (0.4 ml) were incubated anaerobically in light and in the dark respectively at 30°C for 10 min with either [$^{3}$H]-TPP$^{+}$ (8.4 µM) or [$^{14}$C]-benzoate (8.8 µM). Then the cells were centrifuged through 0.5 ml silicone oil mixture (75% v/v fluid 550 and 25% v/v fluid 510/50 cs, Dow Corning Australia Pty. Ltd.) in an Eppendorf microfuge at 13,000 x g for 30 sec. Redistribution of the radioactive probes in the supernatant and pellet fractions were determined in a Packard Tri-Carb 460 CD liquid scintillation spectrometer (Section 2.2.19.1). Details of the experiments are given in Section 2.2.13.3. [$^{3}$H]-TPP$^{+}$ uptake is represented by $\Delta\Psi$ and that of [$^{14}$C]-benzoate by 59 x $\Delta$pH calculated from Nernst equation; $\Delta p = \Delta\psi - 59 \times \Delta pH$. Experiments were conducted in triplicate and results were within 5% error.

△ membrane potential ($\Delta\psi$);
▲ proton motive force ($\Delta p$);
○ pH gradient (59 x $\Delta$pH).

A. Dark.
B. Light.

FIG.39A

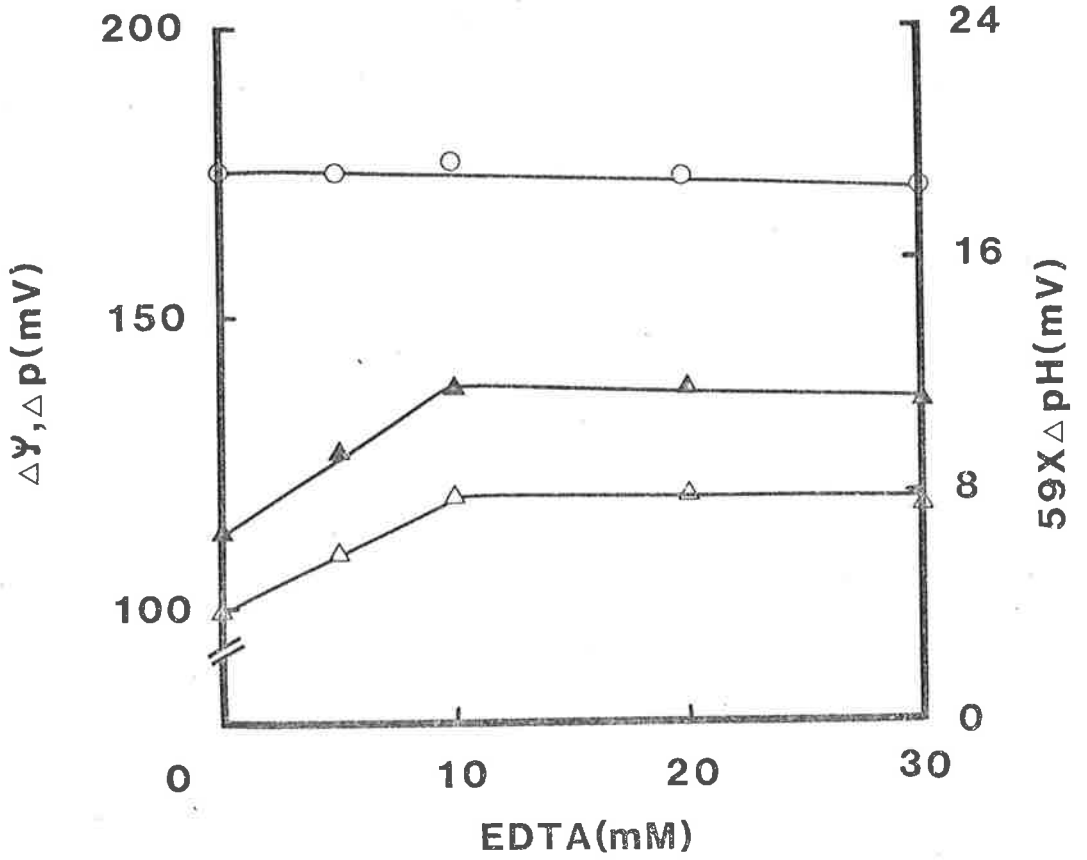



FIG.39B

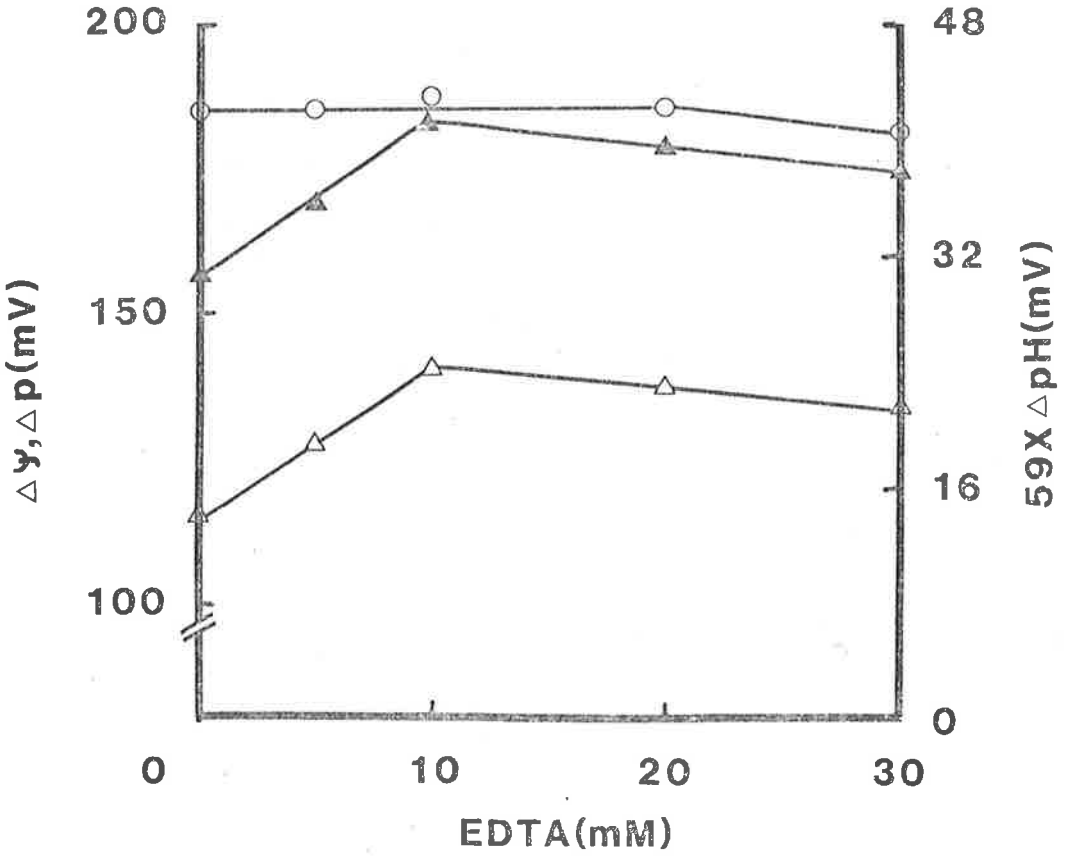


FIG. 40: EFFECTS OF EXTERNAL pH ($pH_{out}$) on pH gradient of EDTA-TREATED CELLS IN LIGHT AND IN THE DARK.

EDTA-treated cells, from photodenitrifying cultures (16h), resuspended (1 to 1.5 mg dry wt./ml) in 50 mM Na-phosphate buffer at the pH values indicated, were incubated anaerobically at 25°C for 10 min with [$^{14}$C]-benzoic acid (8.8 µM) and either $NO_3^-$ (10 mM), $NO_2^-$ (5 mM), $N_2O$ (25 mM) or with $O_2$ (235 µM). Uptake of [$^{14}$C]-benzoate was determined as described in Fig.39. pH gradient, calculated from Nernst equation, is represented by 59 x ΔpH. Experiments were done in triplicate and errors were ⩽ 5%.

- ● control (without added substrate);
- ○ $NO_3^-$;
- ▲ $NO_2^-$;
- △ $N_2O$;
- □ $O_2$.

A. Dark.

B. Light.

FIG.40A

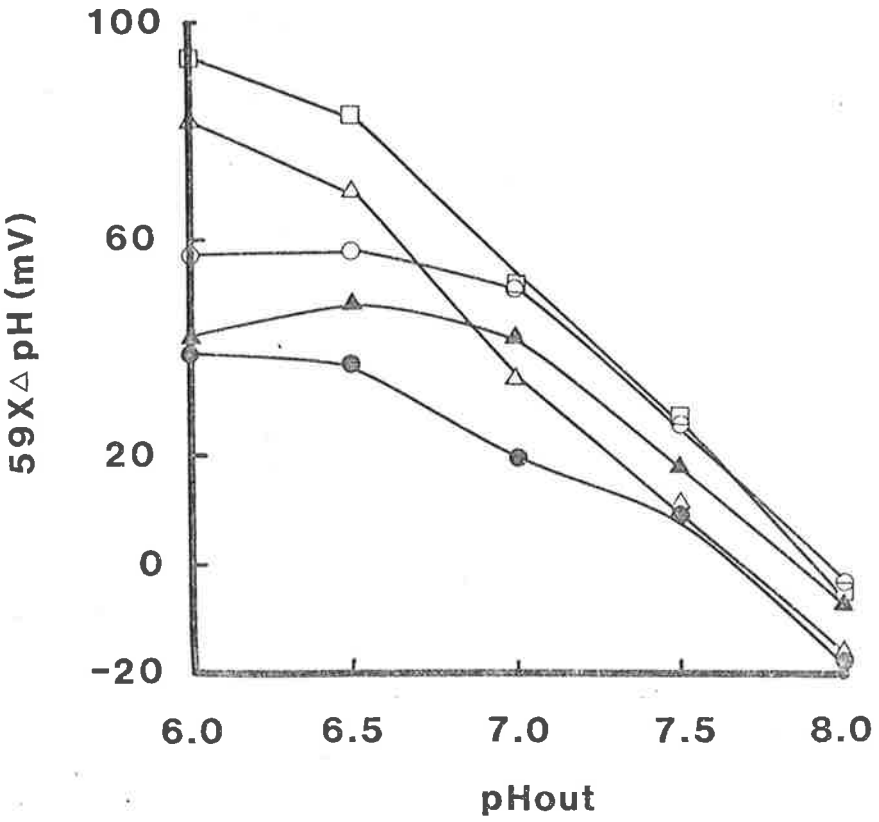
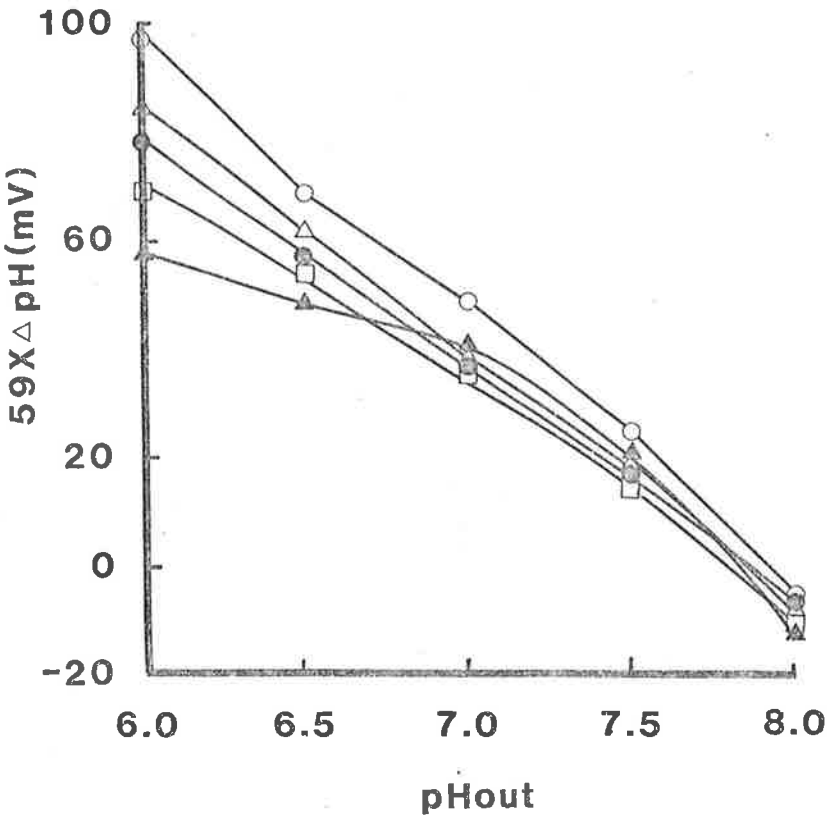



FIG.40B



[$^{14}$C]-benzoic acid accumulated in washed cells supplied with either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ only when $pH_{out}$ was $\leqslant$8.0. At $pH_{out}$ 8.0, the cells became acidic compared to external pH (Table 17). When $pH_{out}$ was varied from 6.0 to 8.0 in the absence of added substrate, $pH_{in}$ of the cells increased from 6.7 to 7.7 in the dark and 6.9 to 7.9 in the light. With either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ as the terminal acceptor, $pH_{in}$ values between 7.6 and 7.9 were recorded in washed cells in light and in the dark at $pH_{out}$ 7.0.

3.5.2.3 Measurement of $\Delta\Psi$

Measurement of ($\Delta\Psi$) in washed cells, grown under photodenitrifying conditions, was determined from the [$^3$H]-$TPP^+$ uptake by these cells. The data in Fig. 41A indicate that the membrane potential (inside negative) of washed cells in the dark was independent of external pH between 6.0-7.5. Further increase in $pH_{out}$ resulted in a decrease in $\Delta\Psi$; 10 to 20 mV lower than the control value (-121 mV), as shown by reduced uptake of [$^3$H]-$TPP^+$ under those conditions. With $NO_2^-$ as the oxidant $\Delta\Psi$ increased from -128 mV at $pH_{out}$ 6.0 to -140 mV at $pH_{out}$ 7.5 and then decreased to -107 mV at $pH_{out}$ 8.0. With either $NO_3^-$, $N_2O$ or $O_2$, $\Delta\Psi$ ranged between -144 mV and -155 mV over a $pH_{out}$ range of 6.0 to 7.0. In the light, however, the membrane potential increased with increasing $pH_{out}$ up to pH 7.0 (Fig. 41B) and then declined sharply to the control value (-132 mV) at pH 8.0. Nitrate, nitrite, $N_2O$ and $O_2$ all developed a $\Delta\Psi$ that ranged between -154 mV and -167 mV when $pH_{out}$ was varied between 6.0 and 7.0.

3.5.2.4 Total proton motive force ($\Delta p$)

Proton motive force is the sum of $\Delta\Psi$ and $\Delta pH$. In the dark, $\Delta p$ was fairly constant; in the absence of substrates (-153 to -161 mV) or with $NO_3^-$ (-203 to -209 mV) over a $pH_{out}$ range of 6.0 to 7.0. Over the same

TABLE 17: DETERMINATION OF INTERNAL pH OF WASHED CELLS AT DIFFERENT VALUES OF EXTERNAL pH IN LIGHT AND IN THE DARK.

Washed cells from photodenitrifying cultures (16h) were treated with 10 mM EDTA as described in Fig. 39. These cells washed once with 50 mM Na-phosphate buffer (pH 7.0) were resuspended (1 to 1.5 mg dry wt./ml) in the same buffer at the pH values indicated, ΔpH was determined in these cells by [$^{14}$C]benzoic acid uptake (Section 2.2.13.3) and $pH_{in}$ values were calculated from $pH_{out}$ and corresponding ΔpH values. Experiments were conducted in triplicate and standard error for ΔpH varied from ± 0.02 to ± 0.11.

| Conditions | $pH_{out}$ | Control (no substrate) | $pH_{in}$ | | | |
|---|---|---|---|---|---|---|
| | | | $NO_3^-$ | $NO_2^-$ | $N_2O$ | $O_2$ |
| Light | 6.07 | 6.91 | 7.75 | 7.04 | 7.51 | 7.26 |
| | 6.59 | 7.56 | 7.76 | 7.50 | 7.65 | 7.51 |
| | 7.05 | 7.68 | 7.87 | 7.75 | 7.70 | 7.64 |
| | 7.50 | 7.80 | 7.93 | 7.85 | 7.83 | 7.75 |
| | 7.97 | 7.86 | 7.86 | 7.77 | 7.85 | 7.79 |
| Dark | 6.07 | 6.74 | 7.03 | 6.90 | 7.46 | 7.64 |
| | 6.59 | 7.27 | 7.57 | 7.40 | 7.75 | 8.00 |
| | 7.05 | 7.48 | 7.91 | 7.76 | 7.63 | 7.92 |
| | 7.50 | 7.67 | 7.93 | 7.80 | 7.68 | 7.95 |
| | 7.97 | 7.68 | 7.90 | 7.86 | 7.68 | 7.89 |

FIG. 41: EFFECTS OF EXTERNAL pH ON MEMBRANE POTENTIAL ($\Delta\psi$) OF EDTA-TREATED CELLS IN LIGHT AND IN THE DARK.

EDTA-treated cells, from photodenitrifying cultures (16h), were resuspended (1 to 1.5 mg dry wt./ml) in 50 mM Na-phosphate buffer at the pH values indicated. They were incubated at 25°C for 10 min with [$^3$H]-$TPP^+$ (8.4 µM) and either $NO_3^-$ (10 mM), $NO_2^-$ (5 mM), $N_2O$ (25 mM) or with $O_2$ (235 µM). Uptake studies of [$^3$H]-$TPP^+$ were done as described in Fig.39. Membrane potential ($\Delta\psi$) was calculated from the Nernst equation. Experiments were conducted in triplicate and errors were $\leqslant 5\%$.

- ● Control (without added substrate);
- ○ $NO_3^-$;
- ▲ $NO_2^-$;
- △ $N_2O$;
- □ $O_2$.

A. Dark.

B. Light.

FIG.41A

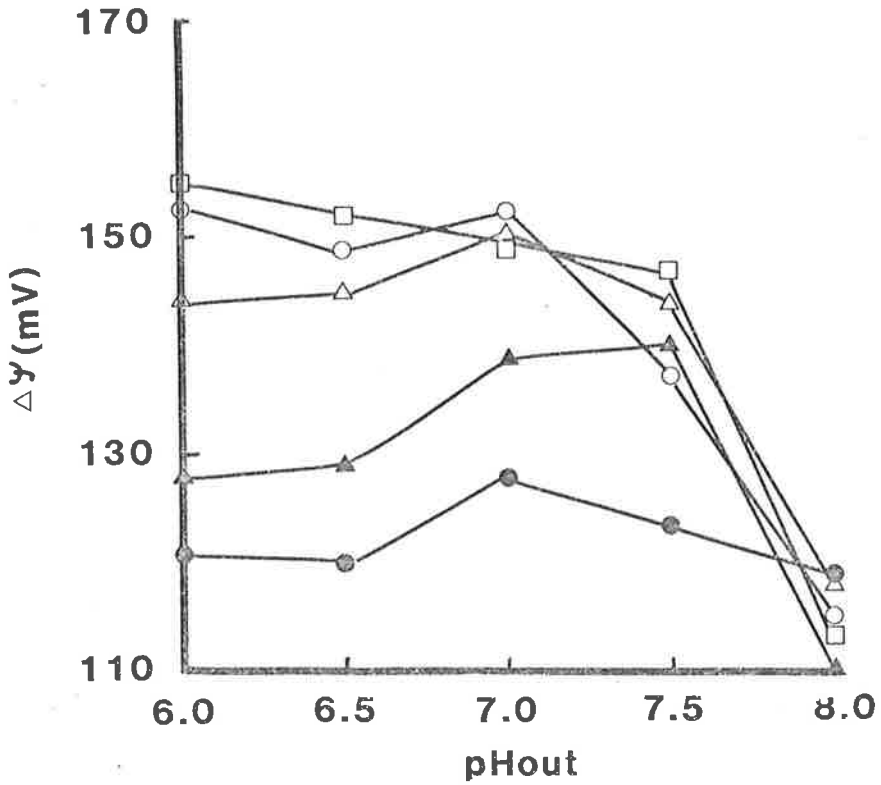
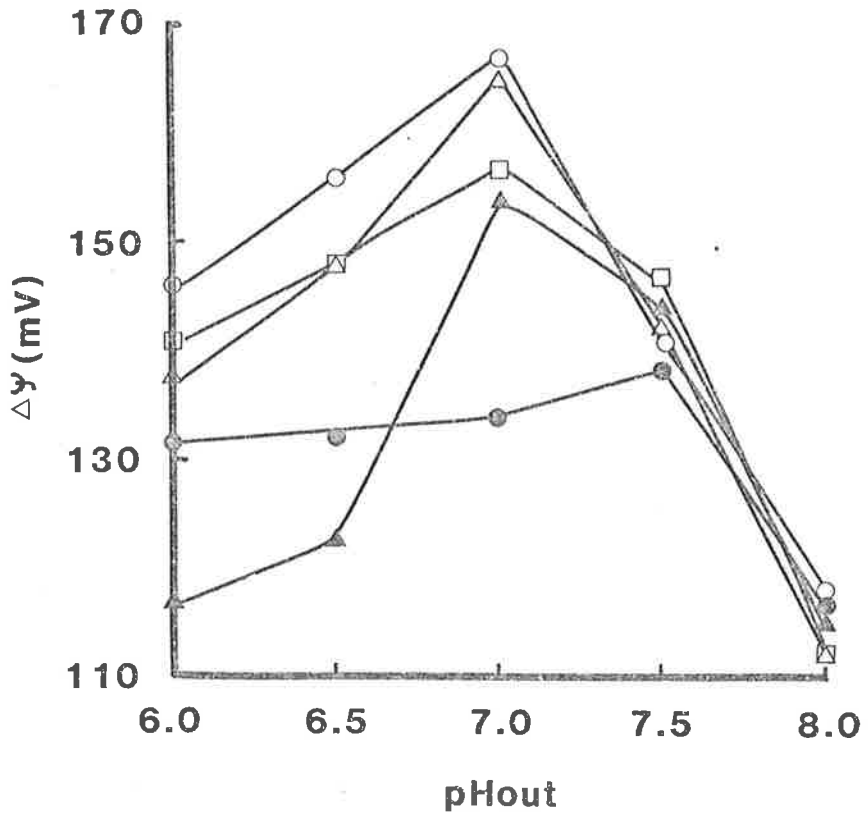



FIG.41B



$pH_{out}$ values, the Δp decreased from -226 to -184 mV with $N_2O$ and from -248 to -200 mV with $O_2$ (Fig. 42A). On the other hand, with $NO_2^-$, the Δp values varied between -177 to -181 mV over the same range of external pH. When the $pH_{out}$ was >7.0 the decrease in ΔpH was not compensated by an increase of ΔΨ resulting in a general diminution of Δp. Except for $NO_2^-$, the Δp values in light (Fig. 42B) were highest at $pH_{out}$ 6.0 (without substrate, -181 mV; with $NO_3^-$, -245 mV; with $N_2O$, -222 mV; with $O_2$, -211 mV). With $NO_2^-$, a maximum Δp value of -195 mV was recorded at pH 7.0.

3.5.2.5 Proton motive force at various stages of growth

Proton motive force was measured in washed cells from cultures grown photosynthetically under denitrifying conditions and harvested at various stages of growth. The intracellular volume measured at four intervals during growth was 1.45 ± 0.2 µl/mg dry wt.

In the dark, Δp values without added substrate increased from -136 to -168 mV (Fig. 43A) until the mid-exponential phase (10-15h) was reached and subsequently a Δp of -163 to -168 mV was maintained through to the stationary phase. When either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ was added to the washed cells, a similar increase in Δp was observed up to the mid-exponential stage (10-15h) and then it declined. The oxidant-induced Δp was maximal during the exponential stage of growth; the values were for $NO_3^-$, -197 mV; $NO_2^-$, -164 mV; $N_2O$, -193 mV and $O_2$, -197 mV. This increase in Δp was accompanied by an increase in both ΔΨ and ΔpH.

When these assays were carried out in light, the values of Δp (-180 mV) in the absence of substrate were about 22 mV higher than those in the dark at the mid-exponential phase (Figs. 43A,B). This Δp value (22 mV) was made up of pH gradient (14 mV) and ΔΨ (8 mV). Here also the levels of Δp were maximal during the exponential stage, irrespective

FIG. 42: EFFECTS OF EXTERNAL pH ON PROTON MOTIVE FORCE ($\Delta p$) of EDTA-TREATED CELLS IN LIGHT AND IN THE DARK.

Proton motive force was calculated from $\Delta p = \Delta\psi - 59 \times \Delta pH$. $\Delta pH$ and $\Delta\psi$ values are those from Figs. 40 and 41 respectively.

- ● Control (without added substrate);
- ○ $NO_3^-$;
- ▲ $NO_2^-$;
- △ $N_2O$;
- □ $O_2$.

A. Dark.

B. Light.

FIG.42A

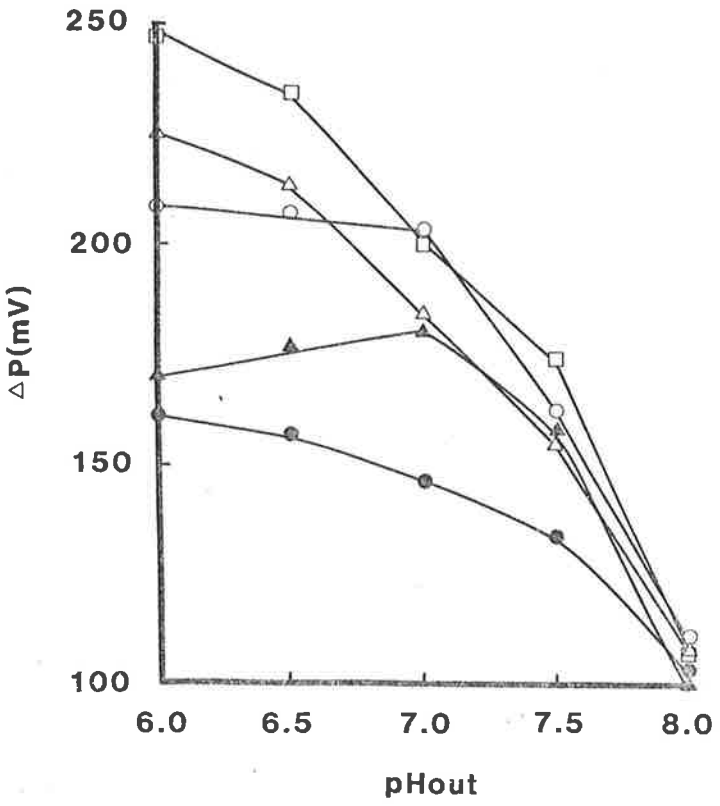



FIG.42B

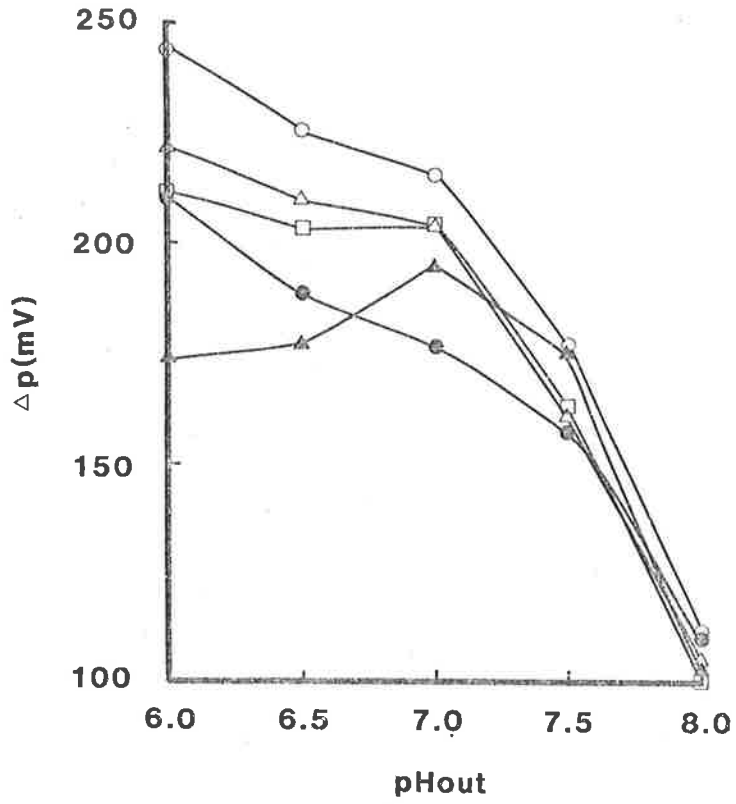


FIG. 43: PROTON MOTIVE FORCE IN WASHED CELLS AT VARIOUS STAGES OF GROWTH.

$\Delta\psi$ and $\Delta pH$ values were measured in EDTA-treated cells (which had been grown under photodenitrifying conditions) from the redistribution of [$^{3}H$]-$TPP^{+}$ and [$^{14}C$]-benzoate respectively, as described in Fig.39. $\Delta p$ was calculated from $\Delta p = \Delta\psi - 59 \times \Delta pH$. Experiments were done in triplicate and results were within 5% error.

- ● Control (without added substrate);
- ○ $NO_3^-$;
- ▲ $NO_2^-$;
- △ $N_2O$;
- □ $O_2$.

