NITROGEN ASSIMILATION AND ENERGY CONSERVATION IN NITROSOBACTER EUROPAEA AND NITROBACTER AGILIS

by

Sharad Kumar, M.Sc.

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

Doctor of Philosophy

Department of Agricultural Biochemistry, Waite Agricultural Research Institute, The University of Adelaide.

October, 1983.
PREFACE

Part of the work described in this thesis has been presented in scientific meetings (Australian Biochemical Society, 1981, 1983; XII International Congress of Biochemistry, 1982). Some of the results have been published or submitted for publication in the journals listed below:

1). Oxygen-dependent nitrite uptake and nitrate production in cells, spheroplasts and membrane vesicles of Nitrobacter agilis.
S. Kumar and D.J.D. Nicholas

2). The uptake of nitrite and oxygen and the production of nitrate by cells, spheroplasts and vesicles of Nitrobacter agilis.
S. Kumar and D.J.D. Nicholas

3). Assimilation of inorganic nitrogen compounds by Nitrobacter agilis.
S. Kumar and D.J.D. Nicholas

4). Respiration-dependent proton translocation in Nitrosomonas europaea and its apparent absence in Nitrobacter agilis during inorganic oxidations.
T.C. Hollocher, S. Kumar and D.J.D. Nicholas

5). A proton-motive force dependent adenosine-5'-triphosphate synthesis in spheroplasts of Nitrosononas europaea.
S. Kumar and D.J.D. Nicholas

6). ATP biosynthesis in the nitrifying bacterium Nitrosomonas europaea.
S. Kumar and D.J.D. Nicholas

7). The proton-electrochemical gradients in washed cells of Nitrosomonas europaea and Nitrobacter agilis.
S. Kumar and D.J.D. Nicholas
8). Definitive $^{15}_N$-NMR evidence that water serves as a source of 'O' during nitrite oxidation by *Nitrobacter agilis*.
S. Kumar, D.J.D. Nicholas and E.J. Williams

9). Adenylylation and deadenylylation of glutamine synthetase in the nitrifying bacteria *Nitrosomonas europaea* and *Nitrobacter agilis*.
S. Kumar and D.J.D. Nicholas

10). Purification, properties and regulation of glutamine synthetase from *Nitrobacter agilis*.
S. Kumar and D.J.D. Nicholas
*J. Gen. Microbiol.* Submitted for publication.

11). NAD$^+$ and NADP$^+$ dependent glutamate dehydrogenases in *Nitrobacter agilis*.
S. Kumar and D.J.D. Nicholas
*J. Gen. Microbiol.* Submitted for publication.

12). Na$^+$ and K$^+$ transport in *Nitrosomonas europaea* and *Nitrobacter agilis*.
S. Kumar and D.J.D. Nicholas
*Biochim. Biophys. Acta.* Submitted for publication.
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Thanks are due to Mr. R.G. Batt for reading the draft of this manuscript, Mr. B.A. Palk for preparing the photographic prints, Mrs. M. Brock for skillful typing and to all others who helped me from time to time.

The award of U.R.G. scholarship by The University of Adelaide is gratefully acknowledged.
DECLARATION

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

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

----------------------------------------
SHARAD KUMAR.
NOMENCLATURE AND ABBREVIATIONS

The major enzymes mentioned in this thesis are listed below with their numbers and systematic names as recommended by Enzyme Commission (Enzyme nomenclature 1978).

<table>
<thead>
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<td>Glutamate dehydrogenase (GDH)</td>
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<td>Glutamine synthetase (GS)</td>
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<tr>
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<td>(ADP forming)</td>
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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 (BBA (1982) 715: 1-23).

**Chemicals**

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<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
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<tr>
<td>AMP</td>
<td>adenosine 5'-monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCCP</td>
<td>carbonyl cyanide (m)-chlorophenyl hydrazone</td>
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<tr>
<td>CDP</td>
<td>cytidine 5'-diphosphate</td>
</tr>
<tr>
<td>CMP</td>
<td>cytidine 5'-monophosphate</td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyl trimethyl ammonium bromide</td>
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<tr>
<td>CTP</td>
<td>cytidine 5'-triphosphate</td>
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<td>DCCD</td>
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<tr>
<td>DESB</td>
<td>diethylstilbestrol</td>
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<tr>
<td>DIECA</td>
<td>diethyl dithiocarbamate (sodium salt)</td>
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<td>2,4 DNP</td>
<td>2,4-dinitrophenol</td>
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<td>DBP</td>
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Chemicals continued...

EDTA  ethylenediamine tetra acetic acid
γ-GH  γ-glutamyl hydroxamate
GDP  guanosine 5'-diphosphate
GMP  guanosine 5'-monophosphate
GTP  guanosine 5'-triphosphate
HEPES  4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
HOQNO  2-heptyl-4-hydroxy quinoline-N-oxide
8-HQ  8-hydroxy quinoline
IDP  inosine 5'-diphosphate
IMP  inosine 5'-monophosphate
ITP  inosine 5'-triphosphate
MSX  L-methionine-DL-sulphoximine
NAD⁺  nicotinamide-adenine dinucleotide (oxidised)
NADH  nicotinamide-adenine dinucleotide (reduced)
NADP⁺  nicotinamide-adenine dinucleotide phosphate (oxidised)
NADPH  nicotinamide-adenine dinucleotide phosphate (reduced)
NBD-chloride  4-chloro-7-nitrobenzo-2-oxa-1,3-diazole
NEM  N-ethyl-maleimide
N-serve  2-chloro-6-trichloromethyl pyridine
pCMB  p-chloro mercuribenzoate
PCP  penta chlorophenol
POPOP  1,4 bis (2,4 methyl-5-phenyl oxazolyl) benzene
PPO  2,5 diphenyloxazole
SDS  sodium-dodecylsulfate
TCA  trichloroacetic acid
TMP  thymidine 5'-monophosphate
2-TMP  2-trichloromethyl pyridine
TPB  tetra phenyl boron
Tris  2-amino-2-hydroxymethyl propane-1,3-diol
TPMP⁺  triphenyl methyl phosphonium cation
TPP⁺  tetra-phenyl phosphonium cation
TTP  thymidine 5'-triphosphate
UDP  uridine 5'-diphosphate
UMP  uridine 5'-monophosphate
UTP  uridine 5'-triphosphate
### Symbols and Units

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<tr>
<td>μg</td>
<td>microgram</td>
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</table>
\( \mu l \) microlitre
\( \mu mol \) micromole(s)
\( \mu M \) micromolar
\( N \) normal
\( nm \) nanometer
\( nmol \) nanamole(s)
\( \% \) percent
\( Pi \) inorganic phosphate
\( pHe \) extracellular pH
\( pHi \) intracellular pH
\( R \) gas constant
\( s \) second(s)
\( S \) substrate concentration
\( t \) time
\( t_1/2 \) half time
\( T \) thermodynamic temperature (Kelvin)
\( v \) volume
\( V \) velocity of the reaction
\( V_{max} \) rate of enzyme catalysed 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
\( m-, p-, 0- \) meta-, para-, ortho-
NMR nuclear magnetic resonance
No. number
/ per
p. (plural pp.) page
PAGE Polyacrylamide gel electrophoresis
S.D. standard deviation
Others continued/...

S.E.M. standard error of means
soln. solution
temp. temperature
Viz. namely
Vs. versus
v/v volume : volume
w/v weight : volume
< less than
\leq less than or equal to
> greater than
\geq greater than or equal to
\approx approximately equal
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1. This thesis embodies results of an investigation on some aspects of nitrogen assimilation and energy conservation in nitrifying bacteria, *Nitrosomonas europaea* and *Nitrobacter agilis*.

2. Electrode techniques have been developed to measure $\text{NO}_3^-$ production and $\text{O}_2$ uptake simultaneously and continuously during $\text{NO}_2^-$ utilization by *Nitrobacter agilis*. The stoichiometry of $\text{NO}_2^-$ oxidation by washed cells was $1\text{NO}_2^- : 0.50_2 : 0.75 \text{NO}_3^-$ compared to $1\text{NO}_2^- : 0.50_2 : 1\text{NO}_3^-$ for spheroplasts and membrane vesicles of *Nitrobacter agilis*. The effects of several metabolic inhibitors on $\text{NO}_2^-$ and $\text{O}_2$ utilization and $\text{NO}_3^-$ production were investigated. Nitrite oxidation was found to be particularly sensitive to the inhibitors of cytochrome oxidase (eg. azide), ATPase (eg. DCCD), -SH group (eg. pCMB) and uncouplers of oxidative phosphorylation (eg. CCCP).