A. Dark.

B. Light.

FIG.43A

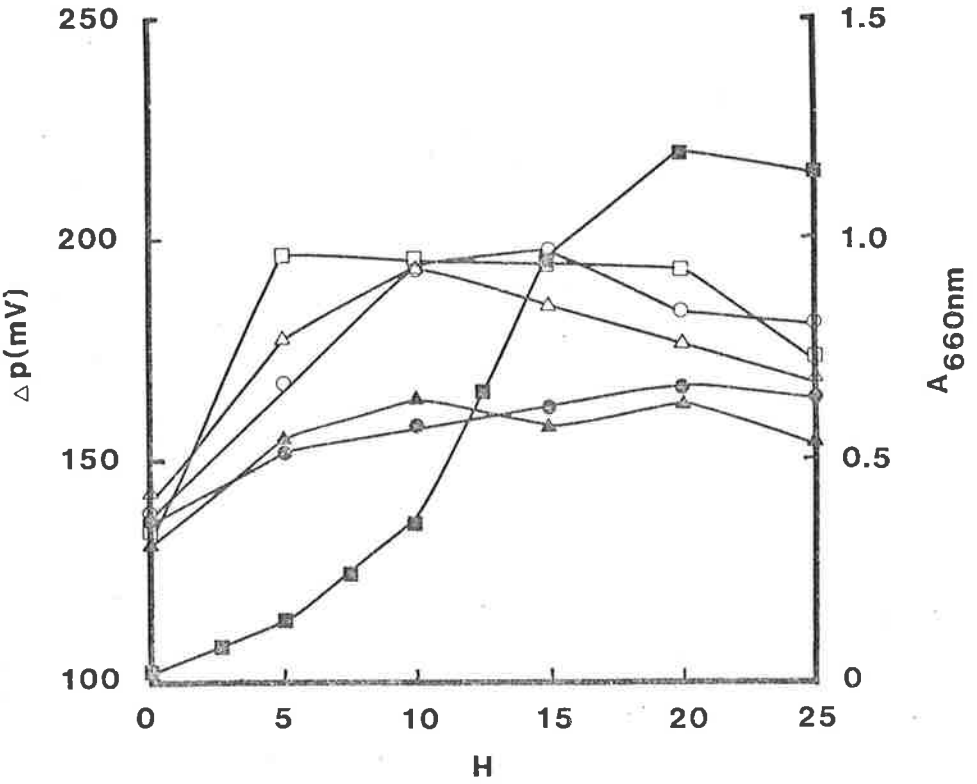
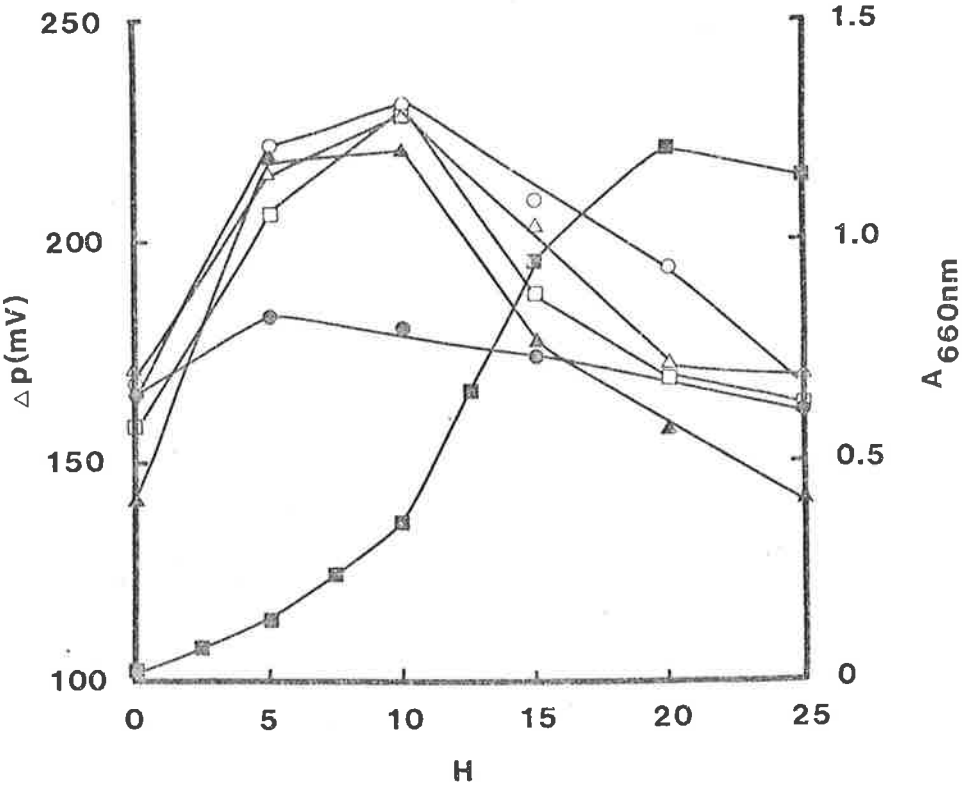



FIG.43B



of the substrates added. The maximum values of Δp were: $NO_3^-$, -230 mV; $NO_2^-$, -220 mV; $N_2O$, -228 mV and $O_2$, -232 mV. Results in Fig. 44A show that nitrate reductase activity in light was always higher than that in the dark throughout the growth period which could account for higher Δp values in light (Fig. 44B). At the mid-exponential phase (10-15h) the rates of reduction of N-oxides (nmol/10 min/mg protein) in light were as follows: $NO_3^-$, 297; $NO_2^-$, 345; $N_2O$, 267 whereas comparable values for those substrates in the dark were 220, 300 and 195 nmol respectively.

3.5.2.6 Effects of various concentrations of $NO_3^-$ and $NO_2^-$ on ΔΨ and ΔpH

The highest values for ΔΨ and Δp were obtained at concentration of 10 mM $NO_3^-$ (-153, -209 mV in the dark and -164, -227 mV respectively in light) or 5 mM $NO_2^-$ (-141, -172 mV in the dark and -157, -187 mV respectively in light). Changes in ΔpH with $NO_2^-$ were less than those with $NO_3^-$ either in light or in the dark (Figs. 45, 46). Further increases in either $NO_3^-$ (>10 mM) or $NO_2^-$ (>5 mM) resulted in a diminution of both ΔpH and ΔΨ in the dark whereas in light ΔpH remained constant with $NO_3^-$ as the terminal acceptor. However, use of $NO_2^-$ (>5 mM) in light resulted in a decrease in ΔpH by 0.3 pH units. The ΔΨ in light declined from -164 mV at 10 mM $NO_3^-$ to -150 mV at 20 mM $NO_3^-$ then remained constant but with $NO_2^-$ >10 mM it gradually decreased from -157 mV to -107 mV at 80 mM.

3.5.2.7 Effects of inhibitors on the components of Δp

The effects of respiratory inhibitors on ΔΨ and ΔpH were examined in washed cells from anaerobic phototrophic cultures grown with $NO_3^-$. The generation of ΔpH by either respiratory or photosynthetic electron transport to either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ was completely inhibited by $NaN_3$.

FIG. 44: RELATION BETWEEN $NO_3^-$ REDUCTION AND PROTON MOTIVE FORCE OF WASHED CELLS IN LIGHT AND IN THE DARK.

Cells were grown anaerobically with $NO_3^-$ in light. Nitrate reductase activity (nmol $NO_2^-$ produced/10 min/mg protein) in EDTA-treated cells at various stages of growth was measured anaerobically at 30°C using succinate as the electron donor (Section 2.2.9.2). $\Delta p$ values in these cells were determined as described in Fig.43. Experiments were conducted in triplicate and errors were $\leqslant 5\%$.

■ Growth;

A. Enzyme activity: □ , light; △ , dark;

B. $\Delta p$ with $NO_3^-$: ○ , light; ● , dark.

FIG.44A

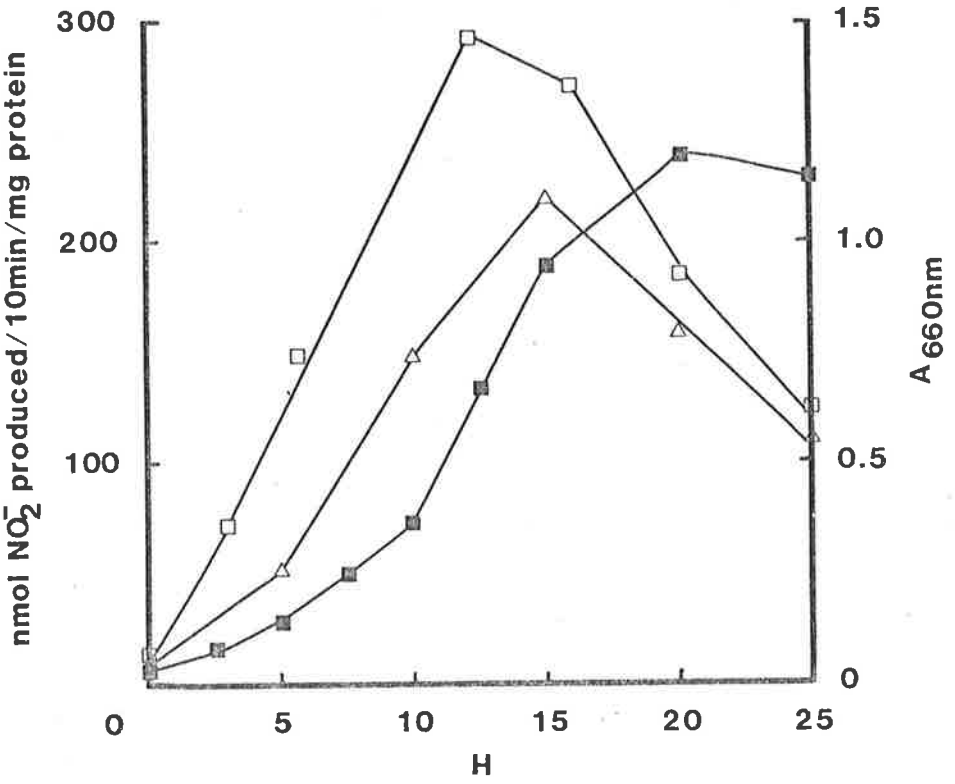



FIG.44B

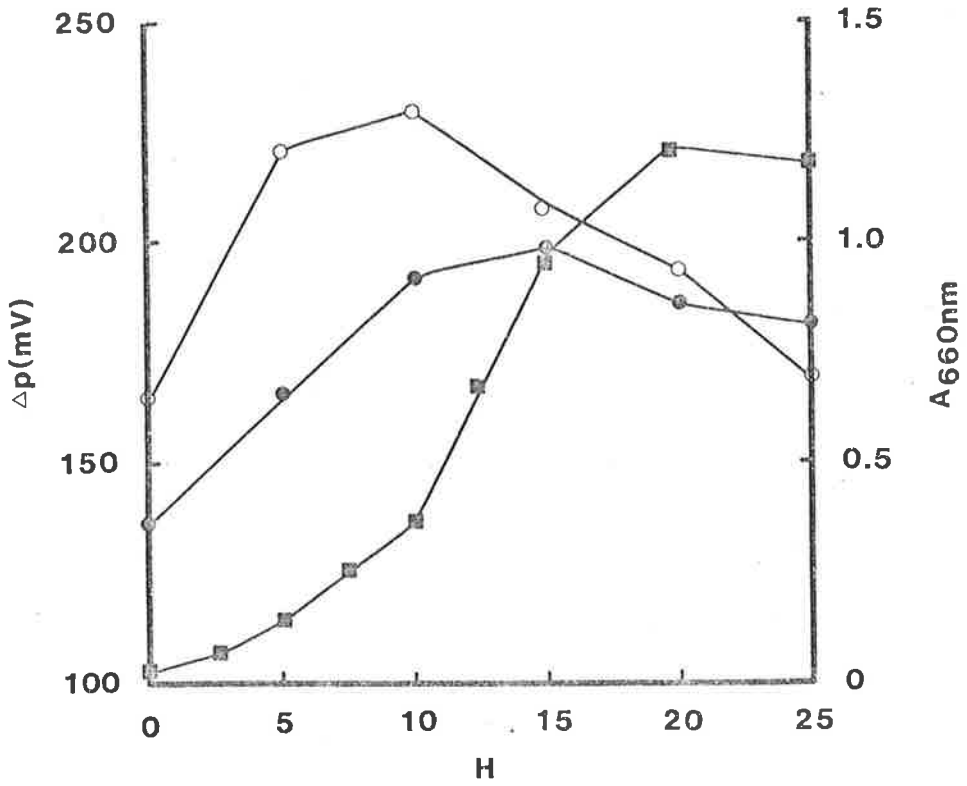


FIG. 45: EFFECTS OF $NO_3^-$ CONCENTRATIONS ON $\Delta\psi$ AND $\Delta$PH OF EDTA-TREATED CELLS IN LIGHT AND IN THE DARK.

EDTA-treated cells were prepared from photodenitrifying cultures (16h) and effects of various concentrations of $NO_3^-$ on the uptake of $[^3H]$-$TPP^+$ and $[^{14}C]$-benzoate were investigated as described in Fig.39. $\Delta\psi$ and $\Delta$pH values were determined from the redistribution of these probes and $\Delta p$ calculated from $\Delta p = \Delta\psi - 59 \times \Delta pH$. Experiments were done in triplicate and errors were $\leqslant 5\%$.

△ $\Delta\psi$;
▲ $\Delta p$;
● $59 \times \Delta pH$.

A. Dark.

B. Light.

FIG.45A

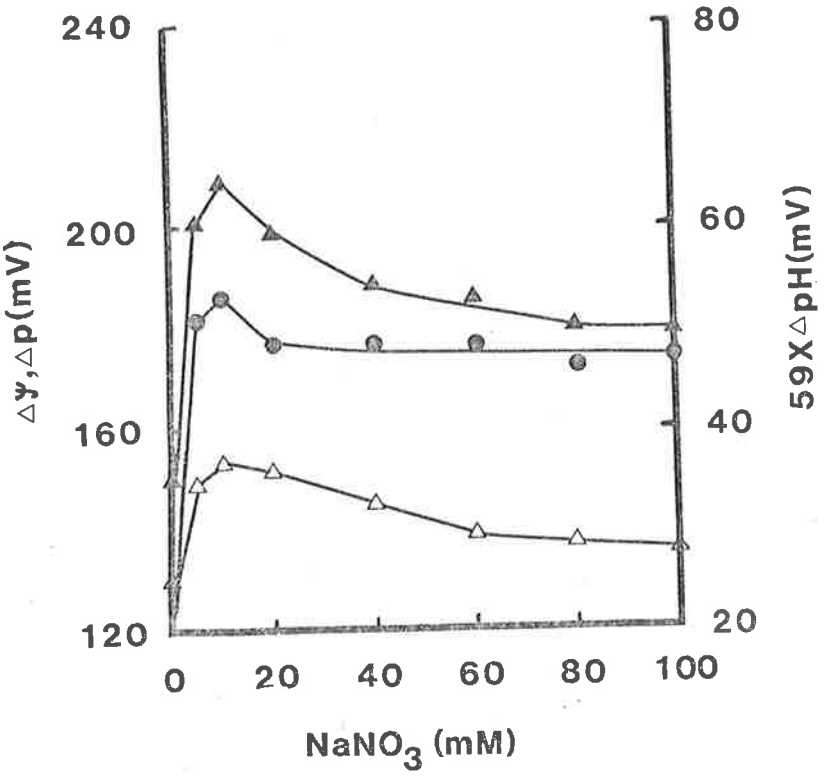



FIG.45B

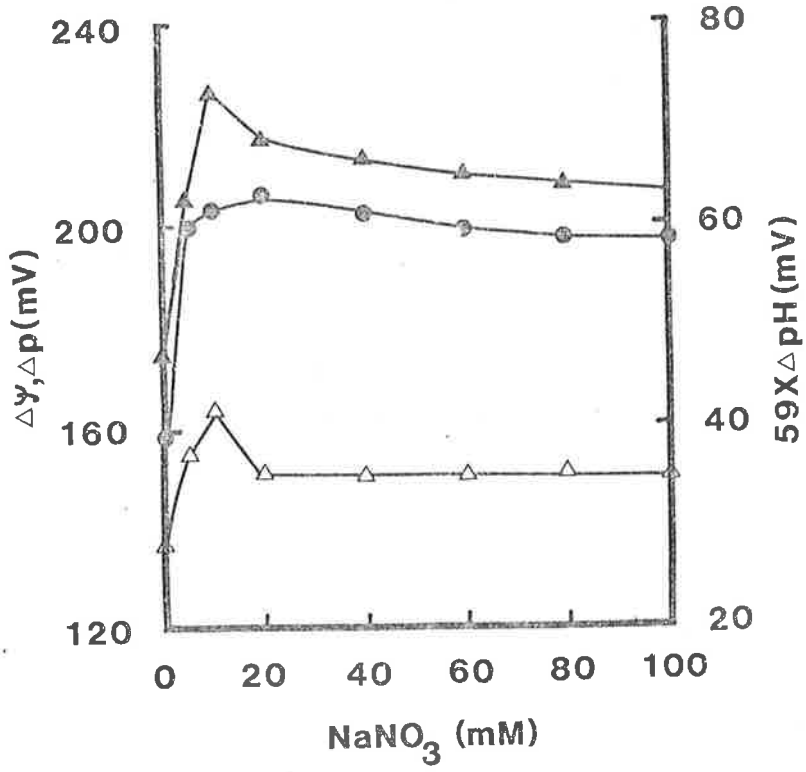


FIG. 46: EFFECTS OF $NO_2^-$ CONCENTRATIONS ON $\Delta\psi$ AND $\Delta pH$ OF EDTA-TREATED CELLS IN LIGHT AND IN THE DARK.

$\Delta\psi$ and $\Delta pH$ values in EDTA-treated cells from photo-denitrifying cultures (16h), were determined with various concentrations of $NO_2^-$ as described in Fig.39. $\Delta p$ values were calculated from $\Delta p = \Delta\psi - 59 \times \Delta pH$. Experiments were conducted in triplicate and results were within 5% error.

△ $\Delta\psi$;

▲ $\Delta p$;

● $59 \times \Delta pH$.

A, Dark.

B, Light.

FIG.46A

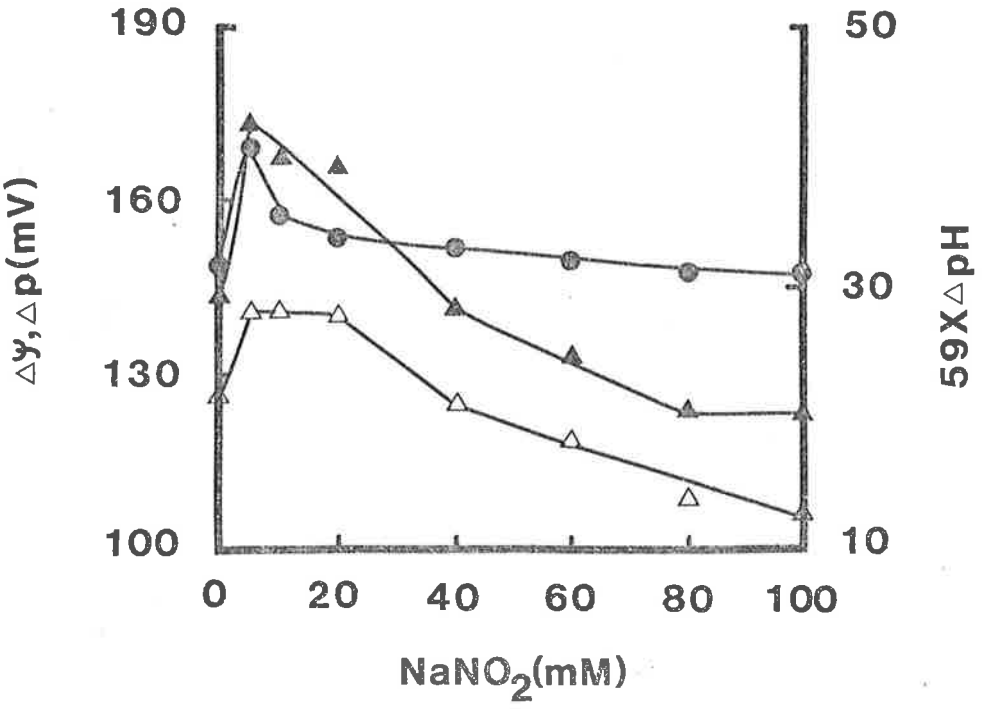
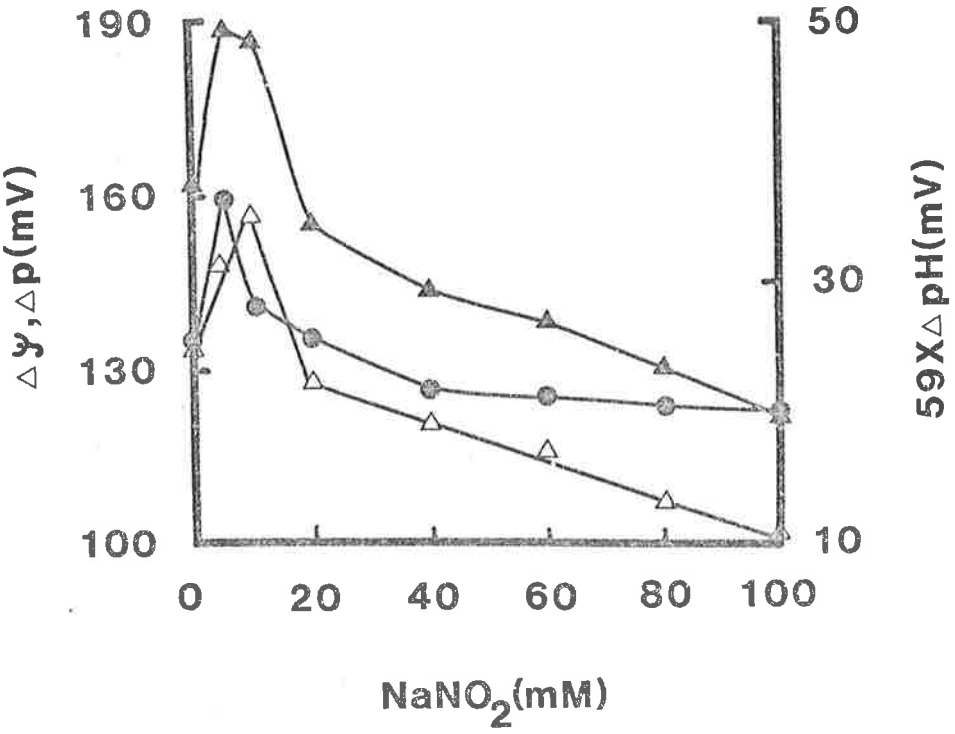



FIG.46B



The ΔpH with either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$, in the presence of $NaN_3$ decreased to 0.2 pH units in the dark and 0.46 in the light, which are normal ΔpH values for cells without $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ (Tables 18-25). In the presence of 0.5 mM $NaN_3$, nitrate-induced Δp decreased by 27 mV (Table 18) in the dark and by 54 mV in light (Table 19) whereas $NO_2^-$, $N_2O$ and $O_2$-induced Δp, either in light or in the dark, was lowered by 40 to 100 mV (Tables 20-25). KCN, an inhibitor of nitrite reductase and cytochrome oxidase, severely restricted (90%) nitrite and $O_2$-induced ΔpH and ΔΨ, both in light and in the dark (Tables 20, 21, 24, 25) whereas with $NO_3^-$ and $N_2O$ these parameters were inhibited by about 60% (Tables 18, 19, 22, 23). In the reaction mixture without added substrate, the addition of either 1 mM KCN or 0.1 mM rotenone did not further reduce the values of ΔΨ and ΔpH indicating that these experiments were conducted under strictly anaerobic conditions. Inhibitors of the electron transport chain, e.g. antimycin A and HOQNO were more effective in reducing the light-dependent electron flow to either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ than electron transfer to those acceptors in the dark. These two inhibitors resulted in a reduction of ΔpH by 30 to 70% in the dark irrespective of substrates used but ΔΨ was markedly reduced (by 47 mV) with HOQNO when $NO_2^-$ only was the acceptor (Tables 18, 20, 22, 24). Light-induced ΔpH was markedly reduced (60-90%) by HOQNO and antimycin A (HOQNO > antimycin A) and that of ΔΨ by 20-70 mV in the presence of either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$, resulting in an overall decrease between 40 to 100 mV for Δp (Tables 19, 21, 23, 25). The uncoupler CCCP at 10 μM was less effective than 1 mM DNP in reducing Δp (Tables 18-25), but CCCP together with 0.1 mM antimycin A in light, lowered the pH to between 0.1 and 0.2 and ΔΨ to about 100 mV. DCCD effectively reduced (50-90%) the ΔpH in light and in the dark, with all the substrates used but ΔΨ was relatively unaffected (<25% inhibition) with $NO_3^-$, $N_2O$ or $O_2$ (Tables 18, 19, 22-25) whereas

TABLE 18: EFFECTS OF INHIBITORS ON NITRATE-DEPENDENT MEMBRANE POTENTIAL (Δψ), pH GRADIENT (ΔpH) AND PROTON MOTIVE FORCE (Δp) IN WASHED CELLS IN THE DARK.

Uptake studies of [$^{3}$H]-TPP$^{+}$ and [$^{14}$C]-benzoate were carried out in EDTA-treated cells (previously grown for 16h under photodenitrifying conditions). Experiments were done in triplicate as described in Section 2.2.13.3.

| Inhibitor | Final conc. (mM) | ΔpH | | | Δψ(-mV) | | | Δp(-mV) |
|---|---|---|---|---|---|---|---|---|
| | | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | |
| -[a] | - | 0.20±0.01 | - | - | 124±2.0 | - | - | 136 |
| KCN[a] | 1.00 | 0.18±0.03 | - | - | 125±3.1 | - | - | 135 |
| Rotenone[a] | 0.10 | 0.25±0.05 | - | - | 120±2.5 | - | - | 135 |
| -[b] | - | 0.53±0.06 | +0.33 | - | 151±1.5 | +27 | - | 182 |
| KCN[c] | 1.00 | 0.35±0.06 | - | +0.15 | 131±1.6 | - | +7 | 152 |
| Rotenone[c] | 0.10 | 0.41±0.03 | - | +0.21 | 136±2.5 | - | +12 | 160 |
| $NaN_3$[c] | 0.50 | 0.24±0.01 | - | +0.04 | 141±2.8 | - | +17 | 155 |
| Antimycin A[c] | 0.10 | 0.34±0.04 | - | +0.14 | 139±1.8 | - | +15 | 159 |
| CCCP[c] | 0.01 | 0.12±0.04 | - | -0.08 | 110±1.5 | - | -14 | 117 |
| DCCD[c] | 0.10 | 0.28±0.01 | - | +0.08 | 142±7.1 | - | +18 | 158 |
| HOQNO[c] | 0.10 | 0.23±0.05 | - | +0.03 | 134±3.1 | - | +10 | 148 |
| DNP[c] | 1.00 | 0.38±0.01 | - | +0.18 | 122±0.8 | - | -2 | 143 |

a = Without added substrate; b = With 10 mM $NaNO_3$; c = With 10 mM $NaNO_3$ + inhibitor;
b~a = Difference between (a) and (b) for ΔpH and Δψ respectively;
c~a = Difference between (a) and (c) for ΔpH and Δψ respectively.

TABLE 19: EFFECTS OF INHIBITORS ON NITRATE-DEPENDENT MEMBRANE POTENTIAL ($\Delta\psi$), pH GRADIENT ($\Delta$pH) AND PROTON MOTIVE FORCE ($\Delta$p) IN WASHED CELLS IN LIGHT.

Uptake studies of $[^3H]$-$TPP^+$ and $[^{14}C]$-benzoate were carried out in triplicate in EDTA-treated cells which had been grown for 16h under photodenitrifying conditions as described in Section 2.2.13.3.

| Inhibitor | Final conc. (mM) | $\Delta$pH | | | $\Delta\psi$(-mV) | | | $\Delta$p(-mV) |
|---|---|---|---|---|---|---|---|---|
| | | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | |
| _a | - | 0.46±0.06 | - | - | 133±2.7 | - | - | 160 |
| KCN[a] | 1.00 | 0.40±0.10 | - | - | 130±3.1 | - | - | 154 |
| Rotenone[a] | 0.10 | 0.41±0.05 | - | - | 129±2.3 | - | - | 153 |
| _b | - | 0.78±0.06 | +0.32 | - | 166±2.0 | +33 | - | 212 |
| KCN[c] | 1.00 | 0.49±0.08 | - | +0.03 | 145±2.6 | - | +12 | 174 |
| Rotenone[c] | 0.10 | 0.45±0.10 | - | -0.01 | 141±3.3 | - | + 8 | 168 |
| $NaN_3$[c] | 0.50 | 0.45±0.02 | - | -0.01 | 131±3.5 | - | - 2 | 158 |
| Antimycin A[c] | 0.10 | 0.36±0.04 | - | -0.10 | 118±3.9 | - | -15 | 159 |
| CCCP[c] | 0.01 | 0.38±0.04 | - | -0.08 | 122±0.2 | - | -11 | 145 |
| DCCD[c] | 0.10 | 0.16±0.03 | - | -0.30 | 155±4.9 | - | +22 | 164 |
| DCMU[c] | 0.01 | 0.52±0.02 | - | +0.06 | 163±2.8 | - | +30 | 194 |
| DNP[c] | 1.00 | 0.07±0.01 | - | -0.39 | 103±0.60 | - | -30 | 108 |
| DBMIB[c] | 0.01 | 0.50±0.08 | - | +0.04 | 152±0.97 | - | +19 | 182 |
| CCCP+Antimycin A[c] | 0.01+0.10 | 0.21±0.03 | - | -0.25 | 100±0.94 | - | -33 | 113 |
| HOQNO[c] | 0.10 | 0.30±0.02 | - | -0.16 | 95±3.04 | - | -38 | 113 |

a = Without added substrate; b = With 10 mM $NaNO_3$; c = With 10 mM $NaNO_3$ + inhibitor;
c~a = Difference between (a) and (b) for $\Delta$pH and $\Delta\psi$ respectively;
c~a = Difference between (a) and (c) for $\Delta$pH and $\Delta\psi$ respectively.