3. A Mg$^{2+}$ dependent ATPase was detected in membrane vesicles of *Nitrobacter agilis* which was inhibited by classical ATPase inhibitors (eg. DCCD) but was unaffected by uncouplers (eg. 2,4-DNP, CCCP). The ATPase activity of membrane vesicles was progressively lost by delipidation of membranes by phospholipase A_2.

4. The growth yields of *Nitrobacter agilis* were increased about 2 fold by growing the bacterium in a $\text{NO}_2^-$ medium supplemented with 2mM-$\text{NH}_4\text{Cl}$. Higher concentrations of $\text{NH}_4\text{Cl}$ however competitively inhibited $\text{NO}_2^-$ oxidation and the growth of the bacterium.

5. Washed cells of *Nitrobacter agilis* readily incorporated $\text{^{15}NH}_4^+$, $\text{^{15}NH}_2\text{OH}$, $\text{^{15}NO}_2^-$ and $\text{^{15}NO}_3^-$ respectively (in decreasing order) into cell nitrogen. Inhibitors of glutamine synthetase (L-methionine DL-sulphoximine) and glutamate synthase (azaserine) did not affect the incorporation of either $\text{^{15}NH}_4^+$ or $\text{^{15}NO}_2^-$ into cell nitrogen, indicating that glutamate dehydrogenase pathway is the main route for the assimilation of $\text{NH}_4^+$ in *Nitrobacter agilis*.

6. Glutamine synthetase was purified 430 fold from *Nitrobacter agilis* and its properties and regulation studied. The enzyme (molecular weight 700,000) which contained 12 subunits of 58,000 each was regulated by feedback inhibition involving amino acids and nucleotides, substrate
inhibition by NH$_4^+$ as well as by an adenyllylation/deadenyllylation mechanism. An isoactivity pH of 7.4 was recorded for the purified enzyme.

7. Glutamine synthetase from *Nitrosomonas europaea* was purified 710 fold. In crude extracts, the Mg$^{2+}$ effect on the γ-glutamyl transferase activity was related to NH$_4^+$ concentration in the growth medium. This enzyme activity was stimulated two fold by Mg$^{2+}$ in crude extracts from cells of culture from which NH$_4^+$ had been depleted. Unlike the *Nitrobacter* enzyme, the γ-glutamyl transferase activity of either crude extracts or the purified enzyme from *Nitrosomonas europaea* was unaffected by snake venom phosphodiesterase treatment.

8. Two isozymes of glutamate dehydrogenase specific for NAD$^+$ and NADP$^+$ respectively were detected in the cytosol fraction of *Nitrobacter agilis*. The NAD$^+$ enzyme functioned in both directions i.e. amination and deamination whereas the NADP$^+$ enzyme was primarily for the amination of α-keto glutarate to glutamate. The NADP$^+$ enzyme was purified 52 fold (free of NAD$^+$ enzyme) by affinity chromatography on 2'-5'ADP Sepharose-4B and some of its properties studied. Substrate activation of the NADP$^+$ enzyme was observed with NH$_4^+$ and NADPH. A comparison was made of the properties of the purified NADP$^+$ enzyme from *Nitrobacter agilis* with that from *Nitrosomonas europaea*.

9. Oxygen pulse experiments were carried out with washed cells of *Nitrosomonas europaea* and *Nitrobacter agilis* and with spheroplasts and everted membrane vesicles prepared from *Nitrobacter agilis*. In addition to thiocyanate, the salting-in anions perchlorate and trichloroacetate and lipophilic cation triphenyl methyl phosphonium (TPMP$^+$) proved to be permeant and effective in allowing respiration-dependent proton translocation in cells of *Nitrosomonas europaea*. The observed $\Delta$$H^+/O$ ratio for NH$_4^+$ oxidation by *Nitrosomonas europaea* was 3.4 and that for both NH$_2$OH and N$_2$H$_5^+$ oxidation was 4.4. These values when corrected for the production of stoichiometric protons and for the fact that the first step in NH$_4^+$ oxidation is mediated by a mono-oxygenase, gave effective $\Delta$$H^+/O$ ratios of about 4 for these three substrates. No convincing evidence was obtained for the operation of a respiratory proton pump in *Nitrobacter agilis* during NO$_2^-$ oxidation.