TABLE 20: EFFECTS OF INHIBITORS ON NITRITE-DEPENDENT MEMBRANE POTENTIAL ($\Delta\psi$), pH GRADIENT ($\Delta$pH) AND PROTON MOTIVE FORCE ($\Delta$p) IN WASHED CELLS IN THE DARK.

Uptake studies of $[^3H]$-$TPP^+$ and $[^{14}C]$-benzoate were carried out in triplicate in EDTA-treated cells from photodenitrifying cultures (16h) as described in Section 2.2.13.3.

| Inhibitor | Final conc. (mM) | $\Delta$pH | | | $\Delta\psi$(-mV) | | | $\Delta$p(-mV) |
|---|---|---|---|---|---|---|---|---|
| | | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | |
| -a | - | 0.20±0.01 | - | - | 124±2.0 | - | - | 136 |
| KCN[a] | 1.00 | 0.19±0.02 | - | - | 126±1.6 | - | - | 137 |
| Rotenone[a] | 0.10 | 0.22±0.01 | - | - | 122±2.1 | - | - | 135 |
| -b | - | 0.53±0.01 | +0.33 | - | 145±2.0 | +21 | - | 176 |
| KCN[c] | 1.00 | 0.30±0.03 | - | +0.10 | 129±1.7 | - | +5 | 147 |
| Rotenone[c] | 0.10 | 0.33±0.02 | - | +0.13 | 132±1.3 | - | +8 | 151 |
| $NaN_3$[c] | 0.50 | 0.24±0.02 | - | +0.04 | 121±1.8 | - | -3 | 135 |
| Antimycin A[c] | 0.10 | 0.32±0.01 | - | +0.12 | 135±4.8 | - | +11 | 154 |
| CCCP[c] | 0.01 | 0.26±0.04 | - | +0.06 | 115±1.7 | - | -9 | 130 |
| DCCD[c] | 0.10 | 0.13±0.03 | - | -0.07 | 111±0.9 | - | -13 | 119 |
| HOQNO[c] | 0.10 | 0.39±0.08 | - | +0.19 | 98±1.0 | - | -26 | 121 |
| DNP[c] | 1.00 | 0.40±0.06 | - | +0.20 | 129±3.3 | - | +5 | 153 |

a = Without added substrate; b = With 5 mM $NaNO_2$; c = With 5 mM $NaNO_2$ + inhibitor;
b~a = Difference between (a) and (b) for $\Delta$pH and $\Delta\psi$ respectively;
c~a = Difference between (a) and (c) for $\Delta$pH and $\Delta\psi$ respectively.

TABLE 21: EFFECTS OF INHIBITORS ON NITRITE-DEPENDENT MEMBRANE POTENTIAL ($\Delta\psi$), pH GRADIENT ($\Delta$pH) AND PROTON MOTIVE FORCE ($\Delta p$) IN WASHED CELLS IN LIGHT.

Uptake studies of [$^3$H]-TPP$^+$ and [$^{14}$C]-benzoic acid were conducted in triplicate (Section 2.2.13.3) in EDTA-treated cells previously grown for 16h under photodenitrifying conditions.

| Inhibitor | Final conc. (mM) | $\Delta$pH | | | $\Delta\psi$(-mV) | | | $\Delta p$(-mV) |
|---|---|---|---|---|---|---|---|---|
| | | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | |
| -[a] | - | 0.46±0.04 | - | - | 134±1.5 | - | - | 161 |
| KCN[a] | 1.00 | 0.42±0.02 | - | - | 135±1.9 | - | - | 160 |
| Rotenone[a] | 0.10 | 0.46±0.01 | - | - | 137±2.1 | - | - | 164 |
| -[b] | - | 0.72±0.03 | +0.26 | - | 152±0.9 | +18 | - | 189 |
| KCN[c] | 1.00 | 0.44±0.03 | - | -0.02 | 137±1.8 | - | +3 | 163 |
| Rotenone[c] | 0.10 | 0.41±0.01 | - | -0.05 | 132±4.1 | - | -2 | 156 |
| $NaN_3$[c] | 0.50 | 0.44±0.01 | - | -0.02 | 127±1.8 | - | -7 | 153 |
| Antimycin A[c] | 0.10 | 0.25±0.01 | - | -0.21 | 132±2.6 | - | -2 | 147 |
| CCCP[c] | 0.01 | 0.21±0.04 | - | -0.25 | 111±3.4 | - | -23 | 124 |
| DCCD[c] | 0.10 | 0.17±0.02 | - | -0.29 | 145±2.4 | - | +11 | 155 |
| DCMU[c] | 0.01 | 0.48±0.02 | - | -0.04 | 155±6.2 | - | +21 | 184 |
| DNP[c] | 1.00 | 0.02±0.01 | - | -0.44 | 102±2.1 | - | -32 | 103 |
| DBMIB[c] | 0.01 | 0.15±0.04 | - | -0.31 | 132±1.0 | - | - 2 | 141 |
| CCCP+Antimycin A[c] | 0.01+0.10 | 0.14±0.03 | - | -0.32 | 97±0.5 | - | -37 | 105 |
| HOQNO[c] | 0.10 | 0.11±0.01 | - | -0.35 | 90±2.2 | - | -44 | 96 |

a = Without added substrate; b = With 5mM $NaNO_2$; c = With 5mM $NaNO_2$ + inhibitor;
b~a = Difference between (a) and (b) for $\Delta$pH and $\Delta\psi$ respectively;
c~a = Difference between (a) and (c) for $\Delta$pH and $\Delta\psi$ respectively.

TABLE 22: EFFECTS OF INHIBITORS ON NITROUS OXIDE-DEPENDENT MEMBRANE POTENTIAL ($\Delta\psi$), pH GRADIENT ($\Delta$pH) AND PROTON MOTIVE FORCE ($\Delta p$) IN WASHED CELLS IN THE DARK.

Uptake rates of [$^3$H]-TPP$^+$ and [$^{14}$C]-benzoate were studied in triplicate (Section 2.2.13.3) in EDTA-treated cells which had been grown for 16h under photodenitrifying conditions.

| Inhibitor | Final conc. (mM) | $\Delta$pH | | | $\Delta\psi$(-mV) | | | $\Delta p$(-mV) |
|---|---|---|---|---|---|---|---|---|
| | | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | |
| -a | - | 0.20±0.01 | - | - | 124±2.0 | - | - | 135 |
| KCN[a] | 1.00 | 0.21±0.03 | - | - | 121±1.9 | - | - | 133 |
| Rotenone[a] | 0.10 | 0.24±0.04 | - | - | 123±2.1 | - | - | 137 |
| -b | - | 0.77±0.08 | +0.57 | - | 143±1.7 | +19 | - | 188 |
| KCN[c] | 1.00 | 0.31±0.05 | - | +0.11 | 129±3.1 | - | +5 | 147 |
| Rotenone[c] | 0.10 | 0.36±0.02 | - | +0.16 | 127±4.2 | - | +3 | 148 |
| $NaN_3$[c] | 0.50 | 0.14±0.02 | - | -0.06 | 128±1.4 | - | +4 | 136 |
| Antimycin A[c] | 0.10 | 0.36±0.02 | - | +0.16 | 133±5.8 | - | +9 | 154 |
| CCCP[c] | 0.01 | 0.17±0.06 | - | -0.03 | 130±3.6 | - | +6 | 140 |
| DCCD[c] | 0.10 | 0.21±0.01 | - | +0.01 | 144±2.0 | - | +20 | 157 |
| HOQNO[c] | 0.10 | 0.36±0.06 | - | +0.16 | 146±3.0 | - | +22 | 168 |
| DNP[c] | 1.00 | 0.29±0.04 | - | +0.09 | 135±1.5 | - | +11 | 152 |

a = Without added substrate; b = With 25 mM $N_2O$; c = With 25 mM $N_2O$ + inhibitor;
b~a = Difference between (a) and (b) for $\Delta$pH and $\Delta\psi$ respectively;
c~a = Difference between (a) and (c) for $\Delta$pH and $\Delta\psi$ respectively.

TABLE 23: EFFECTS OF INHIBITORS ON NITROUS OXIDE-DEPENDENT MEMBRANE POTENTIAL ($\Delta\psi$), pH gradient ($\Delta$pH) and PROTON MOTIVE FORCE ($\Delta$p) IN WASHED CELLS IN LIGHT.

Uptake rates of [$^3$H]-TPP$^+$ and [$^{14}$C]-benzoate were studied in triplicate in EDTA-treated cells from photodenitrifying cultures (16h) as described in Section 2.2.13.3.

| Inhibitor | Final conc. (mM) | $\Delta$pH | | | $\Delta\psi$(-mV) | | | $\Delta$p(-mV) |
|---|---|---|---|---|---|---|---|---|
| | | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | |
| -[a] | - | 0.46±0.06 | - | - | 133±2.7 | - | - | 160 |
| KCN[a] | 1.00 | 0.43±0.02 | - | - | 131±3.1 | - | - | 156 |
| Rotenone[a] | 0.10 | 0.45±0.03 | - | - | 135±1.8 | - | - | 161 |
| -[b] | - | 0.75±0.10 | +0.30 | - | 162±5.1 | +29 | - | 200 |
| KCN[c] | 1.00 | 0.39±0.08 | - | -0.07 | 132±2.3 | - | -1 | 155 |
| Rotenone[c] | 0.10 | 0.41±0.09 | - | -0.05 | 136±3.4 | - | +3 | 160 |
| $NaN_3$[c] | 0.50 | 0.38±0.08 | - | -0.08 | 121±3.2 | - | +12 | 143 |
| Antimycin A[c] | 0.10 | 0.20±0.01 | - | -0.26 | 122±4.3 | - | -11 | 134 |
| CCCP[c] | 0.01 | 0.25±0.02 | - | -0.21 | 98±3.1 | - | -35 | 113 |
| DCCD[c] | 0.10 | 0.22±0.01 | - | -0.24 | 162±4.2 | - | +29 | 175 |
| DCMU[c] | 0.01 | 0.51±0.02 | - | +0.05 | 155±2.8 | - | +22 | 186 |
| DNP[c] | 1.00 | 0.10±0.01 | - | -0.36 | 105±2.9 | - | -28 | 111 |
| DBMIB[c] | 0.01 | 0.17±0.01 | - | -0.29 | 129±2.6 | - | - 4 | 142 |
| CCCP-Antimycin A[c] | 0.01+0.10 | 0.09±0.03 | - | -0.37 | 99±4.1 | - | -34 | 105 |
| HOQNO[c] | 0.10 | 0.08±0.02 | - | -0.38 | 85±3.8 | - | -48 | 90 |

a = Without added substrate; b = With 25 mM $N_2O$; c = With 25 mM $N_2O$ + inhibitor;
b~a = Difference between (a) and (b) for $\Delta$pH and $\Delta\psi$ respectively;
c~a = Difference between (a) and (c) for $\Delta$pH and $\Delta\psi$ respectively.

TABLE 24: EFFECTS OF INHIBITORS ON OXYGEN-DEPENDENT MEMBRANE POTENTIAL ($\Delta\psi$), pH GRADIENT ($\Delta$pH) AND PROTON MOTIVE FORCE ($\Delta p$) IN WASHED CELLS IN THE DARK.

Uptake rates of [$^3$H]-TPP$^+$ and [$^{14}$C]-benzoate were studied in triplicate in EDTA-treated cells previously grown for 16h under photodenitrifying conditions (Section 2.2.13.3).

| Inhibitor | Final conc. (mM) | $\Delta$pH | | | $\Delta\psi$(-mV) | | | $\Delta p$(-mV) |
|---|---|---|---|---|---|---|---|---|
| | | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | |
| _a | - | 0.20±0.01 | - | - | 124±2.0 | - | - | 136 |
| KCN[a] | 1.00 | 0.20±0.02 | - | - | 128±3.1 | - | - | 140 |
| Rotenone[a] | 0.10 | 0.22±0.04 | - | - | 125±3.0 | - | - | 138 |
| _b | - | 1.00±0.15 | +0.80 | - | 152±7.6 | +28 | - | 212 |
| KCN[c] | 1.00 | 0.30±0.06 | - | +0.10 | 135±1.6 | - | +11 | 153 |
| Rotenone[c] | 0.10 | 0.35±0.05 | - | +0.15 | 139±2.7 | - | +15 | 160 |
| $NaN_3$[c] | 0.50 | 0.27±0.05 | - | +0.07 | 102±1.7 | - | -22 | 118 |
| Antimycin A[c] | 0.10 | 0.68±0.05 | - | +0.48 | 122±2.2 | - | - 2 | 162 |
| CCCP[c] | 0.01 | 0.04±0.01 | - | -0.16 | 132±3.9 | - | + 8 | 134 |
| DCCD[c] | 0.10 | 0.12±0.01 | - | -0.08 | 149±2.8 | - | +25 | 156 |
| HOQNO[c] | 0.10 | 0.30±0.03 | - | +0.10 | 155±2.6 | - | +31 | 173 |
| DNP[c] | 1.00 | 0.39±0.04 | - | +0.19 | 128±2.8 | - | + 4 | 151 |

a = Without added substrate; b = With 235$\mu$M $O_2$; c = With 235$\mu$M $O_2$ + inhibitor;
b~a = Difference between (a) and (b) for $\Delta$pH and $\Delta\psi$ respectively;
c~a = Difference between (a) and (c) for $\Delta$pH and $\Delta\psi$ respectively.

TABLE 25: EFFECTS OF INHIBITORS ON OXYGEN-DEPENDENT MEMBRANE POTENTIAL ($\Delta\psi$), pH GRADIENT ($\Delta$pH) AND PROTON MOTIVE FORCE ($\Delta p$) IN WASHED CELLS IN LIGHT.

Uptake rates of [$^3$H]-TPP$^+$ and [$^{14}$C]-benzoate were studied in triplicate in EDTA-treated cells which had been grown for 16h under photodenitrifying conditions (Section 2.2.13.3).

| Inhibitor | Final conc. (mM) | $\Delta$pH | | | $\Delta\psi$(-mV) | | | $\Delta p$(-mV) |
|---|---|---|---|---|---|---|---|---|
| | | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | Measured | Substrate effect (b~a) | Inhibitor effect (c~a) | |
| -[a] | - | 0.46±0.06 | - | - | 133±2.7 | - | - | 160 |
| KCN[a] | 1.00 | 0.42±0.10 | - | - | 128±3.5 | - | - | 153 |
| Rotenone[a] | 0.10 | 0.39±0.08 | - | - | 136±1.1 | - | - | 159 |
| -[b] | - | 0.55±0.08 | +0.09 | - | 161±2.8 | +28 | - | 194 |
| KCN[c] | 1.00 | 0.44±0.05 | - | -0.02 | 130±4.6 | - | -3 | 156 |
| Rotenone[c] | 0.10 | 0.48±0.07 | - | +0.02 | 139±3.9 | - | +6 | 167 |
| $NaN_3$[c] | 0.50 | 0.38±0.08 | - | -0.08 | 121±3.2 | - | -12 | 143 |
| Antimycin A[c] | 0.10 | 0.20±0.01 | - | -0.26 | 122±4.3 | - | -11 | 134 |
| CCCP[c] | 0.01 | 0.25±0.01 | - | -0.21 | 98±3.1 | - | -35 | 113 |
| DCCD[c] | 0.10 | 0.22±0.01 | - | -0.24 | 162±4.2 | - | +29 | 175 |
| DCMU[c] | 0.01 | 0.51±0.02 | - | +0.05 | 155±2.8 | - | +22 | 186 |
| DNP[c] | 1.00 | 0.10±0.01 | - | -0.36 | 105±2.9 | - | -28 | 111 |
| DBMIB[c] | 0.01 | 0.17±0.03 | - | -0.29 | 132±2.6 | - | - 1 | 142 |
| CCCP+Antimycin A[c] | 0.01+0.10 | 0.09±0.02 | - | -0.37 | 99±4.1 | - | -34 | 104 |
| HOQNO[c] | 0.10 | 0.07±0.01 | - | -0.39 | 85±3.8 | - | -48 | 89 |

a = Without added substrate; b = With 235$\mu$M $O_2$; c = With 235$\mu$M $O_2$ + inhibitor;
b~a = Difference between (a) and (b) for $\Delta$pH and $\Delta\psi$ respectively;
c~a = Difference between (a) and (c) for $\Delta$pH and $\Delta\psi$ respectively.

with $NO_2^-$ it was reduced by 34 mV in the dark (Table 20). DCMU did not appreciably (<30%) reduce the $\Delta\Psi$ or $\Delta pH$ in light, whereas DBMIB reduced $\Delta pH$ by 40-60% and $\Delta\Psi$ by about 20-30 mV irrespective of the substrate used (Tables 19, 21, 23, 25).

### 3.5.3 Active transport of proline

The energy required for active transport of metabolites in aerobic bacteria is mainly derived from oxidative processes. The respiratory chain plays an important role in membrane energetics since it is involved in electron transport and extrudes $H^+$ into the surrounding medium thus establishing an electrochemical potential difference of $H^+$, proton motive force, across membranes. The proton motive force is the driving force for ATP synthesis in $H^+$-translocating ATPase and is also involved in active transport of many substrates. Active transport of amino acids is an energy linked process. Proline transport dependent on both aerobic respiration and denitrification of nitrate was studied in washed cells of the photodenitrifier in light and in the dark. The role of proton motive force in the active transport of proline during denitrification of $NO_3^-$, $NO_2^-$ and $N_2O$ to $N_2$ respectively, has also been examined.

#### 3.5.3.1 Proline uptake in light and in the dark

The intracellular volume of washed cells, determined as the inulin impermeable space was found to be 1.47 ± 0.10 µl/mg dry wt. By thin layer chromatography, it was shown that L-proline was not metabolized by these cells over the 10 min incubation period when the rates of transport were calculated.

The washed cells, grown under photodenitrifying conditions (Section 2.2.3) accumulated L-proline at a low rate (3.2 nmol/min/mg dry wt.) under dark-anaerobic conditions (Fig. 47). Addition of 10 mM $NO_3^-$ to these

FIG. 47: PROLINE UPTAKE BY WASHED CELLS IN LIGHT AND IN THE DARK.

Cells (0.8 to 1.0 mg dry wt./ml) from photodenitrifying cultures (18h), after EDTA treatment (Section 2.2.13.1), were suspended in 50 mM potassium phosphate buffer (pH 7.0). The incubation mixture contained 0.4 ml cell suspension and 10 µm [$^{14}$C]-proline in a total volume of 0.5 ml. $NO_3^-$ was added (final conc. 10 mM) to this mixture as indicated. Reactions were conducted anaerobically at 25°C, in triplicate, as described in Section 2.2.14.1. [$^{14}$C]-proline uptake was measured by liquid scintillation spectrometry (Section 2.2.13.3). Experimental errors were ⩽5%.

● Light;
▲ Dark;
○ Light + 10 mM $KNO_3$;
△ Dark + 10 mM $KNO_3$.

FIG. 48: PROLINE UPTAKE IN WASHED CELLS AS A FUNCTION OF EXTERNAL pH ($pH_{out}$).

EDTA-treated cells from photodenitrifying cultures (18h) were suspended (0.8 to 1.0 mg dry wt./ml) in 50 mM potassium phosphate buffer at various pH values (6.0 to 8.0) and the assay was carried out anaerobically in the presence of 10 mM $KNO_3$, in triplicate, as described in Fig. 47. Experimental error was ⩽5%.

○ Light;
● Dark.

FIG.47

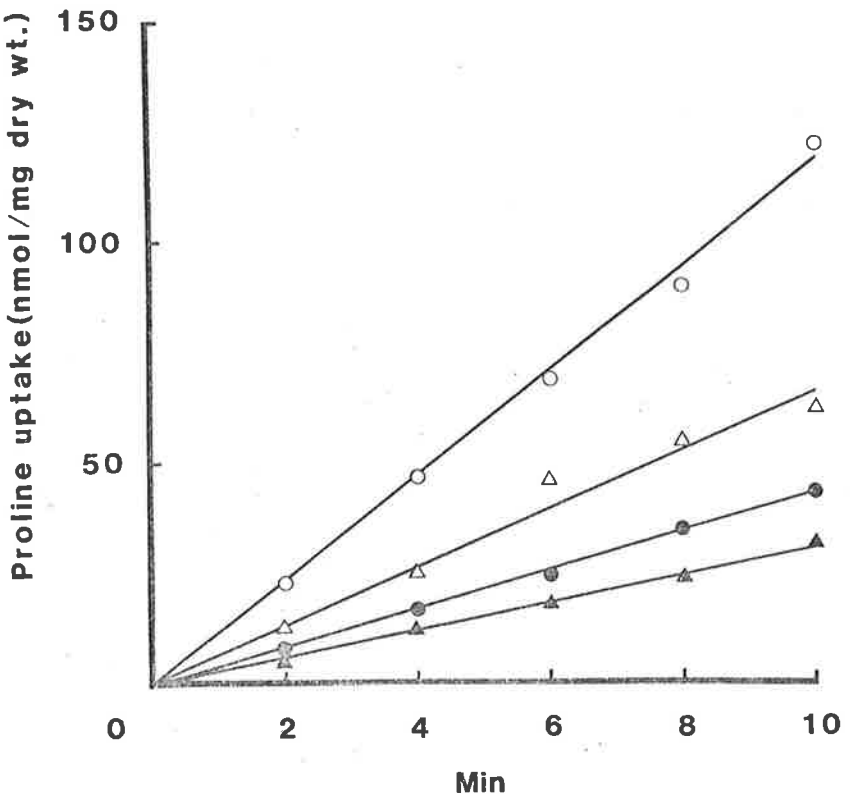
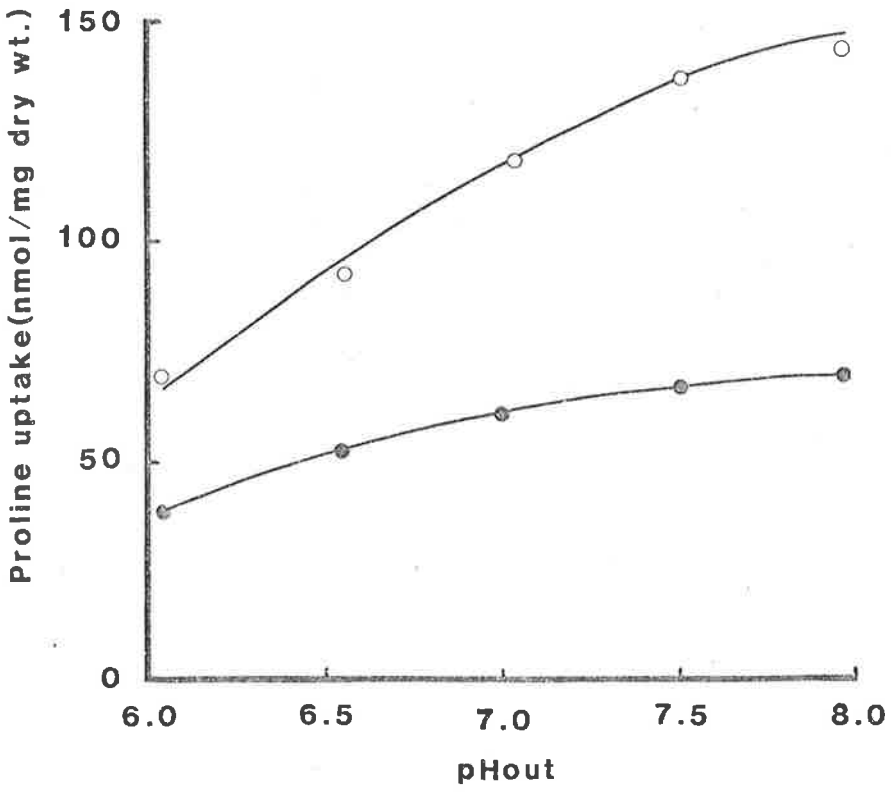

Proline uptake(nmol/mg dry wt.)
150
100
50
0
2
4
6
8
10
Min

FIG.48


Proline uptake(nmol/mg dry wt.)
150
100
50
0
6.0
6.5
7.0
7.5
8.0
pHout

cells in the dark doubled this rate, when $NO_3^-$ was reduced to $N_2$. In light, the proline transport into these cells was stimulated by the light induced electron flow. In the absence of $NO_3^-$, proline uptake was stimulated by 50% on exposure to light whereas addition of $NO_3^-$ (10 mM) resulted in a 4-fold increase in this rate. In all cases proline uptake was linear over the incubation period of 10 min.

3.5.3.2 Effects of pH

The effect of external pH ($pH_{out}$) on the rate of proline uptake by washed cells was studied anaerobically with 10 mM $NO_3^-$ as the terminal electron acceptor (Fig. 48). The rate of increase in Vpro (proline uptake/mg dry wt./min) in the dark was higher at $pH_{out}$ <7.0 compared to that at pH >7.0. In the presence of light proline uptake was higher at all $pH_{out}$ values compared to that in the dark and the rate of increase in Vpro was linear up to pH 7.5.

3.5.3.3 $K_m$ for proline

Proline transport between 2 and 20 µM showed saturation kinetics in washed cells in light with or without added $NO_3^-$ (Fig. 49). When the cells were incubated with 10 mM $NO_3^-$, proline uptake was greatly enhanced. The $K_m$ for proline transport into cells without $NO_3^-$ was 40 µM and $V_{max}$, 20.8 nmol/mg dry wt./min. Addition of $NO_3^-$ (10 mM) reduced the $K_m$ to 5.7 µM and the $V_{max}$ increased to 29.4 nmol/mg dry wt./min.

3.5.3.4 Utilization of various substrates

The washed cells previously grown anaerobically in light with $NO_3^-$ (Section 2.2.3) as the terminal acceptor derive energy from the reduction of either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$. Therefore, it was necessary to demonstrate that proline uptake in these cells is linked to the reduction of these substrates. The incorporation of [$^{14}$C]-proline into these cells was enhanced

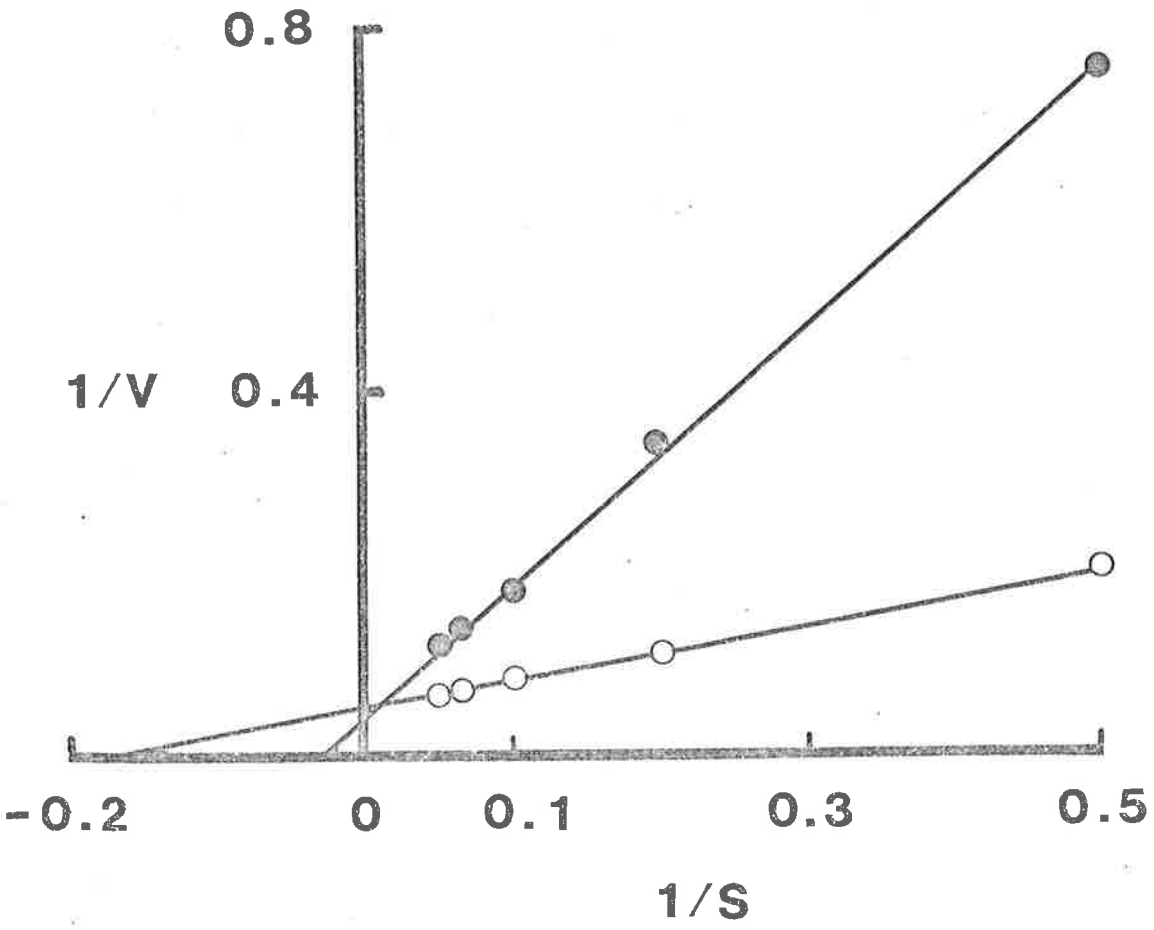
FIG. 49: KINETICS OF PROLINE TRANSPORT IN WASHED CELLS IN LIGHT IN THE PRESENCE AND ABSENCE OF NITRATE.