10. The components of proton-motive force (Δp), namely membrane potential (Δψ) and transmembrane pH gradient (ΔpH) were determined in washed cells of *Nitrosomonas europaea* and *Nitrobacter agilis*. In these bacteria, both Δψ and ΔpH were dependent on external pH (pHe). Thus at pHe 8 cells of
Nitrosomonas europaea and Nitrobacter agilis had $\Delta \psi$ of 173mV and 125mV (inside negative) respectively as determined by the distribution of $[^{3}\text{H}]$ tetraphenyl phosphonium cation (TPP'). Intracellular pH was determined by the distribution of $[^{14}\text{C}]$ benzoic and $[^{14}\text{C}]$ acetyl salicylic acids and $[^{14}\text{C}]$ methylamine. At pH of 7 for Nitrosomonas europaea and 7.5 for Nitrobacter agilis there was no detectable $\Delta \text{pH}$ so that only $\Delta \psi$ contributed to $\Delta \text{p}$. Intracellular pH ($\text{pHi}$) in cells of Nitrosomonas europaea varied from 6.3 at pH 6 to 7.8 at pH 8.5. In Nitrobacter agilis however pH was relatively constant (7.3 to 7.8) over the pH range of 6 to 8.5. The components of $\Delta \text{p}$ ($\Delta \psi$ and $\Delta \text{pH}$) remained constant at various stages of growth of Nitrosomonas europaea so that the metabolic state of the cells did not affect $\Delta \text{p}$. Such an experiment was not possible with Nitrobacter agilis because of low cell yields.

11. Spheroplasts of Nitrosomonas europaea synthesized ATP in response to an artificially created $\Delta \psi$. This ATP synthesis was inhibited by DCCD indicating that it was mediated by an ATPase.

12. Cation (Na$^+$, K$^+$ and NH$_4^+$) transport systems in Nitrosomonas europaea and Nitrobacter agilis were investigated. In K$^+$ depleted cells it was shown that K$^+$ is transported by an electrogenic mechanism in both bacteria and its uptake resulted in partial conversion of $\Delta \psi$ into $\Delta \text{pH}$. NH$_4^+$ was transported essentially as a neutral species (NH$_3$) by a simple diffusion mechanism. Experiments with $^{22}\text{Na}^+$ loaded cells indicated that antiporters for Na$^+$/H$^+$, Na$^+$/K$^+$ and K$^+$/H$^+$ were present in both bacteria. At least one of these antiporters (Na$^+$/K$^+$) required an electrochemical gradient for its operation.

13. Using stable isotopes of $^{15}\text{N}$ ($^{15}\text{NO}_2^-$ and $^{15}\text{NO}_3^-$) and $^{18}\text{O}$ (H$_2$$^{18}\text{O}$, $^{18}\text{O}_2$ and P$^{18}\text{O}_4^-$) it was shown by GC/MS and $^{15}\text{N}$-NMR techniques that the third 'O' in NO$_3^-$ produced by NO$_2^-$ oxidation by cells of Nitrobacter agilis originated from H$_2$O and not from O$_2$ or PO$_4^-$.
1. INTRODUCTION
1. INTRODUCTION

1.1 NITRIFYING BACTERIA

1.1.1 Morphology

Nitrifying bacteria, which belong to family Nitrobacteriaceae, have been classified into seven genera (Breed et al., 1957): Nitrobacter, Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosocystis, Nitrosogloea and Nitrocystis. Watson (1971) and Watson and Waterbury (1971) confirmed the first four genera and added three new ones, namely Nitrospina, Nitrococcus and Nitrosolobus. These bacteria fall into two main groups: (i) ammonia oxidisers and (ii) nitrite oxidisers. The two predominant genera of the family are the ammonia oxidising genus Nitrosomonas and the nitrite oxidiser Nitrobacter.