EDTA-treated cells from photodenitrifying cultures (18h) were suspended (0.8 to 1.0 mg dry wt./ml) in 50 mM potassium phosphate buffer (pH 7.0). Reactions were carried out (in triplicate) in light, with or without 10 mM $KNO_3$ as in Section 2.2.14.1, except that various concentrations of [$^{14}$C]-proline (0 to 20 µM) were used and [$^{14}$C] accumulation in cells were determined as in Section 2.2.13.3. Experimental errors were ⩽5%.

● $-NO_3^-$;

○ $+NO_3^-$.

FIG.49



in the presence of these substrates albeit at different rates (Table 26). Both in light and in the dark, the rate of proline uptake was maximal when $O_2$ was the terminal electron acceptor. With $NO_3^-$ as the substrate, the cells accumulated similar amounts of proline in light and slightly less (20%) in the dark compared with $O_2$ as a terminal electron acceptor. When either $NO_2^-$ or $N_2O$ was used, the rate of incorporation was 20 to 30% lower than that for $NO_3^-$.

3.5.3.5 Energy dependence of proline transport

The energy required for transport of metabolites in aerobic bacteria is usually derived from oxidative processes. To determine the energy requirements for proline transport, the effects of several metabolic inhibitors were examined using $NO_3^-$ as the terminal electron acceptor (Table 27). The inhibitor of electron transport, antimycin A (probably between cytochromes *b* and *c*) severely restricted (70%) the light driven proline uptake whereas a 40% inhibition was recorded in the dark. DBMIB, an inhibitor of photosynthetic electron transport in chloroplasts had no effect on proline accumulation in the dark whereas in light the incorporation of this amino acid was reduced by about 25% by this inhibitor. In the presence of 1 mM KCN, proline uptake was also inhibited by a half either in light or in the dark. Compounds that dissipate $\Delta p$ across the bacterial membranes were also effective inhibitors of proline transport, e.g. the uncoupler of oxidative phosphorylation, DNP severely restricted (60%) the accumulation of proline by the cells whereas the ATPase inhibitor, DCCD, had little effect (about 20% inhibition only).

3.5.3.6 Energy relations during proline transport

The energy-dependent processes in bacteria are dependent on the generation of a proton motive force ($\Delta p$). The generation of $\Delta p$ during

TABLE 26: PROLINE UPTAKE BY WASHED CELLS IN RELATION TO UTILIZATION OF SUBSTRATES.

$NO_3^-$, $NO_2^-$, $N_2O$ and $O_2$ respectively, was used as a substrate and proline uptake by washed cells, previously grown under photodenitrifying conditions for 18h, was assayed (in triplicate) in light and in the dark as described in Section 2.2.14.1. Experimental errors were ≤5%.

| Substrate | Final conc. (mM) | Light | Proline uptake (nmol/mg dry wt.) |
|---|---|---|---|
| - | - | + | 41 |
| $NO_3^-$ | 10 | + | 116 |
| $NO_2^-$ | 5 | + | 99 |
| $N_2O$ | 25 | + | 88 |
| $O_2$ | 23.5 (μM) | + | 128 |
| - | - | - | 21 |
| $NO_3^-$ | 10 | - | 56 |
| $NO_2^-$ | 5 | - | 41 |
| $N_2O$ | 25 | - | 32 |
| $O_2$ | 23.5 (μM) | - | 63 |

TABLE 27: EFFECTS OF INHIBITORS ON PROLINE UPTAKE BY WASHED CELLS.

Reactions were carried out (in triplicate) in washed cells from photodenitrifying cultures (18h) in the presence of 10 mM $KNO_3$ with or without an inhibitor as described in Section 2.2.14.1. Experimental errors were $\leqslant$ 5%.

| Inhibitor | Final conc. (mM) | Proline uptake (nmol/mg dry wt) Dark | Light |
|---|---|---|---|
| - | - | 62 | 111 |
| Antimycin A | 0.10 | 39 | 34 |
| DNP | 1.00 | 20 | 40 |
| DCCD | 0.10 | 52 | 95 |
| DBMIB | 0.01 | 61 | 87 |
| KCN | 1.00 | 30 | 58 |

the reduction of $NO_3^-$ to $N_2$ in light and in the dark was determined by measuring its components, namely, the membrane potential ($\Delta\Psi$) and the pH gradient ($\Delta pH$). The chemical gradient for proline ($\Delta\mu pro$) and the membrane potential were determined from the same experiment using [$^{14}C$]-proline and [$^{3}H$]-$TPP^+$ whereas $\Delta pH$ was measured separately in parallel experiments using [$^{14}C$]-benzoic acid. The proton gradient contributed about 100 mV in light (Fig. 50A) and 60 mV in the dark (Fig. 50B) to the proton motive force developed during the reduction of $NO_3^-$ at $pH_{out}$ 6.0 (-245 mV and -189 mV respectively). With the increase of $pH_{out}$ from 6.0 to 8.0, the $\Delta pH$ fell to zero both in light and in the dark. As the external pH was varied from 6.0 to 7.0 the $\Delta\Psi$ increased by 30 mV in light and by 20 mV in the dark. Further increases in $pH_{out}$ up to pH 8.0 resulted in a 25-50 mV decrease in $\Delta\Psi$. As a result of decreases in $\Delta pH$ and $\Delta\Psi$, the $\Delta p$ also decreased from -245 mV to -128 mV in light and from -189 mV to -126 mV in the dark when the $pH_{out}$ was increased from 6.0 to 8.0. The chemical gradient of proline ($\Delta\mu pro$) increased linearly in light (250 to 320 mV) as well as in the dark (190 to 255 mV) over an external pH range of 6.0 to 8.0. Since $\Delta\mu pro$ was unaffected by pH changes and $\Delta p$ was reduced at higher pH values the $H^+$/proline ratio varied from 1.0 to 2.5 in light and between 1.0 and 2.0 in the dark when $pH_{out}$ was varied between 6.0 and 8.0.

3.5.3.7 Involvement of $Na^+$ in light-dependent proline uptake

Cells grown under photodenitrifying conditions (Section 2.2.3) and washed in a $Na^+$-free buffer e.g. (Tris-HCl, pH 7.5), accumulated L-proline anaerobically at a low rate (2.5 nmol/min/mg dry wt., Fig. 51). Addition of 10 mM $KNO_3$ to these cells increased the proline uptake by about 5-fold. When 20 mM NaCl was added instead of $KNO_3$, there was a 6-fold increase in uptake. Addition of $KNO_3$ in the presence of NaCl increased proline accumulation about 8-fold. In the presence of various concentrations of

FIG. 50: EFFECTS OF EXTERNAL pH ($pH_{out}$) ON THE CHEMICAL POTENTIAL OF PROLINE ($\Delta\mu pro$), PROTON MOTIVE FORCE ($\Delta p$), MEMBRANE POTENTIAL ($\Delta\psi$) AND pH GRADIENT ($\Delta pH$) IN WASHED CELLS IN THE PRESENCE OF NITRATE.

EDTA-treated cells from photodenitrifying cultures (18h) were suspended (0.8 to 1.0 mg dry wt./ml) in 50 mM potassium phosphate buffer at the various pH values indicated. Reaction mixture contained 0.4 ml cell suspension, 10 mM $KNO_3$, [$^3$H]-$TPP^+$ (8.4 µM) and either 10 µM [$^{14}$C]-proline or [$^{14}$C]-benzic acid. Experimental details are given in Sections 2.2.13.3 and 2.2.14.1. Experiments were conducted in triplicate and errors were ≤5%.

△ $\Delta\mu pro$;

○ $\Delta p$;

● $\Delta\psi$;

▲ 59 x $\Delta pH$.

A, Light.

B, Dark.

FIG.50 A

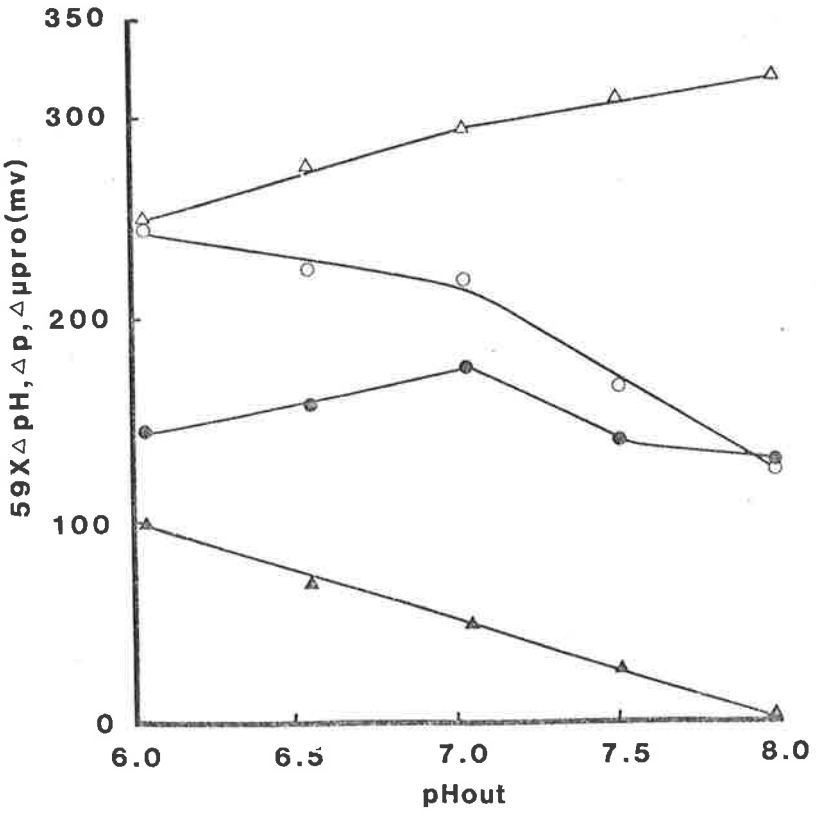



FIG.50B

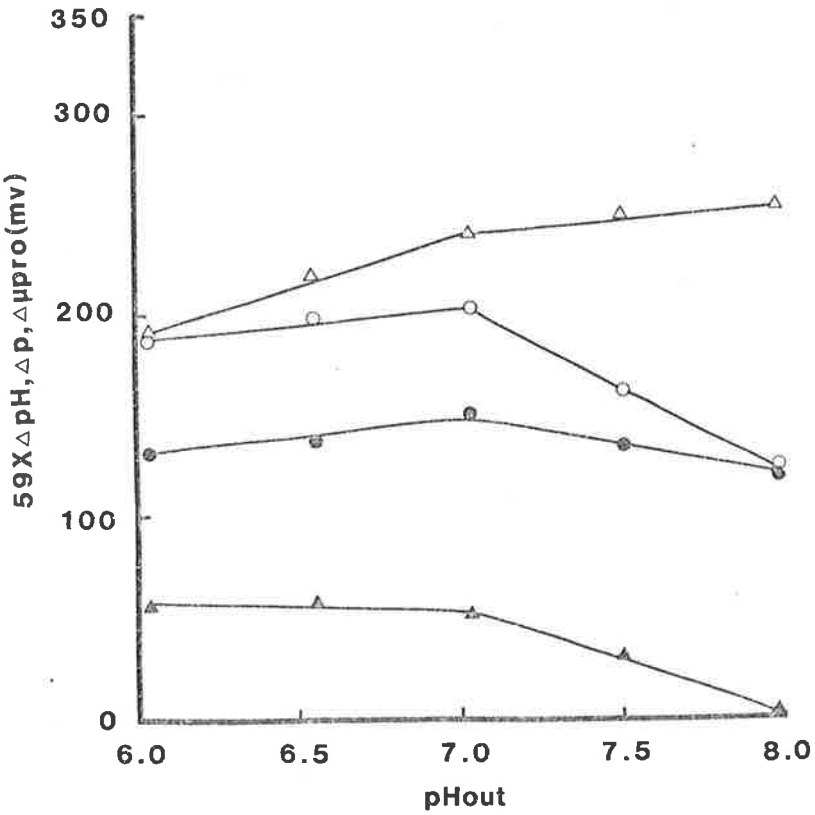
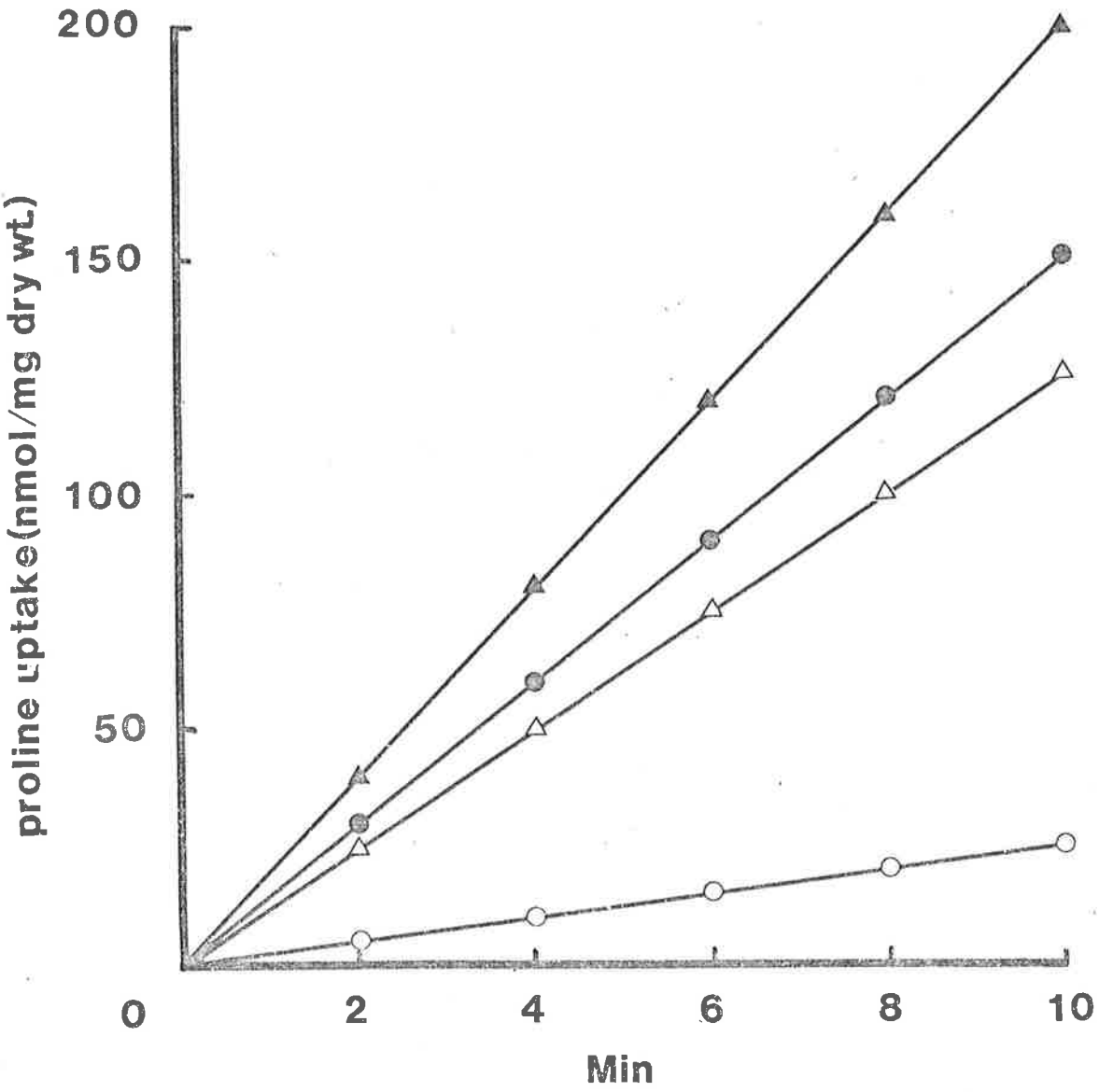


FIG. 51: PROLINE UPTAKE BY CELLS, WASHED IN A $Na^+$ FREE BUFFER, IN LIGHT.

EDTA-treated cells, previously grown (18h) under photodenitrifying conditions, were washed twice and resuspended (0.8 to 1.0 mg dry wt./ml) in 50 mM Tris-HCl buffer (pH 7.5). The uptake studies were carried out (in triplicate) anaerobically at 25°C as described in Sections 2.2.13.3 and 2.2.14.1. Proline (10 µM), $KNO_3$ (10 mM) and NaCl (20 mM) were included in the reaction mixtures as indicated. Experimental error was ⩽5%.

○ $-Na^+ - NO_3^-$;
△ $-Na^+ + NO_3^-$;
● $+Na^+ - NO_3^-$;
▲ $+Na^+ + NO_3^-$.

FIG.51



of $Na^+$ (5 to 100 mM) addition of $KNO_3$ (10 mM) did not appreciably increase proline uptake (Fig. 52A,B). Similarly, addition of $KNO_3$ had no effect on the $K_m$ or $V_{max}$ values for $Na^+$-dependent proline uptake by these cells.

Data for the $Na^+$-dependent uptake of proline by washed cells in relation to various concentrations of proline are presented in Fig. 53. The $K_m$ value for proline in the absence of $Na^+$ was 50 µM whereas in the presence of 20 mM $Na^+$ it decreased to 6.7 µM. Similarly, $Na^+$ increased the $V_{max}$ for proline uptake by about 5-fold.

Sodium loaded cells also accumulated proline at a low rate (2.3 nmol/min/mg dry wt.) in the absence of either $K^+$ or $NO_3^-$ (Fig. 54). The addition of 20 mM KCl to these cells increased the proline uptake 3-fold and the addition of 10 mM $KNO_3$ increased the rate 4-fold. When both KCl and $NaNO_3$ were added together the effect was additive i.e., an 8-fold increase in proline uptake.

#### 3.5.3.8 Effects of uncoupler, electron transport inhibitor and ionophores

Results in Table 28 show that proline uptake by washed cells in light was inhibited by CCCP (80%) and by HOQNO (70%). Addition of nigericin to washed cells in light produce about a 60% stimulation (as for $Na^+$) in the initial rate of proline uptake whereas valinomycin resulted in a substantial inhibition (65%).

### 3.5.4 $Na^+/H^+$ exchange activity

#### 3.5.4.1 ΔpH dependent $Na^+$ uptake

When washed cells were pre-equilibrated in an acidic medium (50 mM MES buffer pH 6.0 with 1 mM EDTA) and then diluted with a more alkaline medium (50 mM HEPES buffer, pH 8.0) containing 50 mM $Na^+$ (labelled with $^{22}Na^+$) they rapidly accumulated $Na^+$ (Fig. 55). In order to prove that a pH gradient (inside acidic) was responsible for this $Na^+$ uptake, various transmembrane

FIG. 52: EFFECTS OF $Na^+$ ON LIGHT-DEPENDENT PROLINE UPTAKE IN WASHED CELLS WITH OR WITHOUT NITRATE.

Assays were done anaerobically in EDTA-treated cells previously grown under photodenitrifying conditions for 18h. These cells were washed twice with 50 mM Tris-HCl buffer (pH 7.5) and then resuspended in the same buffer (0.8 to 1.0 mg dry wt./ml). Proline uptake was followed in the presence of various concentrations of NaCl with or without 10 mM $KNO_3$ as described in Sections 2.2.13.3 and 2.2.14.1. Experiments were done in triplicate and errors were ≤5%.

A, Reaction rates (nmol/mg dry wt.); ○,$-NO_3^-$; ●,$+NO_3^-$.

B, Reaction kinetics; ●,$-NO_3^-$; ○,$+NO_3^-$.

FIG.52A

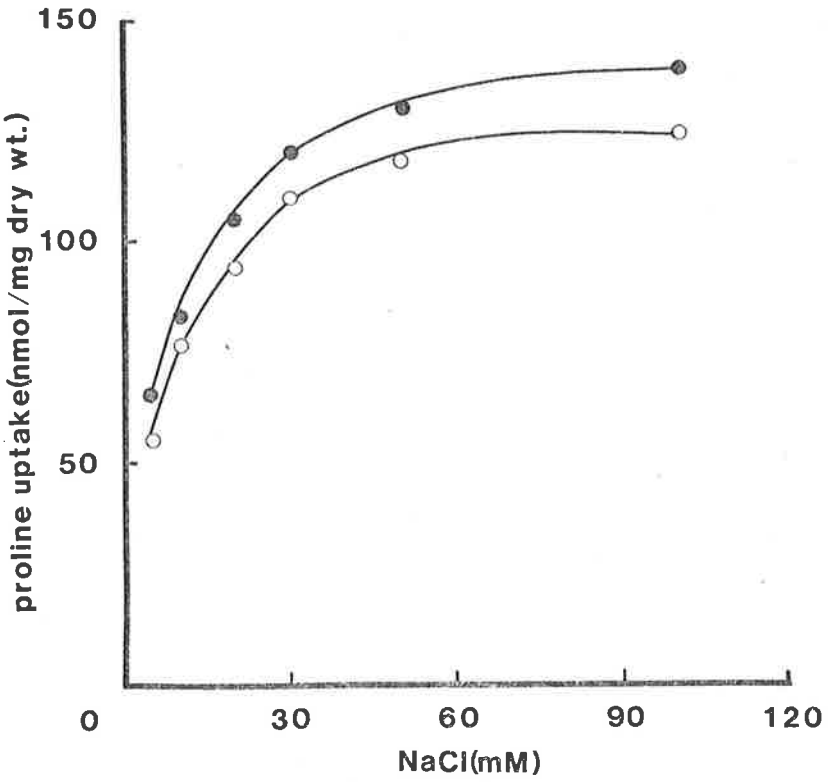



FIG.52B

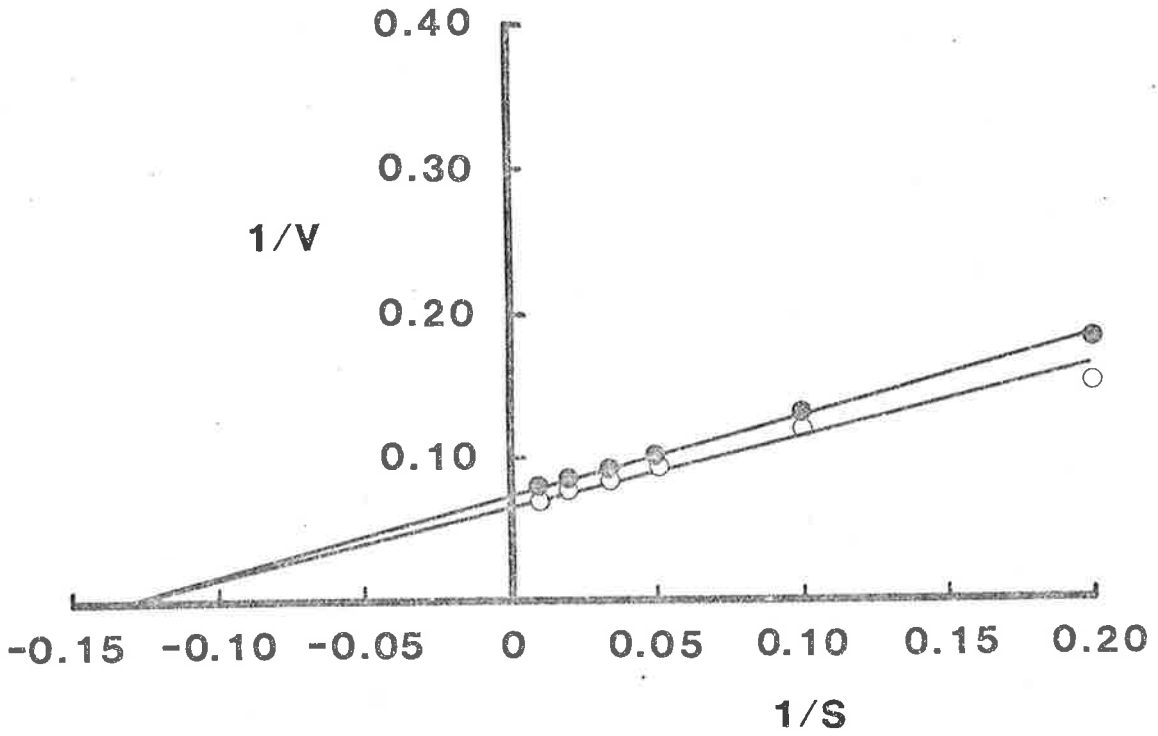


FIG. 53: EFFECTS OF $Na^+$ ON THE KINETICS OF LIGHT-DEPENDENT PROLINE UPTAKE IN WASHED CELLS WITHOUT NITRATE.

Cells from photodenitrifying cultures (18h) washed with 50 mM Tris-HCl, pH 7.5, after EDTA-treatment, were used for the assay of proline uptake (Section 2.2.13.1). Assays were carried out (in triplicate) anaerobically in 50 mM Tris-HCl buffer (ph 7.5), in the absence of $NO_3^-$ using various concentrations of [$^{14}$C]-proline (0 to 20 µM) and NaCl (20 mM) as indicated. (See Fig.47 for details). Experimental error was ⩽5%.

○ $+Na^+$;

● $-Na^+$.

FIG. 54: EFFECTS OF $K^+$ ON LIGHT-DEPENDENT PROLINE UPTAKE IN $Na^+$-LOADED CELLS.

$Na^+$-loading and proline uptake studies in washed cells from photodenitrifying cultures (18h) were carried out (in triplicate) anaerobically in the presence or absence of $NO_3^-$ as described in Sections 2.2.14.2, 2.2.14.1 and 2.2.13.3, respectively. In these experiments 10 mM $NaNO_3$ and 20 mM KCl were included in the assay mixture as indicated. Experimental error was ⩽5%.

○ $-K^+ - NO_3^-$;

● $+K^+ - NO_3^-$;

△ $-K^+ + NO_3^-$;

▲ $+K^+ + NO_3^-$.

FIG.53

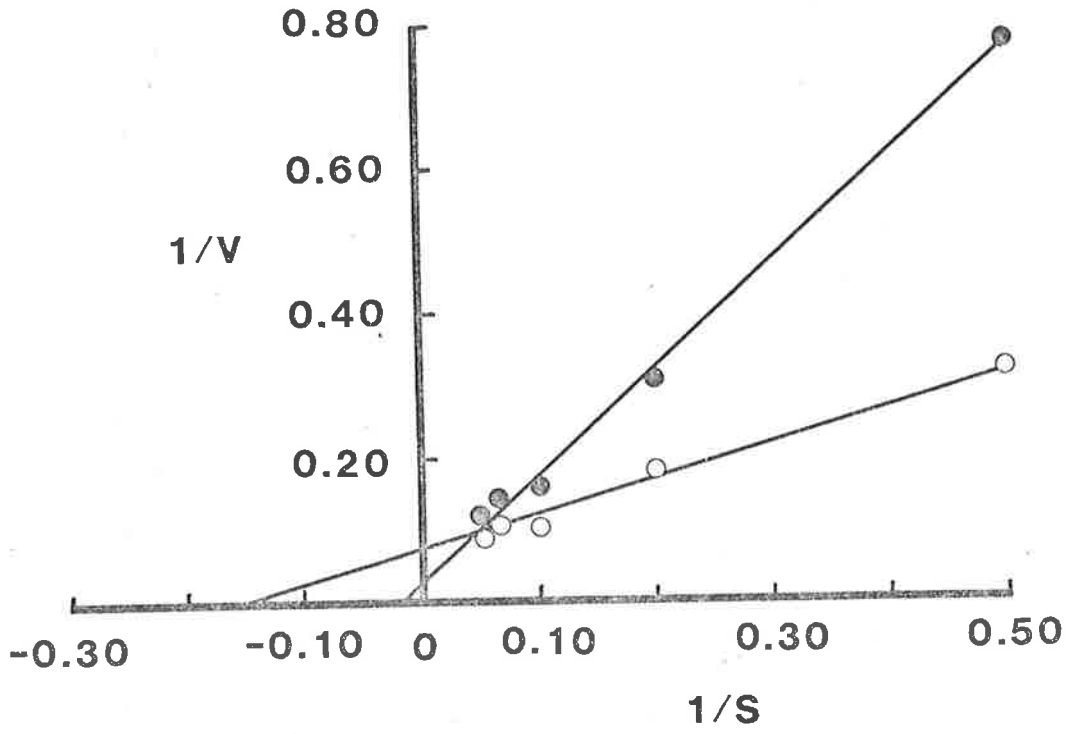

0.80
0.60
1/V
0.40
0.20
-0.30
-0.10
0
0.10
0.30
0.50
1/S

FIG.54

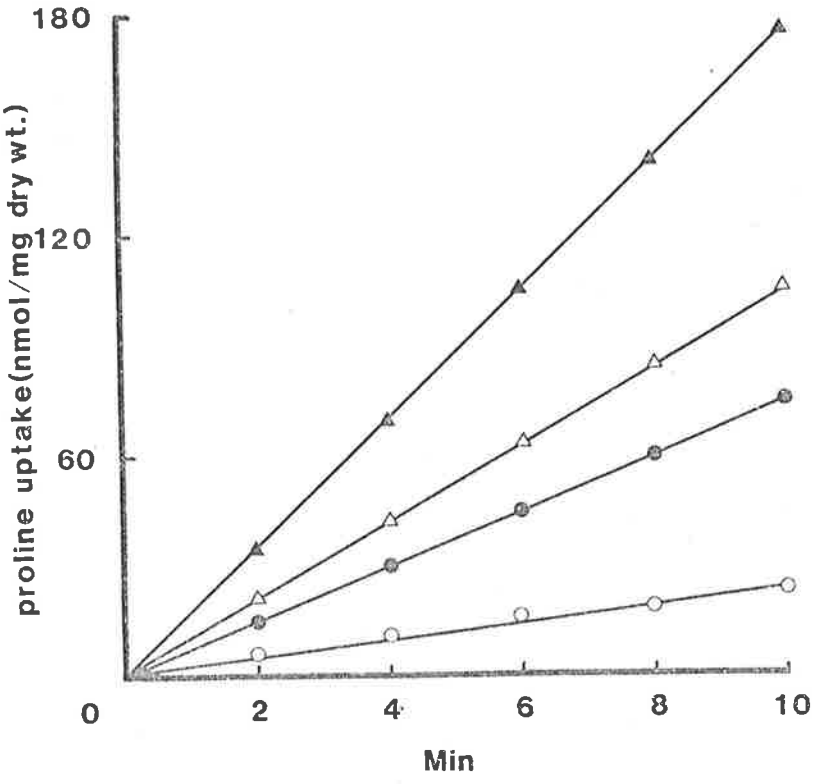

180
120
60
0
proline uptake(nmol/mg dry wt.)
2
4
6
8
10
Min

TABLE 28: EFFECTS OF IONOPHORES, 2-N-HEPTYL-4-HYDROXYQUINOLINE-N-OXIDE AND CARBONYL CYANIDE-M-CHLOROPHENYLHYDRAZONE ON LIGHT-DEPENDENT PROLINE UPTAKE IN WASHED CELLS.

These experiments were carried out (in triplicate) anaerobically with EDTA-treated cells, from photodenitrifying cultures (18h), washed twice in 50 mM Tris-HCl buffer, pH 7.5, and resuspended in the same buffer containing 10 mM $KNO_3$ as described in Section 2.2.14.1. Experimental errors were $\leq$ 5%.

| Treatments | Proline uptake (nmol/mg dry wt.) | % Control |
|---|---|---|
| Control (washed cells) | 140 | 100 |
| +20 mM NaCl | 224 | 160 |
| + 5 µM nigericin | 220 | 157 |
| + 50 µM CCCP | 30 | 21 |
| + 20 µM Valinomycin | 50 | 35 |
| + 100 µM HOQNO | 40 | 29 |

FIG. 55: SODIUM UPTAKE BY WASHED CELLS DRIVEN BY A TRANSMEMBRANE pH GRADIENT.

Washed cells (40 mg wet wt./ml) from photodenitrifying cultures (20h) were pre-equilibrated for 16h at 4°C in either 50 mM MES ($pH_{in}$ = 6.0; ○, ● ) or in 50mM HEPES ($pH_{in}$ = 8.0;△,▲) buffer both containing 1 mM EDTA. The cells were then centrifuged at 20,000 x g for 30 min at 4°C and resuspended in the equilibrating buffer (without EDTA) to 25 mg dry wt./ml. An aliquot (20 µl) of this cell suspension was diluted to 1 ml at 25°C with the following uptake medium:- 50 mM NaCl (2 µCi/ml $^{22}Na^{+}$) in either 50 mM MES ($pH_{out}$ = 6.0;○,▲) or 50 mM HEPES ($pH_{out}$ = 8.0;△,●). At the times indicated, 100 µl aliquots were withdrawn, passed through 0.45 µm Millipore filters and the filters containing cells were radioassayed as described in Section 2.2.15.1. Correction was made for the $^{22}Na^{+}$ retained by the filters in the absence of the cells.

FIG. 56: RELEASE OF ACCUMULATED $Na^{+}$ FROM CELLS BY GRAMICIDIN.

The washed cells (40 mg wet wt./ml), from photodenitrifying cultures (20h), were pre-equilibrated for 16h at 4°C in 50 mM MES, 1 mM EDTA ($pH_{in}$ = 6.0) and concentrated by centrifugation (see Fig.55). These cells (20 µl) were then diluted at 25°C with either the same medium (0.98 ml) without EDTA ($pH_{out}$ = 6.0,○) or 0.98 ml of 50 mM HEPES buffer ($pH_{out}$ = 8.0,●) both containing 50 mM NaCl (2 µCi/ml $^{22}Na^{+}$) as described in Fig.53. After an 8 min incubation at 25°C, 500 µg of gramicidin (in 100 µl, 95% v/v ethanol) was added. Appropriate controls were done to check any effects of alcohol on the release of $Na^{+}$. Aliquots (100 µl) withdrawn before and after the addition of gramicidin, were passed through Millipore filters (0.45 µm). Filters containing the cells were radioassayed as described in Section 2.2.15.1. Correction was made for the $^{22}Na^{+}$ retained by the filters in the absence of the cells.

FIG.55

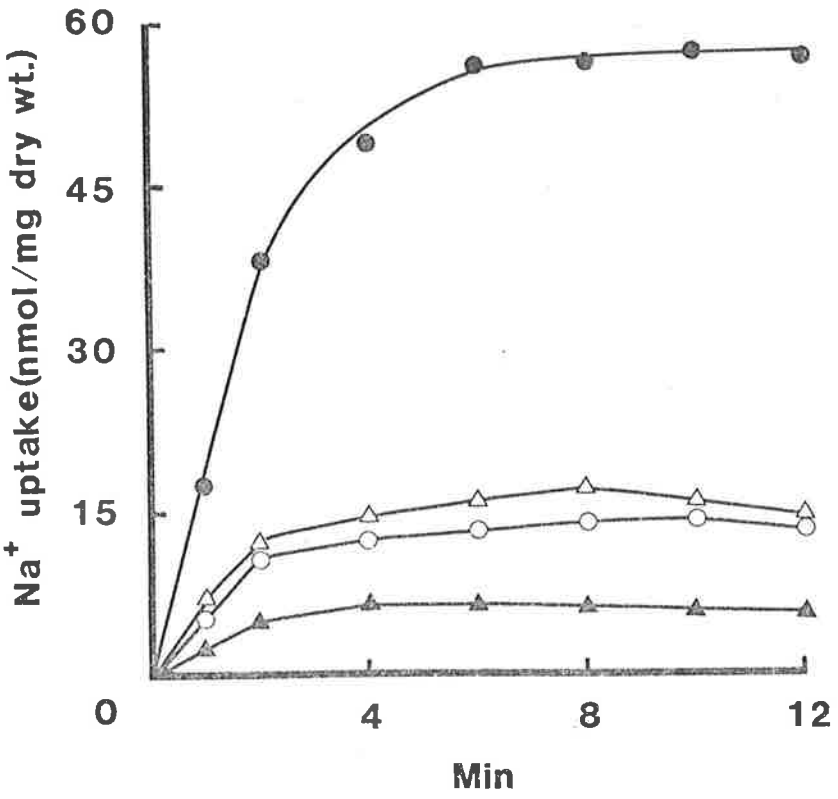
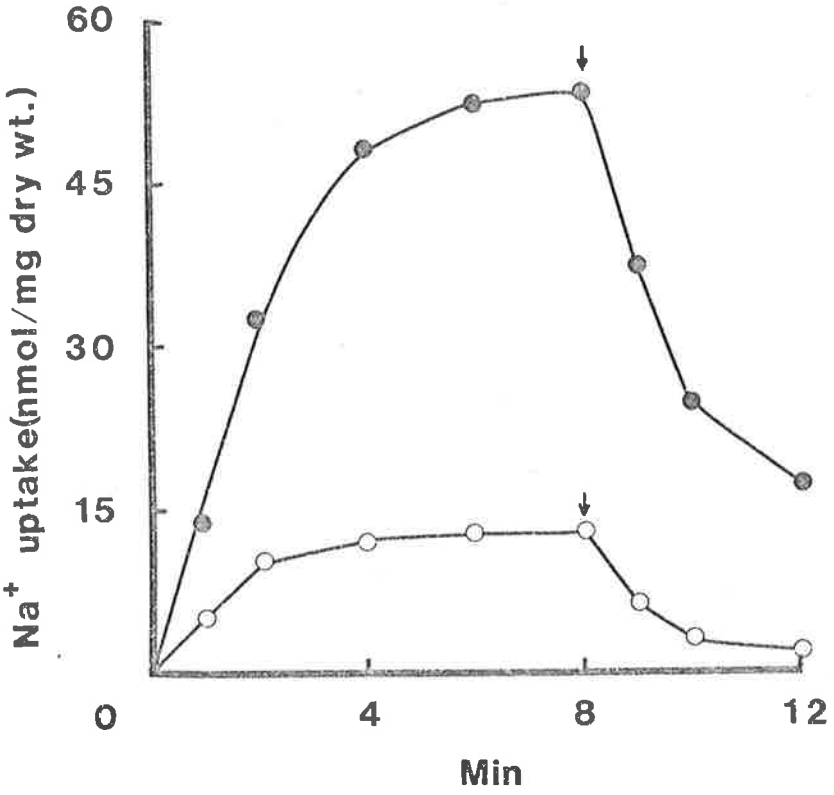

60
45
30
15
0
4
8
12
Min
Na+ uptake(nmol/mg dry wt.)

FIG.56


60
45
30
15
0
4
8
12
Min
Na+ uptake(nmol/mg dry wt.)

pH gradients were developed and the amounts of $Na^+$ accumulated by the cells determined. In the absence of a transmembrane $H^+$ gradient (when cells were pre-equilibrated in MES medium at pH 6.0 and then diluted in the same buffer or when they were pre-equilibrated in HEPES medium at pH 8.0 and then diluted with the same buffer), only 20-25%, $^{22}Na^+$ entered the cells compared with that recorded in the presence of a pH gradient. The $Na^+$ uptake in the presence of a pH gradient (inside acidic) was about 5-times higher than that by passive diffusion. When cells pre-equilibrated at pH 8.0 were diluted with a buffer (MES) at pH 6.0 (producing a trans-membrane pH gradient, inside alkaline), $Na^+$ movement into the cells was about a half of that observed in the absence of a pH gradient. When gramicidin was added to the cells accumulating $Na^+$, the cation was rapidly released (Fig. 56).

3.5.4.2 $Na^+$ pulse responses

Cells with a $Na^+/H^+$ antiporter, eject protons upon adding $Na^+$. As shown in Fig. 57 (A-H) the addition of NaCl to cell suspensions (aerobic or anaerobic) resulted in an efflux of $H^+$ as measured by the acidification of the cell suspensions in light and in the dark. Protons extruded by the cells were dependent on the reaction conditions and the size of the $Na^+$ pulse (Fig. 58). In general, the proton extrusion was 35-45% higher in the dark than that in light under both aerobic and anaerobic conditions. Proton extrusion was 20-30% greater in the presence of $O_2$ than under anaerobic conditions in light and in the dark (Fig. 58).

3.5.4.3 Effects of $Na^+$ on $\Delta\Psi$ and $\Delta pH$

Washed cells had a $\Delta\Psi$ (inside negative) value of -134 mV in light and -124 mV in the dark (anaerobic) whereas in the presence of 10 mM $KNO_3$ the $\Delta\Psi$ values were -171 mV and -152 mV respectively (Table 29). When 200 mM

FIG. 57: ACIDIFICATION OF THE SUSPENSION MEDIUM UPON ADDING $Na^+$ TO WASHED CELLS.

The assay was performed in a 7.5 ml electrode vessel containing 2 ml washed cells from photodenitrifying cultures (20h), resuspended (50 mg dry wt./ml) in 150 mM KCl (pH 7.0). The cell suspension was continuously stirred with a magnetic flea under a stream of either argon or $O_2$ (10 ml/min). Light (4,000 lux), filtered through a flat bottle filled with water, was used for the light reactions. Aluminium foil was wrapped around the reaction vessel when experiments were conducted in the dark. An argon saturated solution of NaCl (50 µl, 2M) was injected into the cell suspension *via* an air-tight microsyringe as indicated ↓. The bar corresponds to 500 ng atom $H^+$.

A,B, dark aerobic; C,D, light aerobic; E,F, dark anaerobic; G,H, light anaerobic; B,D,F,H, with 20 µM CCCP.

FIG. 58: CORRELATION BETWEEN PROTON EXTRUSION AND THE AMOUNT OF $Na^+$ ADDED TO WASHED CELLS IN $Na^+$-PULSE EXPERIMENTS.

$Na^+$-pulse experiments were carried out as described in Fig. 57.

- ○ Light aerobic;
- ● Dark aerobic;
- △ Light anaerobic;
- ▲ Dark anaerobic.

FIG.57

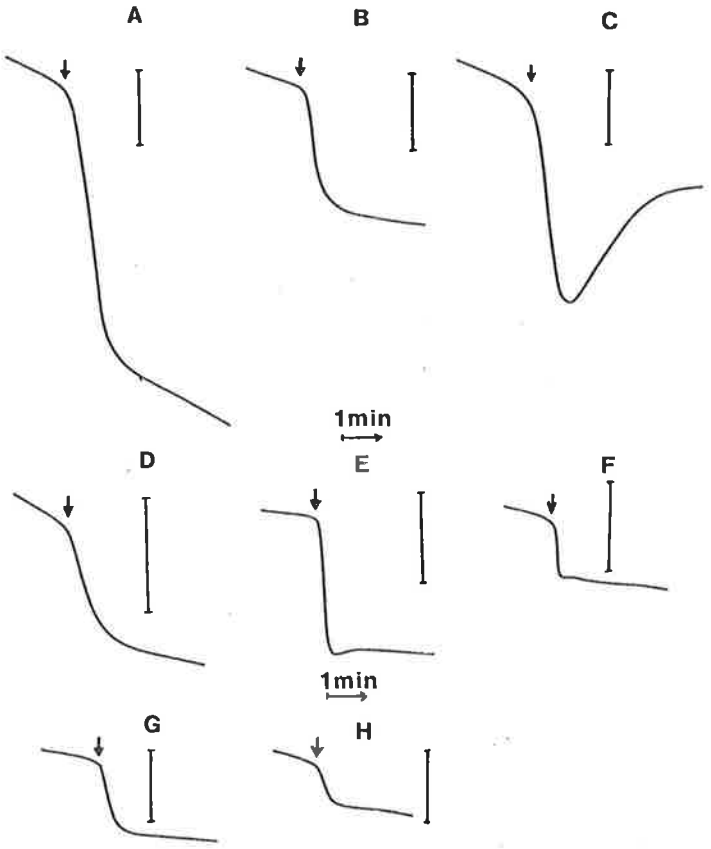

A
B
C
1min
D
E
F
1min
G
H

FIG.58

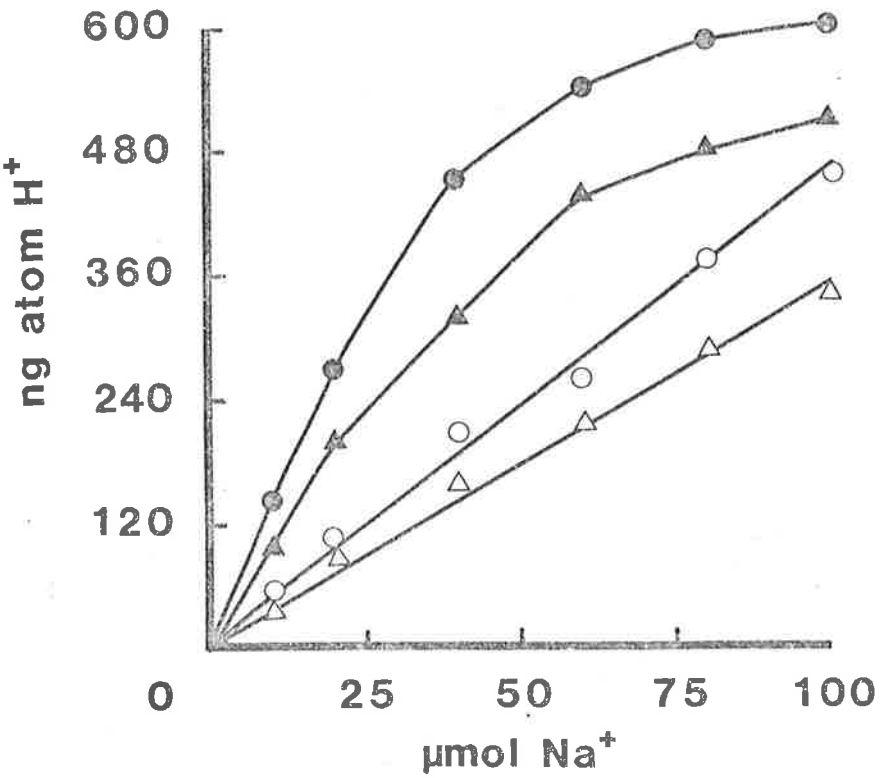

600
480
360
240
120
ng atom H+
0
25
50
75
100
µmol Na+

TABLE 29: EFFECTS OF $Na^+$ ON $\Delta pH$, $\Delta\psi$ AND $\Delta p$ IN WASHED CELLS.

Washed cells from photodenitrifying cultures (20h), after EDTA treatment, were resuspended (1.2 mg dry wt/ml) in 50 mM potassium phosphate buffer (pH 7.0) and uptake studies by these cells were carried out in triplicate as described in Section 2.2.13.3. $\Delta pH$ in mV i.e. 59 x $\Delta pH$ was determined by the uptake of [$^{14}C$]-benzoic acid; $\Delta\psi$ values were calculated from the uptake of [$^{3}H$]-$TPP^+$ and $\Delta p$ was determined from the $\Delta pH$ and $\Delta\psi$ values ($\Delta p = \Delta\psi - 59 \times \Delta pH$). Experimental errors were $\leqslant 5\%$.

| Conditions | Treatments | -mV | | |
|---|---|---|---|---|
| | | 59x$\Delta pH$ | $\Delta\psi$ | $\Delta p$ |
| Dark | 1. Washed cells | 15 | 124 | 139 |
| | 2. [1] + $KNO_3$ (10 mM) | 33 | 152 | 185 |
| | 3. [1] + NaCl (200 mM) | 53 | 103 | 156 |
| | 4. [1] + $KNO_3$ (10 mM) + NaCl (200 mM) | 52 | 100 | 152 |
| Light | 5. Washed cells | 23 | 134 | 157 |
| | 6. [5] + $KNO_3$ (10 mM) | 38 | 171 | 209 |
| | 7. [5] + NaCl (200 mM) | 60 | 106 | 166 |
| | 8. [5] + $KNO_3$ (10 mM) + NaCl (200 mM) | 60 | 110 | 170 |

NaCl was added to the cell suspensions in 50 mM potassium phosphate buffer (pH 7.0) with or without added $NO_3^-$, there was a depolarization of about 50 mV in the dark and 60-65 mV in the light. Concomitant with this depolarization of $\Delta\Psi$, the $\Delta$pH (inside alkaline) value increased by about 20 mV in light and in the dark.

### 3.5.5 $K^+/H^+$ exchange activity

#### 3.5.5.1 $\Delta$pH-dependent $K^+$ uptake

When washed cells were pre-equilibrated by soaking them overnight (16h) in 50 mM MES (pH 6.0) containing 1 mM EDTA followed by a dilution in a more alkaline medium (50 mM HEPES) containing 50 mM KCl, the cells readily accumulated $K^+$ (Fig. 59) indicating that this uptake was $\Delta$pH-dependent. In order to confirm this effect, various transmembrane pH gradients were established and the uptake of $K^+$ by these cells measured (Fig. 59). In the absence of a transmembrane pH gradient (when the cells were pre-equilibrated in 50 mM MES buffer, pH 6.0, containing 1 mM EDTA and then diluted with the same buffer without EDTA but containing 50 mM KCl or when the cells were pre-equilibrated in 50 mM HEPES buffer, pH 8.0, containing 1 mM EDTA and then diluted with the same buffer without EDTA but containing 50 mM KCl) the uptake of $K^+$ was 30 to 35% of that in the presence of a pH gradient (inside acidic). $K^+$ uptake in the presence of a pH gradient, inside alkaline (when cells were pre-equilibrated in 50 mM HEPES buffer, pH 8.0, containing 1 mM EDTA and then diluted with 50 mM MES buffer, pH 6.0 containing 50 mM KCl), was only about 15% of that when it was acidic inside. The $K^+$ accumulated by a pH gradient (inside acidic), was rapidly released into the medium on adding 50 µg/ml gramicidin (Fig. 60).

FIG. 59: POTASSIUM UPTAKE BY WASHED CELLS DEPENDENT ON A TRANSMEMBRANE pH GRADIENT.

Washed cells (40 mg wet wt./ml) from photodenitrifying cultures (20h) were pre-equilibrated by soaking for 16h at 4°C in either 50 mM MES buffer ($pH_{in}$ = 6.0; ○, ●) or in 50 mM HEPES ($pH_{in}$ = 8.0; △, ▲) buffer; both buffers contained 1 mM EDTA. The cells, centrifuged at 20,000 x g for 30 min at 4°C were resuspended (25 mg dry wt./ml) in the appropriate soaking buffer (without EDTA). An aliquot (20 µl) of this cell suspension was diluted at 25°C to 1 ml in the following buffers respectively:

(a) 50 mM MES + 50 mM KCl ($pH_{out}$ = 6.0; ○, ▲),
(b) 50 mM HEPES + 50 mM KCl ($pH_{out}$ = 8.0; △, ●).

At the times indicated, 100 µl aliquots were withdrawn, filtered through 0.45 µm Millipore filters and filters containing the cells were washed with 2 ml 0.1M Tris-choline buffer (pH 7.5). The $K^+$ contents were determined in 5% w/v TCA extracts as described in Section 2.2.16.1. Corrections were always made for the $K^+$ retained by the filters in the absence of the cells.

FIG. 60: RELEASE OF $K^+$ FROM CELLS TREATED WITH GRAMICIDIN.

The cells from photodenitrifying cultures (20h), were pre-equilibrated overnight at 4°C in 50 mM MES, 1 mM EDTA ($pH_{in}$ = 6.0), concentrated by centrifugation (at 20,000 x g for 30 min) and then diluted in either the same medium but without EDTA ($pH_{out}$ = 6.0; ○) or 50 mM HEPES ($pH_{out}$ = 8,0; ●) both containing 50 mM KCl as described in Fig.57. After 8 min incubation, 500 µg of Gramicidin (in 95% v/v ethanol) was added (Final conc. 50 µg/ml). Appropriate controls were done to check for any effects of alcohol on the release of $K^+$. Aliquots (100 µl) withdrawn before and after the addition of Gramicidin were filtered through Millipore filter (0.45 µm). Filters containing the cells were washed with 2 ml buffered choline chloride and the $K^+$ contents were determined in 5% w/v TCA extracts as described in Section 2.2.16.1.

FIG.59

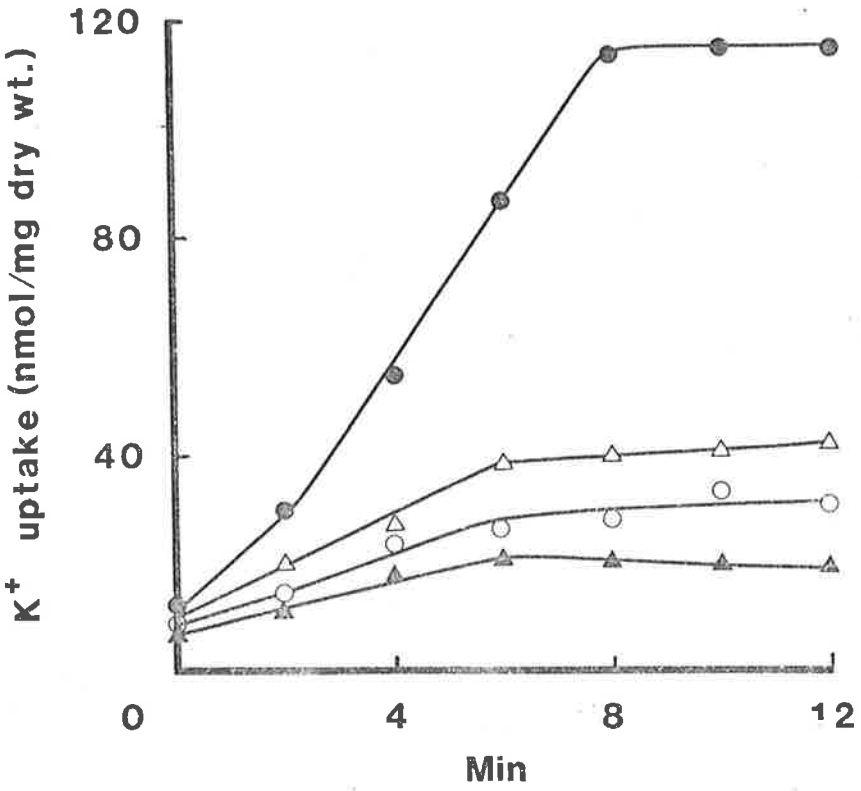
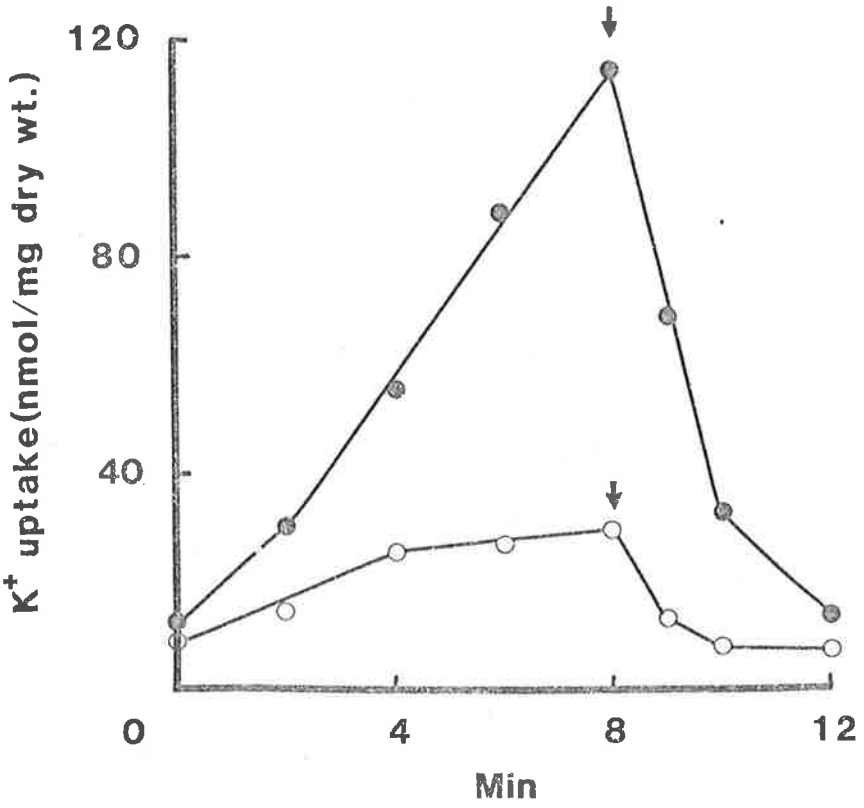

120
80
40
0
4
8
12
Min
K+ uptake (nmol/mg dry wt.)

FIG.60


120
80
40
0
4
8
12
Min
K+ uptake(nmol/mg dry wt.)

3.5.5.2 $K^+$ pulse responses

Cells with a $K^+/H^+$ antiporter should eject protons upon adding $K^+$. As shown in Fig. 61 (A-L), the addition of KCl to a suspension of washed cells (aerobic or anaerobic) resulted in an efflux of $H^+$ either in light or in the dark (Fig. 61A,D,G,J). Acidification increased 2-fold under anaerobic conditions both in light and in the dark. In light, however, the $H^+$ extrusion was about 25% higher than that under dark anaerobic conditions. Aerobically, the photodenitrifier produced about 30% less acidification in light than that in the dark (Fig. 61, G.J). The acidification produced by these washed cells in response to $K^+$ pulses aerobically or anaerobically in light and in the dark was inhibited by CCCP (Fig. 61, C,F,I,L).

Addition of valinomycin to the cells of the photodenitrifier, washed in a $K^+$-free buffer (50 mM Tris-HCl, pH 7.5) and resuspended in 50 mM Tris-HCl, pH 6.5 containing 1 mM dithiothreitol) resulted in $H^+$ influx as shown by alkalinization of the reaction medium (Fig. 61, B,E,H,K). Under similar conditions, these cells produced an acidification of the external medium in respect to $K^+$ pulses (Fig. 61A,D,G,J).

3.5.5.3 Effects of $K^+$ on $\Delta\Psi$ and $\Delta pH$

Results in Table 30 show that washed cells of this photodenitrifier had a higher $\Delta p$ (20-30 mV) in light than in the dark with or without added $NO_3^-$. Addition of $NO_3^-$ to washed cells resulted in an increase of $\Delta pH$ (inside alkaline) by about 15 mV and $\Delta\Psi$ (inside negative) by about 30 mV resulting in an overall increase of $\Delta p$ by about 50 mV both in light and in the dark. When 200 mM KCl was added to the cells in the dark, the $\Delta\Psi$ decreased from -120 mV to -94 mV (without $NO_3^-$) and from -152 mV to -90 mV (with $NO_3^-$). Addition of $K^+$ to the cells in light also resulted in a depolarization (about 60 mV) of $\Delta\Psi$ with a simultaneous increase (20-30 mV) in $\Delta pH$.

FIG. 61: ACIDIFICATION OF THE CELL SUSPENSION UPON ADDING $K^+$ TO WASHED CELLS.

The assay was conducted in a 7.5 ml electrode vessel containing 2 ml cells (50 mg dry wt./ml) from photo-denitrifying cultures (20h), washed and resuspended in 5 mM Tris-HCl, pH 6.5. The cell suspension was continuously stirred with a magnetic flea (200 rpm) under a stream of either argon or $O_2$ (10 ml.min$^{-1}$) at 25°C. Light (4,000 lux), from two 150w tungsten lamps, filtered through a flat bottle filled with distilled water, was used as a source of light. Aluminium foil was wrapped around the reaction vessel when experiments were conducted in the dark. An argon saturated solution of KCl (50 µl 2M) was injected into the cell suspension *via* an air-tight microsyringe as indicated↓ The bar corresponds to 3 µg atom $H^+$.