Nitrosomonas and Nitrosocystis are ovoid and approximately 2 and 2.5μ in length respectively (Murray and Watson, 1965). Nitrobacter, which is smaller (1.5μ) than Nitrosomonas is enlarged at one end, tapering towards the other end (Murray and Watson, 1965). Nitrosomonas europaea contains a few concentric membranes at the periphery of the cell enclosing the cytoplasm while Nitrobacter possess a unique lamellar array of membranes located peripherally at the swollen end of the cell (Murray and Watson, 1965). Polyhedral bodies have been reported in Nitrosomonas europaea (Wullenweber et al., 1977) and Nitrobacter agilis (Pope et al., 1968).

Polar flagella have been reported in species of Nitrobacter (Breed et al., 1957). Nitrosomonas cells are usually motile and possess two sub-terminal flagella (Engel, 1961). Non-flagellar species of Nitrosomonas have been observed by Engel (1961), but it is likely that the flagella were lost during preparative centrifugation of the cells for electron-microscopy.

1.1.2 The concept of chemolithotrophy

Chemolithotrophic bacteria can obtain all the energy needed for growth and carbon assimilation from the oxidation of inorganic compounds eg. inorganic sulfur compounds (Hutchinson et al., 1969; Kelly, 1967), iron metal or ferrous
ion (Peck, 1968), hydrogen (Peck, 1968; Kelly, 1971) ammonia and nitrite (Peck, 1968; Wallace and Nicholas, 1969b; Aleem, 1970; Suzuki, 1974; Aleem, 1977; Hooper, 1978; Nicholas, 1978; Suzuki et al., 1981b; Hooper, 1982). The two important genera of the nitrifying bacteria mainly responsible for the biological oxidation of ammonia to nitrate in soil are *Nitrosomonas* which converts ammonia to nitrite (Meyerhoff, 1917; Lees and Quastel, 1945; Quastel and Scholefield, 1951; Zavarzin, 1960; Alexander, 1965) and *Nitrobacter*, which then oxidises nitrite to nitrate (Lees, 1955). Winogradsky (1890) established that these bacteria are chemolithotrophic types because they derive their energy from the oxidation of reduced inorganic nitrogen compounds and carbon from CO₂. Heterotrophic growth of both *Nitrosomonas europaea* and *Nitrobacter agilis* has been reported (Smith and Hoare, 1968; Pan and Umbreit, 1972; Bock, 1976, 1978; Matin, 1978). The fixation of CO₂ by nitrifiers is well established in *Nitrosomonas* (Rao and Nicholas, 1966), *Nitrobacter* (Aleem, 1965) and *Nitrocystis* (Campbell et al., 1966). It seems that CO₂ fixation is common to all chemoautotrophs (Kelly, 1967, 1971). Delwiche et al. (1963) have shown that *Nitrosomonas* actively fixed ¹⁴C-labelled CO₂ into compounds that were precipitated by trichloroacetic acid. Carboxydismutase, enzymes of the reductive pentose pathway and phosphoenolpyruvate carboxylyase have been described in *Nitrosomonas* (Nicholas and Rao, 1964; Rao and Nicholas, 1966). The triose-phosphate dehydrogenase in both *Nitrosomonas* and *Nitrobacter* was found to be NAD-specific (Aleem, 1965; Rao and Nicholas, 1966). Malic enzyme which carboxylates pyruvate, was not detected in *Nitrosomonas europaea* (Rao and Nicholas, 1966). There was no evidence for glycollic or phosphoglycollic acids as intermediates in the fixation of CO₂ and the enzymes that utilize glucose, fructose, ribose and acetate were not detected (Rao, 1966).