A-C, Dark anaerobic; D-F, Light anaerobic;
G-I, Dark aerobic; J-L, Light aerobic;
B,E,H,K, with 5 µM valinomycin; C,F,I,L, with 20 µM CCCP.

FIG.61

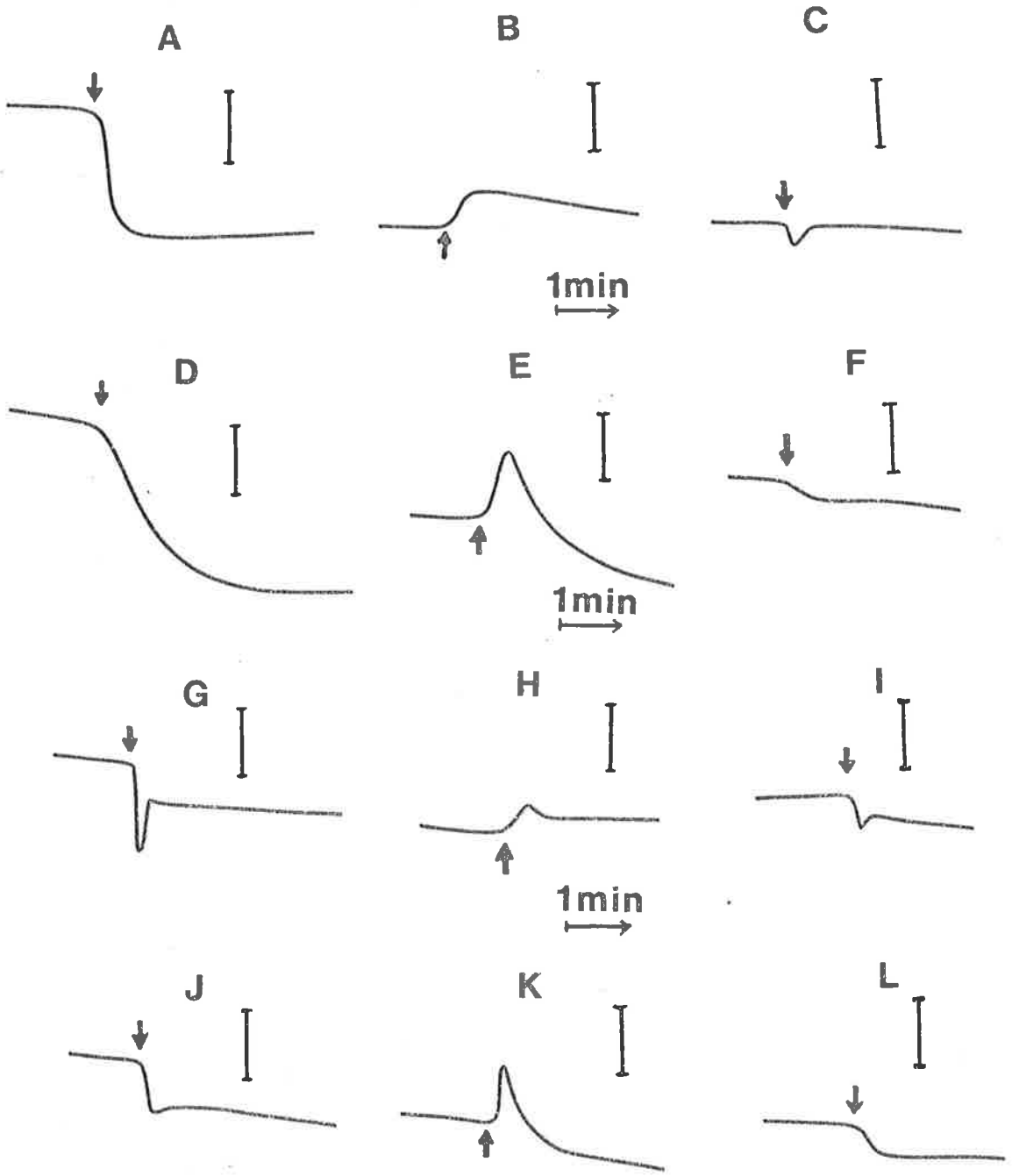


TABLE 30: EFFECTS OF ADDED $K^+$ ON $\Delta pH$, $\Delta\Psi$ AND $\Delta p$ IN WASHED CELLS.

Washed cells from photodenitrifying cultures (16h), after treating with EDTA (Section 2.2.13.1), were suspended (1.0 mg dry wt./ml) in 50 mM sodium phosphate buffer (pH 7.0). Uptake studies with labelled compounds were carried out in triplicate as described in Section 2.2.13.3. $\Delta pH$ in mV i.e. 59 x $\Delta pH$ was determined with [$^{14}$C]-benzoic acid; $\Delta\psi$ values were calculated from the uptake of [$^{3}$H]-$TPP^+$ and $\Delta p$ was determined from $\Delta pH$ and $\Delta\psi$ values ($\Delta p = \Delta\psi - 59 \times \Delta pH$). Experimental errors were $\leqslant 5\%$.

| Conditions | Treatments | -mV | | |
|---|---|---|---|---|
| | | 59x$\Delta pH$ | $\Delta\psi$ | $\Delta p$ |
| Dark | 1. Washed cells | 15 | 120 | 135 |
| | 2. [1] + 10 mM $NaNO_3$ | 32 | 152 | 184 |
| | 3. [1] + 200 mM KCl | 56 | 94 | 150 |
| | 4. [1] + 10 mM $NaNO_3$ + 200 mM KCl | 61 | 90 | 151 |
| Light | 5. Washed cells | 21 | 137 | 158 |
| | 6. [5] + 10 mM $NaNO_3$ | 36 | 168 | 204 |
| | 7. [5] + 200 mM KCl | 58 | 101 | 159 |
| | 8. [5] + 10 mM $NaNO_3$ + 200 mM KCl | 56 | 102 | 158 |

### 3.5.6 Studies on $Na^+$ and $K^+$ transport

#### 3.5.6.1 $Na^+$ and $K^+$ content of cells

In washed cells, the intracellular concentrations of $K^+$ and $Na^+$ varied greatly from one batch to another. Thus, in six batches of cells, $K^+$ varied from 115 to 174 mM and $Na^+$ from 32 to 60 mM (Table 31). A slow extrusion of intracellular $K^+$ was observed on adding 4 ml of a cell suspension (in HEPES-NaOH buffer, pH 7.5) to 21 ml 50 mM Tris-HCl/150 mM NaCl, pH 9.2 (Fig. 62). When aliquots (4 ml) of the same cell suspension was added to 50 mM diethanolamine-HCl/150 mM NaCl there was a rapid loss of $K^+$ from the cells. Thus, after 10 min about a half of the intracellular $K^+$ was lost and after 1h, 4 mM $K^+$ only remained in the cells. The rate of $^{22}Na^+$ uptake during the DEA-treatment was extremely slow even when the buffer contained 150 mM NaCl. Thus after 1h of DEA-treatment, the cells lost 145 mM of $K^+$ whereas only 5.3 mM $^{22}Na^+$ was taken up.

#### 3.5.6.2 Denitrification in $K^+$-depleted cells

The denitrification rates of untreated and DEA-treated cells were determined by measuring the nitrate and nitrite reductase activities of these cells with BVH as the electron donor. The $K^+$-depleted cells (DEA-treated) retained about 90% of the nitrate reductase activity and nitrite reductase activity was not affected (Table 32). The addition of $K^+$ to washed cells did not appreciably increase (14%) nitrate reductase activity whereas $K^+$-depleted cells showed a 24% inhibition in enzyme activity. When $K^+$ was added to either washed cells or $K^+$-depleted cells there was no appreciable change (<10%) in nitrite reductase activity.

#### 3.5.6.3 $^{22}Na^+$ loading of $K^+$-depleted cells

The results in Fig. 62 indicate that there was very little uptake of

TABLE 31: INTRACELLULAR CONCENTRATIONS OF $Na^+$ AND $K^+$ IN WASHED CELLS.

Cells grown for 20h under photodenitrifying conditions were harvested by centrifugation at 20,000 x g and 4°C for 20 min, washed 2 to 3 times in 50 mM Tris-HCl buffer (pH 7.5) and resuspended (25 mg wet wt./ml) in the same buffer. Aliquots (100 µl) filtered through Milipore filters (0.45 µm) were washed once with 2 ml, 0.2M choline chloride. $Na^+$ and $K^+$ were determined in TCA extracts by atomic absorption spectroscopy as described in Section 2.2.17.3. Experiments were conducted in triplicate and results were within 5% error.

| Batch | Intracellular conc. (mM) | |
|---|---|---|
| | $K^+$ | $Na^+$ |
| 1 | 137 | 40 |
| 2 | 141 | 32 |
| 3 | 115 | 35 |
| 4 | 174 | 59 |
| 5 | 147 | 60 |
| 6 | 128 | 52 |

FIG. 62: POTASSIUM EXTRUSION AND $^{22}Na^+$ UPTAKE IN WASHED CELLS DURING DEA-TREATMENT.

Freshly harvested cells (0.5g wet wt.) from photodenitrifying cultures (20h), washed twice with 50 mM Tris-HCl, pH 7.5, were then suspended in 4 ml 50 mM HEPES-NaOH (pH 7.5). This cell suspension was added to 21 ml 50 mM DEA-HCl/150 mM NaCl containing 2 $\mu Ci.ml^{-1}$ $^{22}NaCl$. Samples withdrawn at intervals as indicated were immediately filtered through Millipore filters (0.45 µM). The cells on the filters were washed twice with 0.2M choline chloride. $K^+$ was determined in trichloroacetic acid extracts of the cells by atomic absorption spectrometry as described in Section 2.2.17.3 and $^{22}Na^+$ by liquid scintillation spectrometry (Section 2.2.19.1).

○ $K^+$ extrusion in washed cells; the cell suspension in 4 ml HEPES buffer was diluted with 21 ml 100 mM Tris HCl pH 9.2.

● $K^+$ extrusion during DEA-treatment.

△ $^{22}Na^+$ uptake during DEA-treatment.

FIG.62

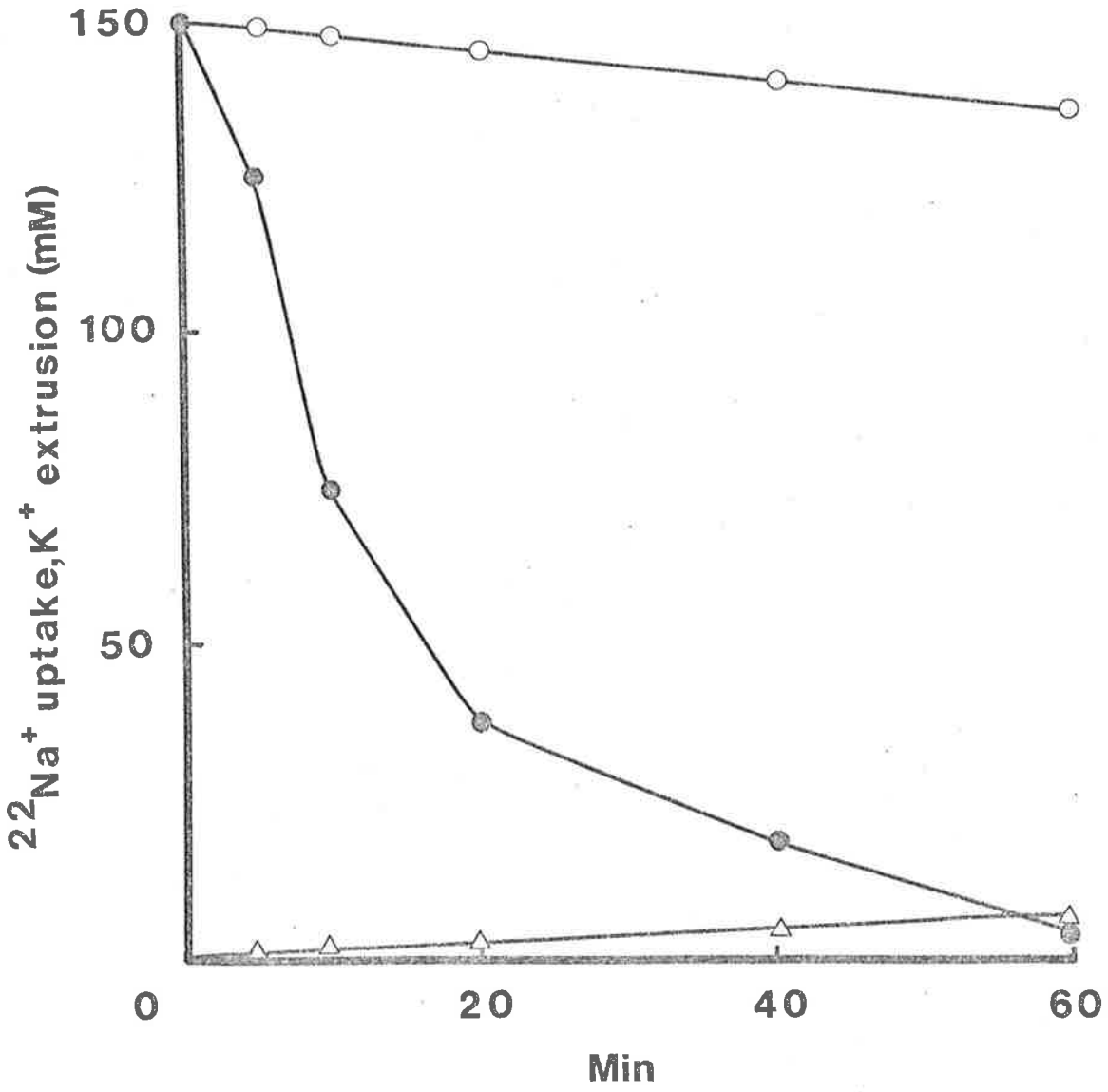


TABLE 32: EFFECTS OF $K^+$-DEPLETION AND $K^+$ ADDITION ON DENITRIFICATION IN WASHED CELLS.

$K^+$-depleted cells (20 mg wet wt./ml) were prepared by DEA-treatment as described in Section 2.2.17.1 and denitrification of $NO_3^-$ and $NO_2^-$ were determined by measuring nitrate and nitrite reductase activities using BVH as the electron donor (Sections 2.2.9.2 and 2.2.9.3). Experiments were done in triplicate and errors were $\leqslant$ 5%.

| DEA-treatment | Substrate ($NO_3^-$ ≡ 5 mM, $NO_2^-$ ≡ 100 µM) | $K^+$ addition (20 mM) | Rate of denitrification (nmol $NO_3^-$ or $NO_2^-$ utilized/mg dry wt.) |
|---|---|---|---|
| NIL | $NO_3^-$ | 0 | 401 |
| NIL | $NO_3^-$ | + | 458 |
| NIL | $NO_2^-$ | 0 | 866 |
| NIL | $NO_2^-$ | + | 916 |
| + | $NO_3^-$ | 0 | 363 |
| + | $NO_3^-$ | + | 305 |
| + | $NO_2^-$ | 0 | 917 |
| + | $NO_2^-$ | + | 929 |

$Na^+$ associated with the exit of $K^+$ in washed cells during DEA treatment. However, when cells were washed and resuspended in a buffer e.g. Tris or HEPES (pH 7.5) containing 150 mM NaCl they readily took up $^{22}Na^+$. The time course for the uptake of $^{22}Na^+$ by $K^+$-depleted cells is shown in Fig.63. In the absence of DEA in the external medium, $^{22}Na^+$ was readily taken up by the cells and the uptake in the dark was almost double that in light under both aerobic and anaerobic conditions. On the other hand, $^{22}Na^+$ uptake was higher aerobically than under anaerobic conditions, both in light (20%) and in the dark (35%).

3.5.6.4 $^{22}Na^+$ extrusion from $^{22}Na^+$-loaded cells

$^{22}Na^+$-loaded cells were prepared under dark aerobic conditions as described in Fig. 63 and an active extrusion of $^{22}Na^+$ was observed using a filtration method (Section 2.2.15.1). When 20 mM KCl was added to $^{22}Na^+$-loaded cells under dark aerobic conditions, about 40% $^{22}Na^+$ was released into the external medium (Fig. 64) and this extrusion was inhibited by 20 µM CCCP. The addition of $NO_3^-$ to $^{22}Na^+$-loaded cells under dark anaerobic conditions did not result in a loss of $^{22}Na^+$ from these cells. When $^{22}Na^+$-loaded cells were exposed to light there was a rapid loss (50%) of $^{22}Na^+$ from cells, both in air and under anaerobic conditions in the presence or absence of $NO_3^-$ (Fig. 65). This loss was further enhanced by adding 20 mM KCl. The extrusion of $^{22}Na^+$ from cells in light in the presence or absence of KCl was inhibited by CCCP.

3.5.6.5 Proton motive force in normal and $K^+$-depleted cells

The effects of external pH on the components of $\Delta p$, *viz.* $\Delta pH$ and $\Delta\Psi$ in normal cells are illustrated in Fig. 66. These cells had higher values of $\Delta\Psi$ (inside negative) and $\Delta pH$ and consequently a higher $\Delta p$ in light (about -200 mV) than in the dark (about -170 mV) at external pH values

FIG. 63: UPTAKE OF $^{22}Na^{+}$ BY $K^{+}$-DEPLETED CELLS IN LIGHT AND IN THE DARK.

$K^{+}$-depleted cells (20 mg wet wt./ml) were prepared from photodenitrifying cultures (20h) as described in Section 2.2.17.1. Uptake studies were carried out aerobically without $NO_3^-$ and anaerobically with 10 mM $KNO_3$ as described previously (Sections 2.2.17.2 and 2.2.19.1).

△ Light aerobic;

□ Light anaerobic;

○ Dark aerobic;

● Dark anaerobic.

FIG.63

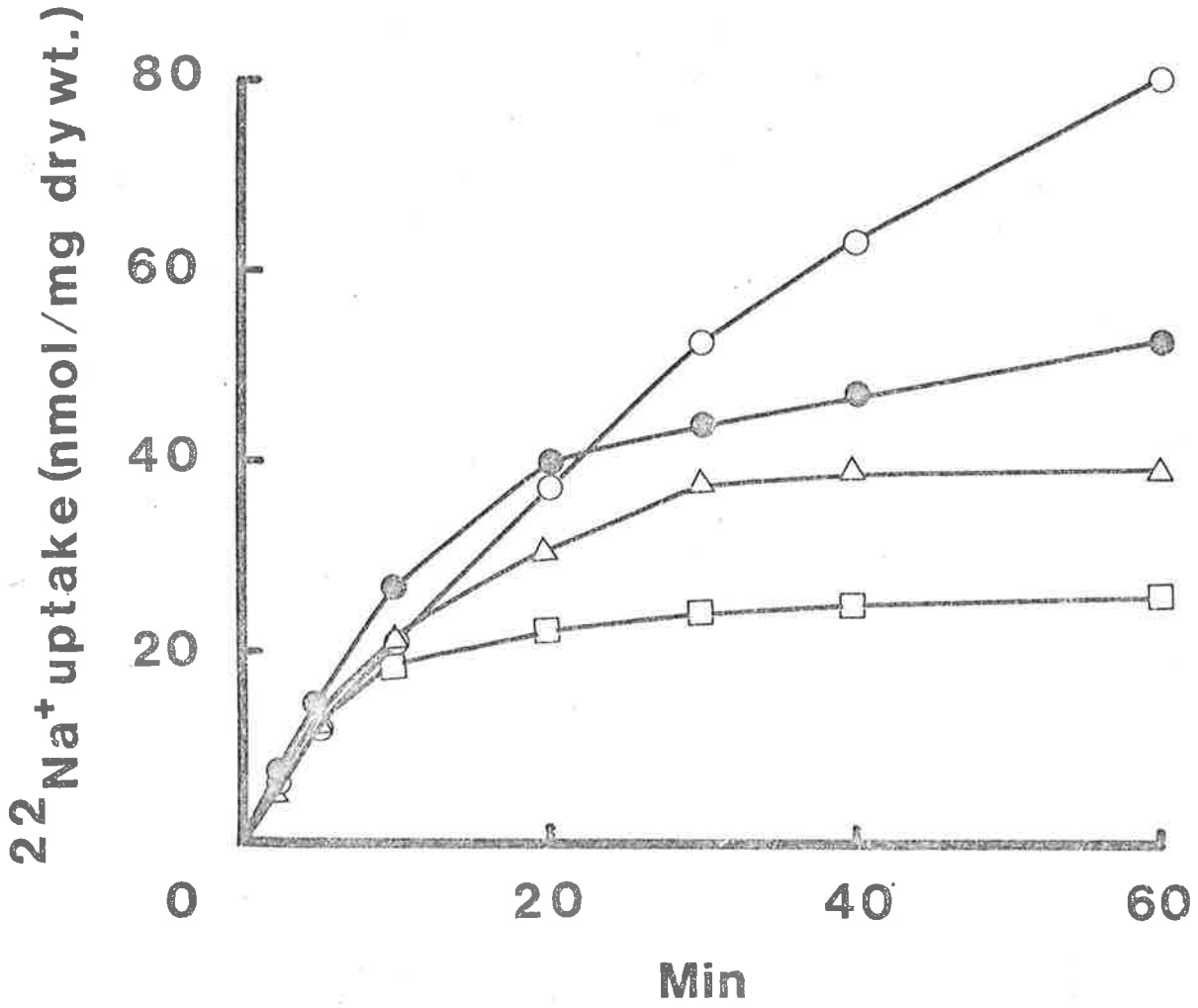


FIG. 64: EXTRUSION OF $^{22}Na^+$ FROM $^{22}Na^+$-LOADED CELLS IN THE DARK.

The $^{22}Na^+$-loaded cells (50 mg wet wt./ml) were prepared from photodenitrifying cultures (20h) aerobically in the dark (Section 2.2.17.2) and the amount of $^{22}Na^+$ retained by these cells, after various treatments in the dark, were determined by a Millipore (0.45 µm) filtration as described in Section 2.2.19.1.

△ Aerobic;

○ Aerobic + 20 mM KCl;

● Aerobic + 20 mM KCl + 20 µm CCCP;

▲ Aerobic + 10 mM $NH_4Cl$;

□ Anaerobic + 10 mM $NaNO_3$.

FIG. 65: EXTRUSION OF $^{22}Na^+$ FROM $^{22}Na^+$-LOADED CELLS IN LIGHT.

The $^{22}Na^+$-loaded cells (50 mg wet wt./ml) were prepared aerobically in the dark from photodenitrifying cultures (20h) as described in Section 2.2.17.2. The amount of $^{22}Na^+$ retained by these cells, after various treatments in light were determined by a Millipore (0.45 µm) filtration as described in Section 2.2.19.1.

△ Dark aerobic;

▲ Light aerobic;

■ Light aerobic + 20 µM CCCP;

○ Light aerobic + 20 mM KCl;

● Light aerobic + 20 mM KCl + 20 µM CCCP;

▽ Light aerobic + 10 mM $NH_4Cl$;

□ Light anaerobic + 10 mM $NaNO_3$.

FIG.64

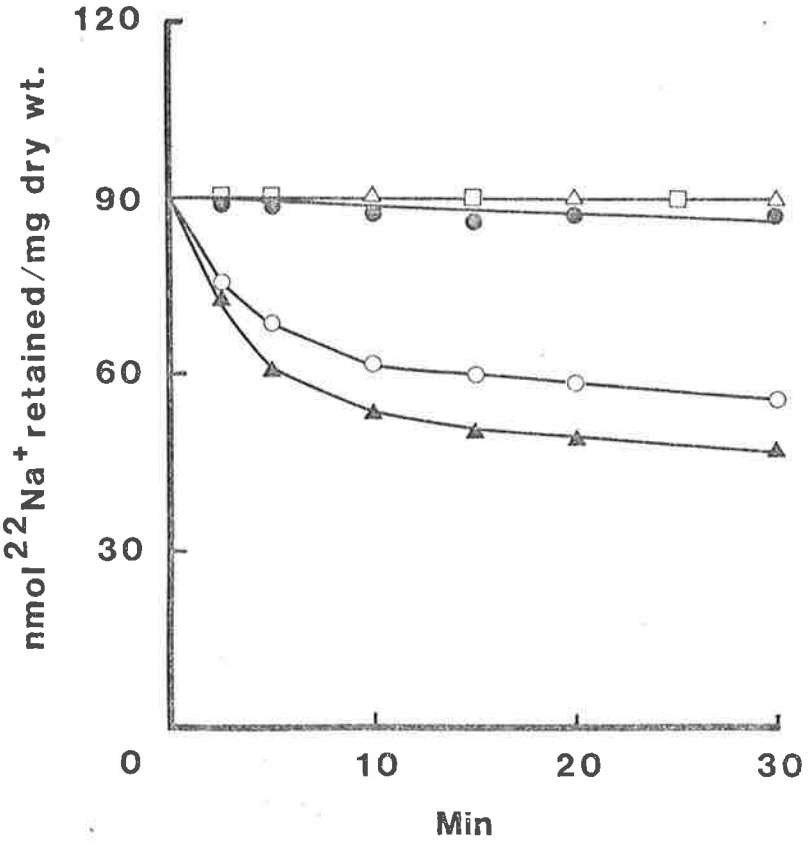

120
90
60
30
0
10
20
30
Min
nmol 22Na+ retained/mg dry wt.

FIG.65

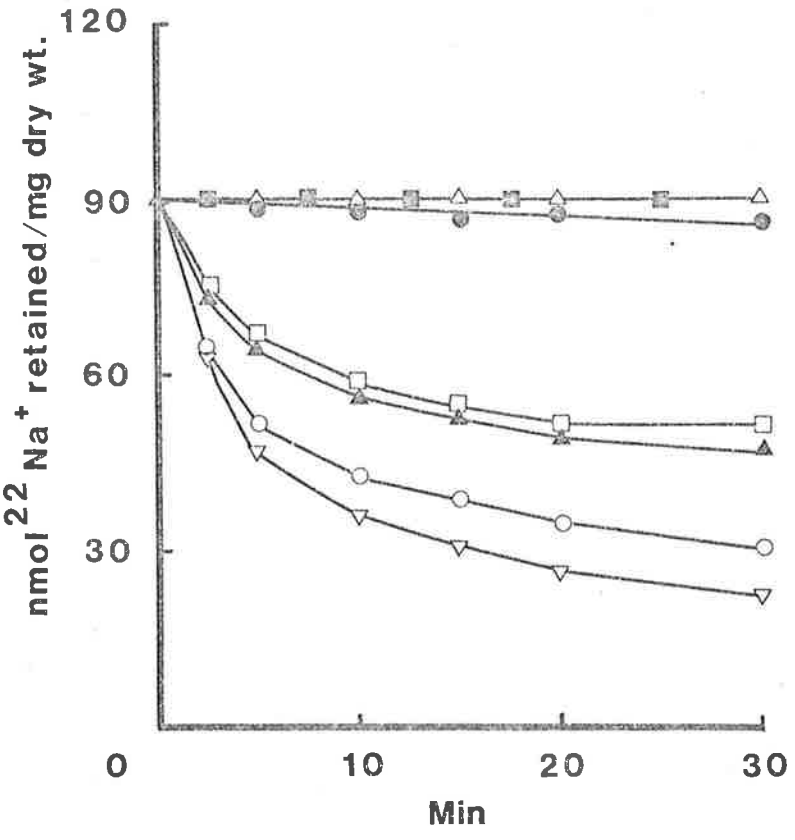
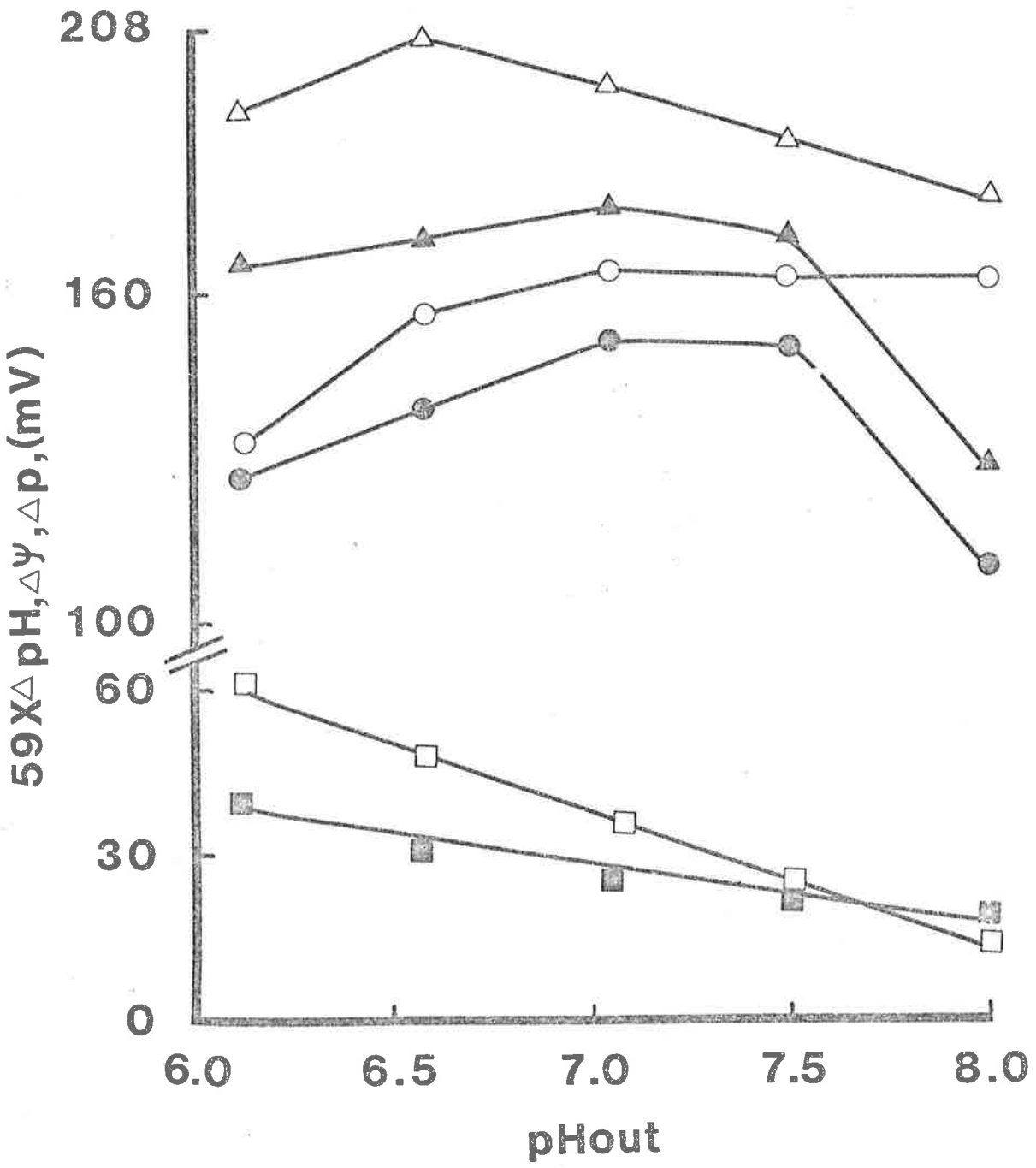

120
90
60
30
0
10
20
30
Min
nmol 22Na+ retained/mg dry wt.

FIG. 66: EFFECTS OF EXTERNAL pH ON THE COMPONENTS OF $\Delta p$ IN WASHED CELLS IN LIGHT AND IN THE DARK.

Washed cells from photodenitrifying cultures (16h), suspended in 100 mM Tris-HCl (pH 9.2), were incubated for 1h at 30°C. These cells were then centrifuged and resuspended (1-1.5 mg dry wt./ml) in 50 mM sodium phosphate buffer at the pH values indicated and $\Delta p$ values determined (Section 2.2.13). $\Delta pH$ (mV) was determined with [$^{14}$C]-benzoic acid; 59 x $\Delta pH$ ( □ , ■ ). $\Delta\Psi$ ( ○ , ● ) values (mV) were calculated from [$^{3}$H]-$TPP^{+}$ uptake; and $\Delta p$ ( △ , ▲ ) from the $\Delta\Psi$ and $\Delta pH$ values ($\Delta p = \Delta\Psi - 59 \times \Delta pH$).

Open symbols, in light; closed symbols, in the dark.

FIG.66



between 6.0 and 7.0. At pH 8.0 in light, the $\Delta p$ dropped to -178 mV whereas in the dark it was reduced to -129 mV. In these cells in light, $\Delta\Psi$ varied from -133 mV to -165 mV and in the dark from -126 mV to -150 mV when the external pH was varied from 6.0 to 7.5. During the external pH changes from 6.0 to 8.0, $\Delta$pH (inside alkaline) for untreated cells in light varied from 61 to 15 mV and in the dark from 40 to 19 mV.

In $K^+$-depleted cells the maximum values of $\Delta p$ (about -210 mV) and $\Delta\Psi$ (about -180 mV) was recorded at pH 7.0 both in light and in the dark (Figs. 67A,B). In these cells, illumination resulted in a higher $\Delta$pH (51 mV at pH 6.0) than those in the dark (37 mV at pH 7.0). The addition of 100 mM KCl in the dark resulted in a depolarization of $\Delta\psi$ by about 10-25 mV and in light by 15-35 mV with higher depolarization at pH $\geqslant$7.0. On the other hand, $\Delta$pH increased by 5-13 mV both in light and in the dark but this effect did not compensate for the depolarization of $\Delta\Psi$, hence $\Delta p$ was reduced by 10-20 mV only. When 100 mM NaCl was added to the $K^+$-depleted cells in the dark, $\Delta\Psi$ was depolarized by 12-33 mV at pH $\geqslant$7.0 (Figs. 68A,B). In light, a maximum depolarization of $\Delta\Psi$ (32 mV) by NaCl was observed at pH 7.0. Concomitant with depolarization of $\Delta\Psi$, there was an increase in $\Delta$pH of 5-9 mV in the dark and 8-11 mV in light. This increase in $\Delta$pH did not compensate for the depolarization of $\Delta\Psi$ and as a result the $\Delta p$ was lowered by 10-20 mV in the dark and 8-37 mV in light at pH $\geqslant$7.0.

FIG. 67: EFFECTS OF $K^+$ ON THE COMPONENTS OF $\Delta p$ IN $K^+$-DEPLETED CELLS.

After $K^+$-depletion by DEA-treatment (Section 2.2.17.1), the cells from photodenitrifying cultures (20h) were washed once in 50 mM sodium phosphate buffer (pH 7.0) and resuspended (1 to 1.5 mg dry wt./ml) in the same buffer at the various pH values indicated. $\Delta\psi$ and $\Delta pH$ values were then determined in these cells as described in Section 2.2.13.

△,▲ $\Delta p$;
○,● $\Delta\psi$;
□,■ 59 x $\Delta pH$.

Open symbols, without KCl; closed symbols, + 100 mM KCl.

A, Dark.
B, Light.

FIG.67A

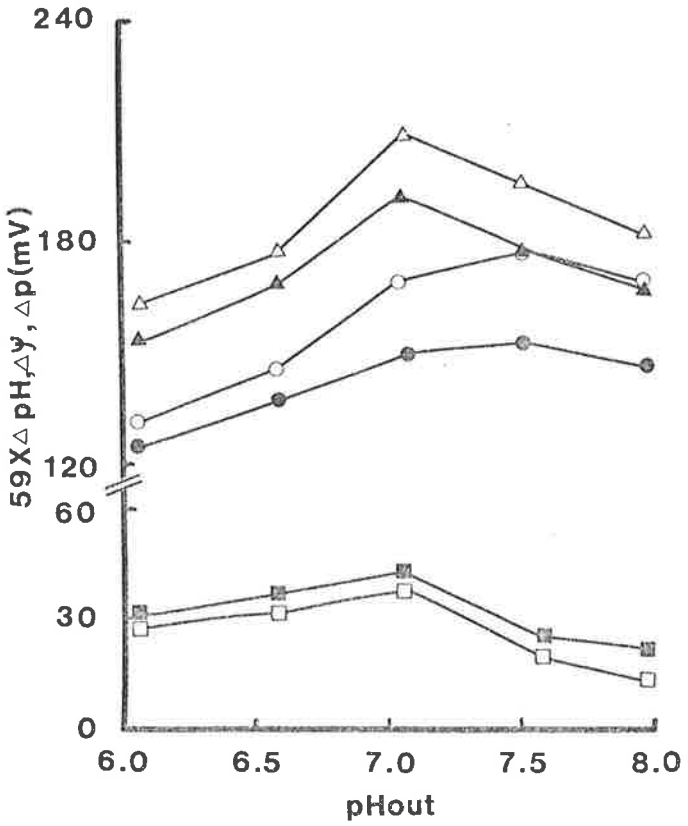



FIG.67B

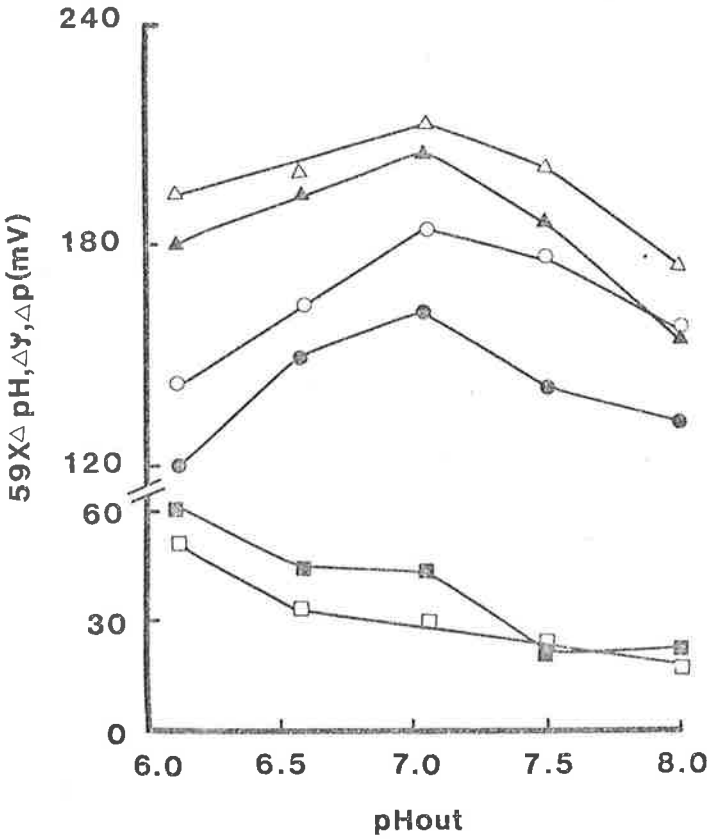


FIG. 68: EFFECTS OF $Na^+$ ON THE COMPONENTS OF $\Delta p$ IN $K^+$- DEPLETED CELLS.

After $K^+$-depletion by DEA-treatment (Section 2.2.17.1), the cells from photodenitrifying cultures (20h) were washed once in 50 mM potassium phosphate buffer (pH 7.0) and resuspended (1 to 1.5 mg dry wt./ml) in the same buffer at the various pH values indicated. $\Delta\psi$ and $\Delta pH$ values were then determined in these cells as described in Section 2.2.13.

△,▲ $\Delta p$;
○,● $\Delta\psi$;
□,■ 59 x $\Delta pH$;

Open symbols, without NaCl; closed symbols, + 100 mM NaCl.

A, Dark.

B, Light.

FIG.68A

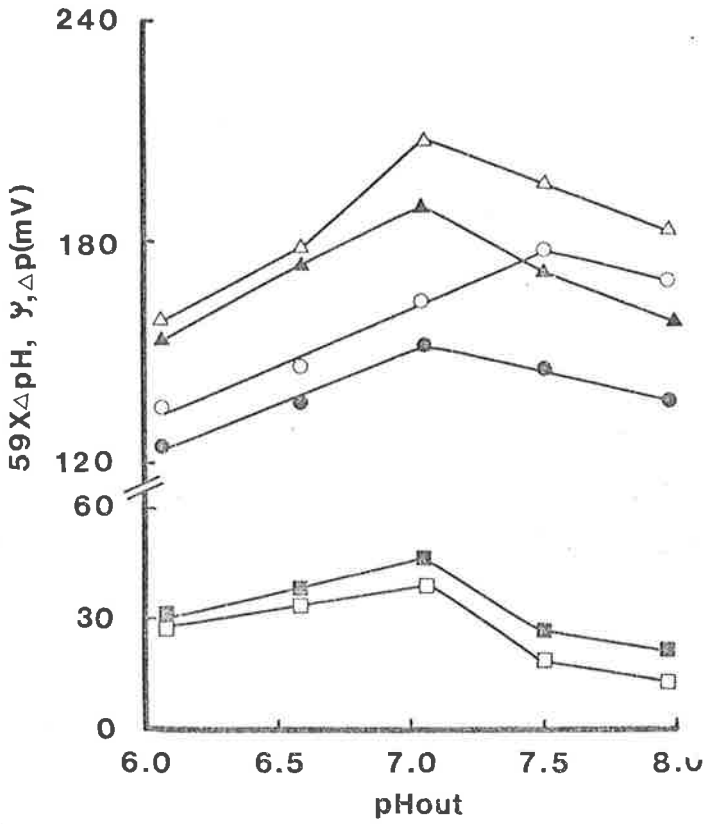
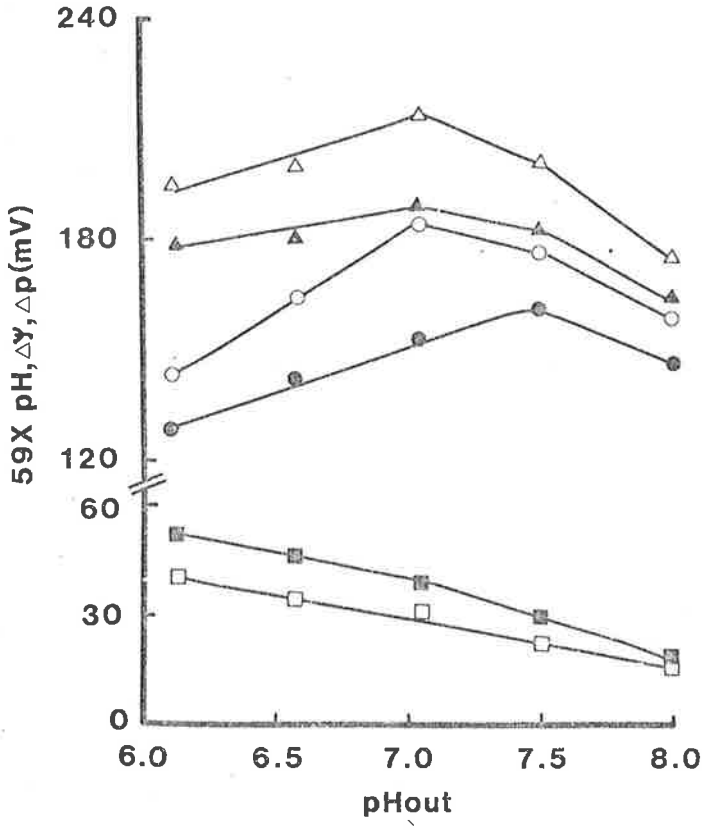



FIG.68B



# 4. DISCUSSION

# 4. DISCUSSION

## 4.1 COMPARISON OF DENITRIFICATION IN *R. SPHAEROIDES* F. SP. *DENITRIFICANS* AND IN OTHER BACTERIA

### 4.1.1 Denitrification in cultures and in washed cells

The purple non-sulphur bacterium *R. sphaeroides* f. sp. *denitrificans* can grow anaerobically with $NO_3^-$ as the terminal electron acceptor both in light and in the dark (Section 3.1.1). The photodenitrifying growth was much faster (maximum growth at about 24h) compared to the dark denitrifying growth (maximum growth at about 168h) indicating that the photosynthetic products were utilized by these cells as electron donors for $NO_3^-$ reduction. Denitrifying cultures of this bacterium, either in light or in the dark, accumulated $NO_2^-$ in the culture medium (Section 3.1.2). In dark denitrifying cultures, $NO_2^-$ accumulated in the medium until all the $NO_3^-$ had been utilized and then $NO_2^-$ was further reduced to $N_2O$ and $N_2$. This resembles the denitrifying growth of *Paracoccus denitrificans* (Whatley, 1981). In the photodenitrifying cultures, however, $NO_2^-$ was not detected in the medium until 6h and it remained at a low concentration until 12h after inoculation because of a high nitrite reductase activity (Section 3.1.3). After 30h growth, 10 mM $NO_3^-$ was utilized by the photodenitrifying cells producing 5 mM $NO_2^-$ and 0.8 mM $N_2O$ and 1.7 mM $N_2$. Only a few photosynthetic bacteria have hitherto been shown to denitrify $NO_3^-$ to $N_2O$ and $N_2$ (Satoh *et al.*, 1976; Klemme *et al.*, 1980). Most of the purple bacteria do not grow anaerobically in the dark with $NO_3^-$ as the terminal acceptor (Malofeeva *et al.*, 1974; Klemme, 1979).

Washed cells, from photodenitrifying cultures, effectively utilized succinate, lactate and formate as electron donors for the reduction of $NO_3^-$, $NO_2^-$ and $N_2O$ to $N_2$ (Section 3.2.1). These organic acids are known to

donate electrons to ubiquinone in *Pa. denitrificans* and *Escherichia coli*. Malate, which enters the electron transport chain at the NADH level in these two bacteria, also resulted in similar nitrite and nitrous oxide reductase activities in the photodenitrifier but nitrate reductase activity with this donor was comparatively low.

Washed cells of the photodenitrifier incubated with either $K^{15}NO_3$ or $K^{15}NO_2$ produced $^{15}N_2O$ and $^{15}N_2$ as well as $^{15}N_2O$ and $^{14}N_2$ but $^{15}NO$ and $^{14}NO$ were not detected (Section 3.2.2). After a 3h incubation all the $^{15}NO_3^-$ and $^{15}NO_2^-$ utilized were fully recovered in $^{15}N_2O$ and $^{15}N_2$. The stoichiometries for either $NO_3^-$ or $NO_2^-$ reduction to $N_2$ was 2:1 and that of $N_2O$ to $N_2$, 1:1. These results are in agreement with the data for *Pa. denitrificans* (Hollocher, 1982), *Pseudomonas aeruginosa* (St. John and Hollocher, 1977; Kim and Hollocher, 1983) and for *Rhizobium japonicum* (Bhandari and Nicholas, 1984). Results in Section 3.2.2 indicate that NO is not an obligatory intermediate during denitrification in this photodenitrifier. The following scheme of denitrification is proposed for this bacterium: $NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$.

### 4.1.2 Properties of nitrate and nitrite reductases

#### 4.1.2.1 Nitrate reductase

A 130-fold purified nitrate reductase from the photodenitrifier contained Mo which was confirmed by tungstate inhibition of the enzyme *in vivo* (Section 3.4.3.1). The inhibitory effects of bathophenanthroline and KCNS on the enzyme suggested that this enzyme requires metals for its activity (Section 3.2.3). These results are consistent with the fact that the dissimilatory nitrate reductases of other bacteria are also Mo-Fe proteins (Forget, 1971, 1974; Rosso *et al.*, 1973; Stouthamer, 1976). *p*-CMB at 0.5 mM inhibited the enzyme activity by 40% which was partially

reversed by adding cysteine indicating the presence of -SH groups. Although the purified enzyme did not contain any cytochrome *c* it was present in the partially purified enzyme (absorption peaks at 418,521 and 551 nm; Section 3.4.3.1). This indicates a likely role for the $c_{551}$-type cytochrome in $NO_3^-$ reduction. A functional association between a soluble cytochrome $c_{554}$ and a nitrate reductase has been shown in a halotolerant *Micrococcus* grown anaerobically in the presence of $NO_3^-$ (Horio, 1963). Fewson and Nicholas (1961) demonstrated a requirement for bacterial or mammalian cytochrome *c* for the activity of particulate nitrate reductase from *Ps. aeruginosa*. Sadana and McElroy (1957) found that a purified nitrate reductase from *Achromobacter fischeri* had a cytochrome *c* type spectrum. Molecular weight determinations for the purified nitrate reductase from the photodenitrifier were variable since the enzyme was present in a variety of aggregated forms e.g. 70 Kd, 100 Kd and 112 Kd. The apparent $K_m$ for $NO_3^-$ was 1.6 mM, which is similar to that of nitrate reductase from *E. coli* (Forget, 1974) and from *Micrococcus halodenitrificans* (Rosso *et al.*, 1973).

4.1.2.2 Nitrite reductase

The 168-fold purified nitrite reductase of the denitrifying phototroph had a molecular weight of 82 Kd with two subunits (40 Kd) (Section 3.4.3.2). These results are similar to those of Sawada *et al.*, (1978) for this enzyme. Gradient gel electrophoresis or two dimensional gel electrophoresis of the SDS-treated enzyme confirmed that there were two subunits present in the enzyme with isoelectric points at 5.2 and 5.0, (Michalski and Nicholas, 1985). The enzyme contained two copper atoms per mole, one in each subunit (Section 3.4.3.2). This enzyme resembles nitrite reductases from *Alcaligenes* sp. (Iwasaki *et al.*, 1963) and *A. cycloclastes* (Iwasaki and Matsubara, 1972; Iwasaki *et al.*, (1975) in that; (a) it is a soluble protein; (b) it has similar molecular weight

and (c) it contains two copper atoms per mole of enzyme. However, the reaction product of $^{15}NO_2^-$ catalyzed by this enzyme and identified by GC/MS was $^{15}N_2O$ (Section 3.4.3.2). Based on manometric studies Sawada *et al.*(1978) claimed that the product of nitrite reductase in this bacterium was NO, but subsequent work from that laboratory showed that NO is not an intermediate in denitrification (Urata *et al.*, 1983). Purified nitrite reductase did not utilize reduced forms of either baker's yeast lactate dehydrogenase (cytochrome $b_2$), cytochrome $c_2$ (prepared from this bacterium) or horse heart cytochrome *c* as electron donors. Inhibition of this enzyme activity in washed cells by HOQNO indicated the involvement of cytochromes *b* and *c* as electron carriers (Section 3.2.3). Cytochromes are known to participate in the electron flow from substrate to the nitrogenous acceptor in denitrification (Payne, 1973). A Cu containing nitrite reductase from *Alcaligenes* sp. utilized reduced cytochrome $c_{553}$ (or $c_{552}$) from the same bacterium as an electron donor (Miyata and Mori, 1969). It is also known that nitrite reductase is associated with a *cd* type cytochrome in *Pa. denitrificans* (Lam and Nicholas, 1969c), *Ps. aeruginosa* (Shimada and Orii, 1975), *Ps. perfectomarinus* (Zumft and Vega, 1979) and in *T. denitrificans* (Sawhney and Nicholas, 1978). The cytochrome *cd*-type nitrite reductase from *Ps. aeruginosa* oxidized reduced cytochrome $c_{551}$ from the same bacterium (Yamanaka and Okunuki, 1963).

## 4.2 EFFECTS OF $O_2$ ON THE PRODUCTION AND ACTIVITIES OF DENITRIFYING ENZYMES

When washed cells, grown aerobically either in light or in the dark (without nitrate) were incubated anaerobically (again without $NO_3^-$), small activities of nitrate and nitrite reductases were recorded after a lag period of about 30 min (Sections 3.3.1 and 3.3.3). However, the producton

of these enzymes were greatly enhanced in the presence of $NO_3^-$ (Section 3.3.1). If $O_2$ was present during this incubation ($\geqslant$1% v/v) these enzymes were not produced (Section 3.3.4). Similar results have been obtained for denitrifying enzymes in other microorganisms (Stouthamer, 1976; Payne, 1981; O'Hara *et al.*, 1983; Kapralek *et al.*, 1982). Chloramphenicol and rifampicin (inhibitors of protein synthesis and messenger RNA respectively) also inhibited the production of nitrate and nitrite reductases in washed cells from aerobically grown cultures (without $NO_3^-$) of the photodenitrifier. Thus, when chloramphenicol was added to these washed cells incubated anaerobically with $NO_3^-$, the production of nitrate and nitrite reductases ceased immediately, whereas when rifampicin was supplied under similar conditions, the synthesis of these enzymes continued for 15 min at a slow rate and then it ceased (Section 3.3.3). Under these conditions, the effect of $O_2$ resembled that of chloramphenicol indicating that $O_2$ inhibited the *de novo* synthesis of the denitrifying enzymes. These results are in agreement with those of Kapralek *et al.* (1982) for *E. coli*.

In washed cells from photodenitrifying cultures, 10% (v/v) $O_2$ inhibited the activity of the preformed denitrifying enzymes, *viz.* nitrate, nitrite and nitrous oxide reductases by about a half (Section 3.3.4). The effects of $O_2$ on the preformed enzymes are known to vary depending on the microorganism; thus in *Rhizobium japonicum*, there was a rapid loss of denitrifying activity at $\geqslant$10% (v/v) $O_2$ (O'Hara *et al.*, 1983) whereas in *Flavobacterium* sp. 10% (v/v) $O_2$ did not inhibit the reduction of $NO_3^-$ to $N_2O$ and $N_2$ (Betlach and Tiedje, 1981). However, it has been reported that derepression of dissimilatory nitrate reductase in *Ps. aeruginosa* occurred at 5% (v/v) $O_2$ (Sacks and Barker, 1949) and that the dissimilatory nitrite reductase in *Thiobacillus denitrificans* was partially derepressed at 7.8% (Justin and Kelly, 1978).

## 4.3 ENERGY RELATIONS DURING DENITRIFICATION

### 4.3.1 Proton translocation

In *R. sphaeroides* f. sp. *denitrificans* the electron transport and proton translocation linked to the reduction of nitrogenous substrates *viz.* $NO_3^-$, $NO_2^-$ or $N_2O$ are more complex than in non-photosynthetic denitrifying bacteria since both photosynthetic and respiratory systems are involved in the photosynthetic types. The $\rightarrow H^+/2e^-$ ratios determined by oxidant pulse experiments in washed cells in light resulted from a joint contribution from the respiratory and photosynthetic proton translocation (Sections 3.5.1.1 and 3.5.1.2). In the dark, however, respiration dependent proton translocation only was recorded (Sections 3.5.1.1 and 3.5.1.2). In washed cells, grown under photodenitrifying conditions with endogenous substrates appreciable increases in $\rightarrow H^+$/oxidant ratios were observed when $NO_3^-$ and $NO_2^-$ were reduced to $N_2$, and $O_2$ reduced to $H_2O$ compared to those in the dark (Section 3.5.1.1). However, $\rightarrow H^+/N_2O$ values were similar in light and in the dark. This is probably associated with less electrons required for the reduction of $N_2O$ to $N_2$ than for either $NO_3^-$ or $NO_2^-$ to $N_2$.

An alkaline transient was observed on adding $NO_2^-$ to washed cells of the photodenitrifier in the dark (Section 3.5.1.1) as was also recorded for *Pa. denitrificans* (Kristjansson *et al.*, 1978). When exposed to light or in the presence of the $K^+$ transporter, valinomycin, the initial alkaline phase was not observed in the photodenitrifier. This alkaline transient may be attributed to the periplasmic consumption of $H^+$ during $NO_2^-$ reduction. Exposing washed cells to either light or valinomycin enhanced the initial rate of $H^+$ extrusion in response to $NO_2^-$ reduction by eliminating the membrane potential.

In the presence of valinomycin as a permeant ion, the $^{\rightarrow}H^+$/oxidant ratios were also higher in light than those in the dark (Section 3.5.1.2). The following corrected $^{\rightarrow}H^+/2e^-$ data were obtained for illuminated cells: 4.82 ($NO_3^- \rightarrow 0.5\ N_2$); 5.19 ($NO_3^- \rightarrow 0.5\ N_2O$); 4.56 ($NO_3^- \rightarrow NO_2^-$); 5.43 ($NO_2^- \rightarrow 0.5\ N_2$); 5.95 ($NO_2^- \rightarrow 0.5\ N_2O$); 6.2 ($N_2O \rightarrow N_2$) and 6.43 ($O_2 \rightarrow H_2O$); these values agree with the theoretical values. The $^{\rightarrow}H^+/2e^-$ ratios for the reduction of $NO_3^- \rightarrow 0.5\ N_2$; $NO_2^- \rightarrow 0.5\ N_2$ and $N_2O$ to $N_2$ in the dark were 3.99, 4.10 and 4.17 respectively, which are similar to the comparable values of 4.3, 3.7 and 4.5 respectively, for *Pa. denitrificans* (Kristjansson *et al.*, 1978). The $^{\rightarrow}H^+/2e^-$ ($NO_2^- \rightarrow 0.5\ N_2$) ratio of 4.1 compares favourably with a value of 4.5 in *Pa. denitrificans* but not with the higher ratio of 6.53 reported by Boogerd *et al.* (1981). The ratios of $^{\rightarrow}H^+/2e^-$ for $NO_3^- \rightarrow NO_2^-$, 3.90; and $NO_2^- \rightarrow 0.5\ N_2O$, 4.88; in the dark, agree with those given by Urata *et al.* (1983), namely 4.05 and 4.95 in this photodenitrifier. Based on these stoichiometric $^{\rightarrow}H^+/2e^-$ values obtained in illuminated and non-illuminated cells, it is clear that in the phototrophic denitrifier, light plays a direct role in the denitrification process by reducing membrane potential and generating a $H^+$ gradient across the bacterial membranes. This would explain the stimulation of denitrification of $NO_3^-$ and $NO_2^-$ by light in this bacterium (Satoh, 1977)

The observed alkalinization of the external aqueous phase in response to the addition of either $NO_3^-$, $NO_2^-$ or $N_2O$ to washed cells in the presence of BVH, NEM and HOQNO (Section 3.5.1.3) with or without valinomycin in the dark indicates the net proton consumption during the reduction of these substrates. NEM and HOQNO inhibited the extrusion of protons from endogenous substrates. The observed ratios $^{\rightarrow}H^+/NO_3^-$ ($\rightarrow 0.5\ N_2$) -6.04 and -6.12, $^{\rightarrow}H^+/NO_2^-$ ($\rightarrow 0.5\ N_2$) -3.99 and -3.46, $^{\rightarrow}H^+/N_2O$ ($\rightarrow N_2$) -2.02 and -1.87 with and without valinomycin, respectively, are in

good agreement with the calculated values. The $^{\rightarrow}H^+/2e^-$ values for $NO_3^-$ $\rightarrow$ $NO_2^-$, -1.93; $NO_2^-$ $\rightarrow$ 0.5 $N_2O$, -2.87; and $N_2O$ $\rightarrow$ $N_2$, -1.87 with valinomycin, are similar to -1.95, -3.03 and -2.02 without the compound. The $^{\rightarrow}H^+$/ oxidant ratios obtained for the reductions of $NO_3^-$ $\rightarrow$ $N_2$ (-6.0), $NO_2^-$ $\rightarrow$ $N_2$ (-5.0), $NO_2^-$ $\rightarrow$ $N_2O$ (-3.0) and $N_2O$ $\rightarrow$ $N_2$ (-2.0) with BVH as the electron donor indicate that these reductions occur in the periplasmic region. The $^{\rightarrow}H^+$/ oxidant ratios (2-6) for these reactions with endogenous substrates supports this proposition because if protons were consumed from the cytoplasm the expected ratios would be higher (6-11). Studies with the subcellular distribution of denitrifying enzymes also confirmed the periplasmic location of these enzymes (Section 3.4.1).