Based on the work with the sulfur oxidising bacterium *Thiobacillus*, it has been proposed that chemolithotrophic bacteria have a defective tricarboxylic acid cycle, since they do not appear to have α-ketoglutarate dehydrogenase and NADH oxidase enzymes (Smith et al., 1967). In *Nitrobacter* however, Kiesow (1964) found an active NADH oxidase which was linked to O₂. Aleem (1968) also studied NADH oxidase in *Nitrobacter*, but was unable to detect α-ketoglutarate dehydrogenase in either this or other chemolithotrophic bacteria (Wallace et al., 1970). Thus there is some evidence now that the Krebs cycle
in chemolithotrophs is impaired at the level of α-ketoglutarate.

1.1.3 Ammonia and hydroxylamine oxidation by Nitrosomonas

1.1.3.1 Ammonia Oxidation

The oxidation of ammonia to nitrite involves a net transfer of 6 electrons. Hofman and Lees (1952, 1953) confirmed the postulate of Kluyver and Donker (1926) that the oxidation of ammonia to nitrite occurs in two steps:

(i) \[ \text{NH}_3 + 2e^- \rightarrow \text{NH}_2\text{OH} \]

(ii) \[ \text{NH}_2\text{OH} + 4e^- \rightarrow \text{NO}_2^- \]

Engel and Alexander (1958a, b) reported that washed cells of Nitrosomonas europaea progressively lost their ammonia oxidising ability upon ageing. Oxidation of ammonia to hydroxylamine is inhibited by a variety of inhibitors (Lees, 1952a, b, 1955; Campbell and Aleem, 1965; Hooper and Terry, 1973; Ashworth et al., 1977; Bhandari and Nicholas, 1979a, b). Lees (1946, 1952a, 1962) postulated that a metal enzyme is involved in ammonia oxidation and this metal could be Cu (Hofman and Lees, 1953; Lees, 1955; Nicholas et al., 1962; Aleem, 1965; Hooper and Terry, 1973; Hooper, 1978; Nicholas, 1978; Bhandari and Nicholas, 1979a, b). It was then suggested by Anderson (1964a, 1965b) that ammonia oxidation requires a form of "high energy" oxygen generated by the oxidation of cuprous copper. Based on inhibitor studies Hooper and Terry (1973) indicated that ammonia oxidation requires, in addition to Cu, a CO-binding factor namely P-460 as well as an intact membrane.

Hooper (1969b) reported that an initial lag phase of ammonia oxidation was eliminated on adding a small quantity of NH$_2$OH to washed cells of Nitrosomonas. Suzuki et al. (1974) established that $K_m$ values for ammonia oxidation in washed cells were pH dependent, ranging from 4 to 0.3mM over the pH range 7.0 to 8.5. Similar $K_m$ values were also reported by Laudelout et al. (1976) and Drozd (1976).
Suzuki and Kwok (1969) prepared spheroplasts from the cells of *Nitrosononas europaea* which oxidised ammonia only in the presence of \( \text{Mg}^{2+} \) or \( \text{NH}_2\text{OH} \). Rees and Nason (1966) found a small (7%) incorporation of \(^{18} \text{O} \) from \(^{18} \text{O}_2 \) into \( \text{NO}_2^- \) (uncorrected for possible loss by exchange reactions) during ammonia oxidation by cells of *Nitrosononas*. Suzuki and Kwok (1969) then suggested that a mixed function oxygenase catalysed the oxidation of ammonia to hydroxylamine, which is coupled to the oxidation of \( \text{NH}_2\text{OH} \) to \( \text{NO}_2^- \). Dua et al. (1979) reported an incorporation of \(^{18} \text{O} \) from \(^{18} \text{O}_2 \) (0.3 atom % excess) into \( \text{NH}_2\text{OH} \) produced by the oxidation of ammonia by cells in the presence of hydrazine, but not from \( \text{H}_2^{18} \text{O} \). This experiment was repeated with the yields of >92 atom % enrichment (Hollocher et al., 1981). Using \(^{18} \text{O} \) isotope shift in \(^{15} \text{N}-\text{NMR} \), Andersson et al. (1982) reported that \( \text{NO}_2^- \) produced by cells from \( \text{NH}_3 \) or \( \text{NH}_2\text{OH} \) has the isotopic oxygen composition of water. Cells were found to catalyse rapid exchange of both the oxygen atoms of \( \text{NO}_2^- \) with water. Thus the origin of second oxygen atom in \( \text{NO}_2^- \) was uncertain. All this evidence indicates that \( \text{NH}_3 \) oxidation to \( \text{NH}_2\text{OH} \) involves an oxygen insertion reaction via a monooxygenase. The origin of second oxygen in \( \text{NO}_2^- \) produced in the second step of \( \text{NH}_3 \) oxidation (\( \text{NH}_2\text{OH} \rightarrow \text{NO}_2^- \)) is still unclear.