Most of the dissimilatory nitrate reductases are associated with cell membranes and it is likely that they are located on the cytoplasmic side of the membrane in *Pa. denitrificans* (John, 1977; Kristjansson *et al.*, 1978; Kristjansson and Hollocher, 1979; Jones *et al.*, 1980). However, Garland *et al.* (1975) found that $NO_3^-$ was reduced on the outer side of the cytoplasmic membrane in *E. coli*. The fact that the nitrate reductase of the photodenitrifier appeared in the pellet after ultracentrifugation at 240,000 x g does not necessarily indicate a membrane location for the enzyme (Section 3.4.2.1) because after lysozyme-EDTA treatment it appeared in the periplasmic fraction (Section 3.4.1). However, it is possible that this enzyme is attached to the outer side of the cytoplasmic membrane and this attachment is released by lysozyme treatment, so that $NO_3^-$ reduction is located in the periplasmic space. A periplasmic location of nitrite reductase has also been proposed in *Pa. denitrificans* based on proton consumption stoichiometries associated with nitrite reduction by ascorbate (Meijer *et al.*, 1979; Alfounder and Ferguson, 1980; Boogerd *et al.*, 1981). Kristjansson and Hollocher

(1980) considered that $N_2O$ reductase in *Pa. denitrificans* is a soluble cytoplasmic enzyme whereas Boogerd *et al.* (1981) proposed a periplasmic orientation for the enzyme in this bacterium.

4.3.2 The proton motive force

The washed cells of *R. sphaeroides* f. sp. *denitrificans*, with or without added substrate in light, developed a 20-30 mV (shared by $\Delta pH$ and $\Delta\Psi$) higher $\Delta p$ compared to those in the dark (Sections 3.5.2.4 and 3.5.2.5). This increase in $\Delta p$ during illumination can be accounted for by higher rates of reduction of the nitrogenous oxides. Thus the rates of reduction in nmol/10 min/mg protein were as follows: $NO_3^-$, 297; $NO_2^-$, 345; $N_2O$, 267; in light compared with 220, 300 and 195 nmol respectively, in the dark. The increased rates for the denitrifying enzymes in light is in accord with the $^{\rightarrow}H^+$/oxidant ratios for $NO_3^-$, $NO_2^-$ and $N_2O$ which are also higher in light (Sections 3.5.1.1 and 3.5.1.2).

Cells grown under photodenitrifying conditions accumulated $NO_2^-$ in the culture medium (Section 3.1.2). The fact that the membrane potential was maximal over the exponential phase of growth (Section 3.5.2.5) can be explained in two ways: (a) $NO_2^-$ in the culture medium would produce $HNO_2$, which could act as a protonophoric uncoupler (McCarthy *et al.*, 1981 and (b) the rate of proton pumping is low during the lag and stationary phase of growth, so that maximal $\Delta\Psi$ cannot be maintained throughout the growth period.

The data reported herein indicate that both $\Delta\Psi$ and $\Delta pH$ were dependent on the external pH since $\Delta\Psi$ decreased at $pH_{out}$ >7.0 in the dark (>7.5 in light) and the intracellular pH became acidic compared to the external one at $pH_{out}$ >8.0 (Sections 3.5.2.2, 3.5.2.3 and 3.5.2.4). The decrease in $\Delta pH$ with increasing external pH suggests

that these cells have a limited buffering capacity to maintain a constant internal pH. These results are in agreement with those reported for *Clostridium pasteurianum* (Riebeling *et al.*, 1975), *Halobacterium halobium* (Bakker *et al.*, 1976), *E. coli* (Padan *et al.*, 1976; Ramos and Kaback, 1977a,b; Kashket, 1981a), *Salmonella typhimurium* (Tokuda and Kaback, 1977), *Bacillus acidocaldarius* (Krulwich *et al.*, 1978), *Micrococcus lysodeikticus* (Friedberg and Kaback, 1980), *Methanospirillum hungatei* (Jarrel and Sprott, 1981) and *Nitrosomonas europaea* (Kumar and Nicholas, 1983). The fact that the $\Delta\Psi$ was markedly reduced at pH >7.5 in the dark and at pH >7.0 in light in the presence of N-oxides (Section 3.5.2.3) can be attributed to two factors: (a) $\Delta\Psi$ decreased markedly at alkaline pH even in the absence of added substrate (in light and in the dark) and (b) the rates for the reduction of substrates, e.g. $NO_3^-$, $NO_2^-$, $N_2O$ and $O_2$ also declined rapidly at pH values >7.5. Thus, at pH 8.0, $O_2$ respiration decreased by 40% in the dark and both nitrate and nitrite respiration by a half in light and in the dark.

In the photodenitrifier, endogenous respiration is coupled to proton translocation (Sections 3.5.1.1 and 3.5.1.2) and therefore it is likely that this enables the cells to maintain a high $\Delta p$ in the absence of added substrate or when the exogenous respiration is inhibited. This is supported by the experimental observations that the addition of either 1 mM KCN or 0.1 mM rotenone to cells without added substrate did not reduce the $\Delta\Psi$ values further (Section 3.5.2.6). The extrusion of protons from the respiratory chain produces $\Delta p$ which promotes the influx of protons

back into the cell *via* the membrane bound ATPase (Harold, 1972). However, the equilibrium of the ATPase reaction is such that it shifts towards ATP production as a result of the formation of $\Delta p$. When the $\Delta p$ is decreased, the equilibrium shifts and the ATPase degrades ATP because of a net outflow of protons. The uncoupler CCCP (10 µM) did not completely collapse $\Delta\Psi$, in agreement with results obtained with washed cells of *Nitrosomonas europaea* and *Nitrobacter agilis* (Kumar and Nicholas, 1983). The increase in $\Delta\Psi$ recorded in cells supplied with $NO_3^-$ (compared with those without $NO_3^-$) was abolished by the addition of either 10 µM CCCP or 1 mM DNP (Section 3.5.2.6). Similar results were obtained with $NO_2^-$, $N_2O$ and $O_2$. The effects of these uncouplers on the inhibition of light-induced $\Delta pH$ and $\Delta\Psi$ was enhanced by 100 µM antimycin A. It is of interest that in *Pseudomonas aeruginosa* and *Pseudomonas denitrificans* the effects of uncouplers on respiration were not linked to a collapse of $\Delta p$ but were attributed to their detergent-like action on cell membranes (Walter *et al.*, 1978).

Antimycin A and HOQNO, known to inhibit electron transport between cytochromes *b* and *c*, affected $\Delta pH$ and $\Delta\Psi$ to a greater degree in light than in the dark (Section 3.5.2.6). These observations indicate that these two inhibitors also affect light-dependent electron transport. A similar observation was made by Cotton *et al.* (1983) in *R. capsulata* and they concluded that antimycin A is a very weak inhibitor of respiration but a strong inhibitor of the photosynthetic electron transport chain.

Dicyclohexylcarbodiimide (DCCD), an ATPase inhibitor, restricted the reduction of either $NO_3^-$, $NO_2^-$ or $N_2O$ in washed cells of the phototrophic denitrifier by about a half in light and by 30% in the dark. The generation of $\Delta pH$ by $NO_3^-$, $NO_2^-$ or $N_2O$ was also inhibited by DCCD during dark respiration as well as photodenitrification (Section 3.5.2.6). In cells with added

nitrogenous substrate ($NO_3^-$, $NO_2^-$ or $N_2O$) in the presence of DCCD, the increase in $\Delta\Psi$ was either only partially affected or remained unaltered. Such a phenomenon was also observed in *N. agilis* (Kumar and Nicholas, 1983). DCCD-treated cells of *R. sphaeroides* were used to investigate the effects of $\Delta\Psi$ during alanine transport (Elferink *et al.*, 1983). Again, with another ATPase inhibitor, DESB, Kumar and Nicholas (1983) observed a stimulation of $\Delta\Psi$ in *N. europaea* and they suggested that in washed cells these inhibitors affect metabolic functions other than ATPase. Since lower concentrations of these inhibitors were without effect, higher levels have been used in biochemical and bioenergetic studies in this bacterium (Satoh, 1977) as well as in other bacteria (Kumar and Nicholas, 1983).

Dichlorophenyl-dimethyl urea (DCMU), known to inhibit electron transport in light between quinone and plastoquinone in chloroplasts (Hauska and Trebst, 1977) did not show any appreciable effect on the light-induced changes of $\Delta pH$ or $\Delta\Psi$ in response to any of the substrates ($NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$). The electron transfer between plastoquinone and cytochrome *f* in chloroplasts is inhibited by DBMIB (Hauska and Trebst, 1977). DBMIB appreciably reduced the $\Delta pH$ and $\Delta\Psi$ in light in the photodenitrifier irrespective of substrates added. This quinone antagonist (inhibits quinone oxidation in chloroplasts, Bohme *et al.*, 1971) was also found to be effective in the chromatophores of *Rhodospirillum rubrum* (Stouthamer, 1978) and its site of inhibition appeared to be similar to that of antimycin A. *R. sphaeroides* f. sp. *denitrificans* generated a $\Delta p$ of about -175 to -200 mV during dark denitrification and -200 to -245 mV in the light. The development of $\Delta p$ in response to the reduction of $NO_3^-$, $NO_2^-$ and $N_2O$ as well as $O_2$ indicate a mechanism whereby this bacterium can effectively produce ATP by denitrification as it does during $O_2$ respiration. The values of $\Delta\Psi$ for the photodenitrifier of about -150 mV for $NO_3^-$ and $N_2O$ and -140 mV for $NO_2^-$

in the dark are in good agreement with those obtained for *Pa. denitrificans* (McCarthy *et al.*, 1981). The light-induced $\Delta\Psi$ in response to the addition of $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ ranged between -152 to -167 mV. These values of $\Delta\Psi$ are similar to the light induced $\Delta\Psi$ of -160 mV for $O_2$ in *R. capsulata* (Clark and Jackson, 1981). Thus the activity of the proton pump of *R. sphaeroides* f. sp. *denitrificans* is similar to that in other photosynthetic bacteria, namely, *R. capsulata* (Clark and Jackson, 1981) and *R. sphaeroides* (Ferguson *et al.*, 1979).

4.3.3 Proline transport

Washed cells of *R. sphaeroides* f. sp. *denitrificans* prepared from cultures grown anaerobically under photodenitrifying conditions accumulated [$^{14}$C]-proline at a low rate under anaerobic-dark conditions (Section 3.5.3.1). This effect was stimulated by a half on exposure to light and a four-fold increase was observed on adding $NO_3^-$. A similar stimulation of alanine uptake by light was demonstrated by Hellingwerf *et al.* (1975) and Elferink *et al.* (1983) in *R. sphaeroides* during $O_2$ respiration. It is shown in this thesis that this photodenitrifier can effectively incorporate proline when either $NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$ is the terminal acceptor in light and in the dark (Section 3.5.3.4). This implies that the active transport can be energized by either photosynthetic or respiratory electron transfer to various oxides of nitrogen ($NO_3^-$, $NO_2^-$ and $N_2O$) or $O_2$. In denitrifying cells of *Pa. denitrificans*, NO supported the active transport of proline but failed to do so with aerobically grown cells (Garber *et al.*, 1982). In light, addition of $NO_3^-$ to washed cells of the photodenitrifier decreased the $K_m$ for proline from 40 µM to 5.7 µM (Section 3.5.3.3) indicating that proline uptake is linked to denitrification of $NO_3^-$. Irrespective of the substrate used ($NO_3^-$, $NO_2^-$, $N_2O$ or $O_2$) the rates of proline transport into washed cells were higher in light

than in the dark. This effect is related to either the formation of Δp on exposure to light as in *R. sphaeroides* (Elferink *et al.*, 1983) or higher rates of reduction of these substrates in light (Section 3.5.2.5).

Proline uptake in washed cells in the presence of $NO_3^-$ increased as the external pH varied from 6.0 to 8.0 both in light and in the dark (Section 3.5.3.2). This increase in proline uptake was higher at lower pH values (pH 6.0 to 7.0) than at pH >7.0 because Δp was similarly affected under these conditions (Section 3.5.3.6). Uptake of this amino acid by washed cells in the presence of $NO_3^-$ was markedly inhibited by KCN, antimycin A and HOQNO (Sections 3.5.3.5 and 3.5.3.8). This uptake was more severely restricted by antimycin A and HOQNO in light (about 70%) than in the dark (about 35%). DBMIB, an inhibitor of photosynthetic electron transport in chloroplasts (Hauska and Trebst, 1977) restricted proline transport in washed cells of the photodenitrifier by about 25%. Inhibition of proline transport, in this bacterium, by CCCP and DNP in the dark (linked to denitrification) is similar to that reported for *Mycobacterium phlei* (Hirata *et al.*, 1974) and *Coxiella burnetii* (Hendrix and Mallavia, 1984). Inhibition was also obtained with these uncoupling agents in light indicating that the active transport of proline is also coupled to photosynthesis in this photodenitrifier. The fact that DCCD did not inhibit proline transport indicates that this process was unlikely to be dependent on ATPase.

The stoichiometry of $H^+$/proline varied between 1 and 2.5 depending on the external pH (6.0 - 8.0). These results support the view that the stoichiometry between protons and substrate during respiration-linked active transport increased as a function of pH (Ramos and Kaback, 1977a,b). In the photodenitrifier, Δμpro increased and Δp was reduced as a result of diminution in ΔpH and $\Delta\Psi$ at pH>7.0 (Section 3.5.3.6) resulting in an

increase in $H^+$/proline ratio. Thus, at pH 6.0, $H^+$/proline stoichiometry was 1:1 (in light and in the dark) and at pH 8.0, 1:2 (in the dark) and 1:2.5 (in light). The symport of a completely coupled system of a neutral substrate e.g. proline at steady-state may be expressed by the equation: $\Delta\mu pro + n\Delta p = 0$ (Rottenberg, 1976), where $\Delta\mu pro$ is the electrochemical potential of proline and n is the stoichiometry of the reaction (number of protons translocated in symport with proline). From this equation it is clear that any change in the ratio $\Delta\mu pro/\Delta p$ would result in a change in n ($H^+$/proline ratio).

Cells grown under photodenitrifying conditions, washed in a $Na^+$ free buffer e.g. Tris-HCl (pH 7.5), accumulated proline in light at a low rate (Section 3.5.3.7). Addition of 10 mM $KNO_3$ to these cells increased the proline uptake by about 5-fold. When 20 mM NaCl was added there was a 6-fold increase in the uptake. The addition of $KNO_3$ in the presence of NaCl increased proline accumulation about 8-fold indicating that the uptake was driven simultaneously by the proton motive force developed during $NO_3^-$ respiration and the $Na^+$ gradient. The stimulation of proline uptake by $Na^+$, observed in the photodenitrifier (Section 3.5.3.7) is similar to those for α-aminoisobutyric acid (AIB) and alanine uptake in cells of *C. vinosum* in light (Pettitt *et al.*, 1982) and proline uptake in *E. coli* (Stewart and Booth, 1983). This stimulation of proline uptake by $Na^+$ indicates that a proline-$Na^+$ symport is operating in the photodenitrifier. In the presence of various concentrations of $Na^+$ (5 to 100 mM), addition of 10 mM $KNO_3$ did not appreciably increase proline uptake. Thus, under these conditions the uptake of proline was mainly driven by the $Na^+$ gradient. Further evidence in favour of this view is that $KNO_3$ (10 mM) had no effect on the $K_m$ or $V_{max}$ values for $Na^+$-dependent proline uptake by these cells in light. The $K_m$ value for proline in the

absence of both $Na^+$ and $NO_3^-$ was 50 μM whereas in the presence of 20 mM $Na^+$ it decreased to 6.7 μM. Similarly $Na^+$ increased the $V_{max}$ for proline uptake by about 5-fold. These effects may be explained in terms of the ternary complex model of Lyon and Crane (1966) where the sodium symport transport systems appear to behave as ternary complexes (between carrier protein, $Na^+$, and metabolite) in which binding of one substrate increases the affinity of the carrier protein for the other substrate. This concept has also been applied to the $Na^+$-dependent α-aminoisobutyrate transport in *C. vinosum* (Pettitt, *et al.*, 1982).

Addition of either $K^+$ or $NO_3^-$ to $Na^+$-loaded cells of the denitrifying phototroph increased the proline uptake and these effects were additive (Section 3.5.3.7). This stimulation of proline uptake by $K^+$ can be explained by the functioning of a $Na^+/K^+$ exchange activity restoring the $Na^+$ gradient necessary for high rates of proline uptake. Such a stimulation of proline uptake by $K^+$ was also observed in *E. coli* (Stewart and Booth, 1983). There are also reports that bacterial cells can extrude $Na^+$ from the cytoplasm concomitant with $K^+$ accumulation (Thompson and MacLeod, 1973; Harold and Altendorf, 1974).

Results in Section 3.5.3.8 show that proline uptake in the photo-denitrifier is energy-dependent since the uncoupler, CCCP, inhibited the uptake by about 80%. In addition, HOQNO, an inhibitor of cyclic electron flow in photosynthetic bacteria (Knaff and Buchanan, 1975; Van Grondelle *et al.*, 1977; Knaff, 1978; Knaff *et al.*, 1979), also restricted light-dependent proline uptake by about 70%. These results indicate that Δp generated by light-driven cyclic electron flow is involved in providing the energy for proline uptake. Addition of nigericin to washed cells in light produced a 60% stimulation (as for $Na^+$) in the initial rate of proline uptake whereas valinomycin (which decreases ΔΨ but increases ΔpH) resulted

in a substantial inhibition (65%). Since, nigericin increased $\Delta\Psi$ while eliminating $\Delta pH$, these results point to the presence of an electrogenic $H^+$/proline symport. Thus, proline transport in the photodenitrifier can be effectively operated either jointly or separately by the $\Delta p$ produced during denitrification and by the $Na^+$ gradient. However, the results suggest that under most conditions $\Delta p$ rather than $Na^+$ gradient produces most of the driving force for proline uptake *via* an electrogenic $H^+$/proline or $Na^+$/proline symport.

4.3.4 $Na^+/H^+$ and $K^+/H^+$ exchange

The $\Delta pH$-dependent $Na^+$ uptake in washed cells of *R. sphaeroides* f. sp. *denitrificans* has been described in Section 3.5.4.1. In the absence of a pH gradient ($pH_{in} = pH_{out} = 6.0$ or $pH_{in} = pH_{out} = 8.0$), only 20-25% $Na^+$ entered the cells compared with that recorded in the presence of a pH gradient (acidic inside). This small uptake of $Na^+$ in the absence of a pH gradient is probably associated with passive diffusion. On the other hand, when the pH gradient was reversed (inside alkaline), $Na^+$ movement into the cells was about a half of that observed in the absence of a pH gradient. This decrease in $Na^+$ uptake may be explained by the operation of a $H^+$ gradient driving passively accumulated $Na^+$ out of the cells. These results indicate that the $Na^+/H^+$ exchange system is reversible. A similar type of $\Delta pH$-dependent $K^+$ uptake has also been observed in this bacterium (Section 3.5.5.1). In both cases either $Na^+$ or $K^+$ which accumulated *via* a pH gradient (inside acidic) was rapidly released from the cells into the medium on adding Gramicidin. This compound translocates ions by forming conducting channels in membranes (Hladky and Haydon, 1970, 1972; Urry, 1971, 1972). A similar $\Delta pH$-dependent $Na^+$ uptake has been demonstrated in purified canine cardiac sarcolemmal vesicles (Seiler *et al.*, 1985).

Washed cells of the photodenitrifier ejected protons upon adding either $Na^+$ or $K^+$, aerobically or anaerobically, in light and in the dark, resulting in an acidification of the cell suspension (Sections 3.5.4.2 and 3.5.5.2). This acidification was either partially or totally inhibited by CCCP indicating that $Na^+/H^+$ and $K^+/H^+$ antiport systems are energy dependent. Similar energy dependent $Na^+/H^+$ exchange activities have been described in *Desulfovibrio vulgaris* (Varma *et al.*, 1983) and in *Methanobacterium thermoautotrophicum* (Schonheit and Beimborn, 1985). $K^+$ uptake in *C. vinosum* was dependent on ATP (Davidson and Knaff, 1982) and ATP synthesis was driven by a $K^+$ diffusion potential in *M. thermoautotrophicum* (Schonheit and Perski, 1983). A light driven, uncoupler-sensitive $Na^+$ uptake has also been observed in chromatophores of *R. sphaeroides*, *R. rubrum* and *C. vinosum* (Hellingwerf *et al.*, 1975; Knaff *et al.*, 1981).

The amount of protons extruded, in response to $Na^+$ pulses, was related to the reaction conditions and to the size of the pulse (Section 3.5.4.2). In general, the $H^+$ extrusion was about 45% higher in the dark than in light under both aerobic and anaerobic conditions. This apparent inhibition of $H^+$ extrusion in the photodenitrifier in light in response to $Na^+$ pulses can be attributed to the fact that photosynthetic bacteria e.g., *C. vinosum* and *R. rubrum* eject $Na^+$ in light (Knaff *et al.*, 1981). Proton extrusion in the phototrophic denitrifier in response to $Na^+$ pulses was about 30% greater in the presence of $O_2$ than under anaerobic conditions either in light or in the dark (Section 3.5.4.2). The inhibitory effect of light on the rate of $O_2$ respiration of purple bacteria is well known. The extent of this inhibition by light, however, are highly variable and depend on many parameters (Van Neil, 1941; Keister, 1978). Thus, the apparent stimulation of $H^+$ extrusion under aerobic conditions is probably

associated with $O_2$ respiration in the dark and an incomplete inhibition of $O_2$ respiration in light.

Maximum acidification, in response to $K^+$ pulses, was produced by the photodenitrifier in light in the absence of air followed by dark anaerobic, dark aerobic and finally light aerobic conditions (Section 3.5.5.2). This result concurs with the observation that washed cells of *R. rubrum* accumulated and maintained a high level of $K^+$ anaerobically in light, whereas $K^+$ was lost under dark anaerobic conditions (Stenn, 1968). Since, under these conditions non-cyclic phosphorylation and anaerobic and oxidative mechanisms are minimal, it is likely that cyclic phosphorylation provided energy for $K^+$ uptake in the photodenitrifier. Washed cells in air produced about 30% less acidification in light than in the dark (Section 3.5.5.2). This effect is probably associated with the light inhibition (70% in the photodenitrifier) of $O_2$ respiration in purple bacteria (Keister, 1978; Cotton *et al.*, 1983).

Addition of valinomycin to the cells of the photodenitrifier, washed in a $K^+$-free buffer, e.g. 50 mM Tris-HCl (pH 7.5), and resuspended in 5 mM Tris-HCl (pH 6.5) containing 1 mM DTT, resulted in a $H^+$ influx as indicated by the alkalinization of the suspension medium. On the other hand, these cells acidified the external medium in response to $K^+$ pulses. These results indicate that the uptake of $K^+$ resulted in an exchange with internal $H^+$ *via* a $K^+/H^+$ antiporter, thus, preventing the acidification of the cell contents. A role of $K^+/H^+$ antiporter in intracellular pH regulation has been described in *C. vinosum* (Davidson and Knaff, 1982), *E. coli* (Brey *et al.*, 1978, 1980) and in *Bacillus acidophilus* (Mandel *et al.*, 1980).

Studies involving the effects of $Na^+$ and $K^+$ on the components of $\Delta p$ have been carried out in washed cells of the photodenitrifier to establish

the electrogenicity of the $Na^+/H^+$ and $K^+/H^+$ antiporter (Sections 3.5.4.3 and 3.5.5.3). When either NaCl or KCl (200 mM) was added to the cell suspensions in light and in the dark, with or without $NO_3^-$, the $\Delta\Psi$ was depolarized by 20-60 mV with a concomitant increase in $\Delta$pH of 20-40 mV indicating that these antiporters are electrogenic. A similar depolarization (60 mV) of $\Delta\Psi$ by $K^+$ occurred in *Streptococcus faecalis* (Bakker and Mangerich, 1981) but the extent of depolarization of $\Delta\Psi$ by $K^+$ was much less in *E. coli* (Bakker and Mangerich, 1981), *N. europaea* and in *N. agilis* (Kumar and Nicholas, 1984). An electrogenic $K^+$ uptake which is not dependent on either $\Delta$pH or ATP has been observed in *R. sphaeroides*. $Na^+$ was transported in this bacterium *via* an electroneutral exchange system against protons, i.e. it was dependent on $\Delta$pH (Hellingwerf *et al.*, 1982). In the photodenitrifier, the presence of such an electroneutral $Na^+$ transport is highly unlikely because increase in $\Delta$pH was always associated with the depolarization of $\Delta\Psi$.

### 4.3.5 $Na^+$ and $K^+$ transport

Analysis of the $K^+$ and $Na^+$ contents of washed cells of *R. sphaeroides* f. sp. *denitrificans* indicate that cellular $K^+$ (115 to 174 mM) was appreciably higher than $Na^+$ (32 to 60 mM). It is well established that bacterial cells accumulate high concentrations of $K^+$ and extrude $Na^+$ (Schultz and Solomon, 1961; Rhoads *et al.*, 1977; Nakamura *et al.*, 1982; Kumar and Nicholas, 1984). The $K^+$ depletion of cells of the photodenitrifier by DEA-treatment was not accompanied by $Na^+$ uptake, in agreement with the results for *Vibrio alginolyticus*, *E. coli* (Nakamura *et al.*, 1982) and *N. europaea* and *N. agilis* (Kumar and Nicholas, 1984). This loss of $K^+$ from cells at alkaline pH may be explained by the functioning of a $K^+/H^+$ antiporter in the photodenitrifier as found in *E. coli* (Brey *et al.*, 1980). Light-induced $K^+$ flux in cells and chromatophores of *C. vinosum* was also

shown to occur *via* a $K^+/H^+$ antiport (Davidson and Knaff, 1982). It is likely that the $K^+/H^+$ antiporter functions as a regulator of cytoplasmic pH in the photodenitrifier as in other bacteria (Padan *et al.*, 1981).

The rate and extent of $^{22}Na^+$ accumulation in $K^+$-depleted cells was dependent on the presence or absence of DEA in the external medium. In the presence of 50 mM DEA there was no appreciable uptake of $Na^+$ (Section 3.5.6.1) whereas in its absence the cells readily accumulated the cation (Section 3.5.6.3). This entry of $Na^+$ into $K^+$-depleted cells can be explained by the functioning of a $Na^+/H^+$ antiporter. Further evidence in favour of this proposal is the extrusion of $^{22}Na^+$ in exchange for protons from $^{22}Na^+$ loaded cells exposed to light (Section 3.5.6.4). This effect has also been observed in washed cells of *C. vinosum* and *R. rubrum* (Knaff *et al.*, 1981). In the dark a similar effect was recorded in the photodenitrifier since on adding $NH_4Cl$ there was an uptake of $H^+$ and extrusion of $Na^+$ *via* the antiporter (Section 3.5.6.4).

The $K^+$-depleted cells had 90% of the nitrate reductase activity of normal cells and nitrite reductase activity was unaffected (Section 3.5.6.2). The adition of $K^+$ to $K^+$-depleted cells had no effect on the activity of nitrite reductase whereas nitrate reductase was inhibited by about 25%, moreover, the extrusion of $^{22}Na^+$ from $^{22}Na^+$ loaded cells either in light or in the dark was not dependent on denitrification. Thus, the addition of $NO_3^-$ did not result in an extrusion of $^{22}Na^+$ in the dark (Section 3.5.6.4). A respiration-dependent primary electrogenic $Na^+$ extrusion system operated at alkaline pH in *V. alginolyticus* (Tokuda and Unemoto, 1981), but not in the nitrifying bacteria e.g., *N. europaea* and *N. agilis* (Kumar and Nicholas, 1984).

Studies on the effects of $Na^+$ or $K^+$ on the components of $\Delta p$ are of interest in establishing whether these transport systems are electrogenic.

The addition of either NaCl or KCl to the $K^+$-depleted cells resulted in a depolarization of $\Delta\Psi$ and a concomitant increase in $\Delta pH$ both in light and in the dark (Section 3.5.6.5). A maximum depolarization of about 35 mV was recorded in light when either KCl or NaCl was added at pH$\geqslant$7.0. The depolarization of $\Delta\Psi$ can be explained by the functioning of a $K^+/Na^+$ antiporter and its electrogenic nature is indicated by the following facts for DEA-treated cells: (a) they take up $^{22}Na^+$ when suspended in $Na^+$-HEPES buffer (3.5.6.3); (b) addition of $K^+$ to $^{22}Na^+$-loaded cells results in an extrusion of $^{22}Na^+$ indicating the operation of a $Na^+/K^+$ antiporter (Section 3.5.6.4); (c) when $K^+$ concentration (100 mM) in the external medium was higher than that of $Na^+$ (50 mM), an electrogenic $K^+$ transport is indicated by the depolarization of $\Delta\Psi$ (Section 3.5.6.5); (d) a similar depolarization of $\Delta\Psi$ by the addition of $Na^+$ (100 mM) in the presence of 50 mM $K^+$ in the suspending medium (Section 3.5.6.5) confirms the operation of an electrogenic system. The addition of $K^+$ to $K^+$-depleted cells also resulted in a depolarization of $\Delta\Psi$ by 60 mV in *S. faecalis*, 21 mV in *E. coli* (Bakker and Mangerich, 1981), and 5 and 10 mV respectively, in *N. europaea* and *N. agilis* (Kumar and Nicholas, 1984). In these bacteria the depolarization was compensated by an approximately equivalent increase in $\Delta pH$. In experiments with the photodenitrifier, however, the depolarization of $\Delta\Psi$ was not totally compensated for by an increase in $\Delta pH$ (up to 13 mV). This depolarization of $\Delta\Psi$ and a concomitant increase in $\Delta pH$ indicate that transport of $Na^+$ and $K^+$ is electrogenic and requires a proton motive force for its operation.

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# APPENDIX

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