The first attempts to prepare cell free-extracts of *Nitrosononas* were made by Imshenetskii and Ruban (1952, 1954a,b, 1956, 1957). Very slow rate of ammonia oxidation was observed when the autolysates of *Nitrosononas* were incubated for 24h at 40°C. Subsequently Engel and Alexander (1959) attempted preparations of cell free extracts, but these were relatively inactive in oxidising \( \text{NH}_3 \) to \( \text{NO}_2^- \). The cell-free preparations by Nicholas and Jones (1960), however oxidised \( \text{NH}_2\text{OH} \) to \( \text{NO}_2^- \) provided an electron carrier such as cytochrome c or phenazine methosulphate was added. Attempts were also made to demonstrate ammonia oxidation in cell free systems of *Nitrosononas europaea* (Suzuki and Kwok 1970, 1981; Suzuki et al., 1974, 1976, 1981a,b; Tokuyama and Asano, 1978a,b,c; Bhandari and Nicholas, 1980). Achievement of high rates of ammonia oxidation in extracts has proved to be extremely difficult.
Hooper (1982) suggested that ammonia oxidation requires 5 possible components in an appropriate aggregate: ammonia oxidase protein(s), hydroxylamine oxidoreductase, cytochromes c554 and c552 and a terminal oxidase. Suzuki and Kwok (1981) attempted the separation and reconstitution of the ammonia oxidising system. Crude fractions were separated by gel filtration on a Sepharose-6B column. A membranous fraction 1, which contained hydroxylamine oxidoreductase, cytochrome a and cytochrome oxidase activity catalyzed the oxidation of NH$_2$OH. Two of the fractions contained a hydroxylamine oxidoreductase and a cytochrome c552 respectively. Maximum rates for ammonia oxidation were recorded when the three fractions were reconstituted or when fraction one was added to purified cytochrome c554. The latter cytochrome (c554) ($K_m$ 3.3 μM) was suggested to be an electron donor for ammonia hydroxylation. Using reconstituted membrane fraction with reduced cytochrome c554 Tsang and Suzuki (1982) reported that the reoxidation of cytochrome c554 and O$_2$ utilization takes place when either CO or NH$_3$ is added to the membrane fraction.

1.1.3.2 Hydroxylamine Oxidation

The NH$_2$OH produced from NH$_4^+$ by ammonia mono-oxygenase (Suzuki et al., 1974; Hollocher et al., 1981) is further oxidized to NO$_2^-$ by hydroxylamine oxidoreductase (HAO). Hofman and Lees (1952, 1953) found that the oxidation of NH$_2$OH by whole cells was inhibited by hydrazine and under these conditions NH$_2$OH was accumulated. Three two-electron steps for the oxidation process were then postulated (Lees, 1952b; Hofman and Lees, 1953):

\[
\text{NH}_4^+ + 2e^- \rightarrow \text{NH}_2\text{OH} + \text{NO}_2^- 
\]

Chromatographic methods were used to identify NH$_2$OH as an intermediate during the oxidation of NH$_4^+$ to NO$_2^-$ (Yoshida and Alexander, 1964).

Hydroxylamine oxidation in cell-free extracts was first achieved by Nicholas and Jones (1960). Cell-free extracts oxidised NH$_2$OH only in the presence of air and an electron carrier such as cytochrome c, phenazine methosulfate, methylene blue, benzyl viologen or ferricyanide (Nicholas and Jones, 1960; Aleem and Lees, 1963; Falcone et al., 1963; Anderson, 1964a; Aleem, 1970). Nicholas and Jones (1960) demonstrated
that the added cytochrome c was reduced by NH$_2$OH as well as by hydrazine but N$_2$ gas instead of NO$^-$ was produced in the presence of hydrazine. They demonstrated that hydrazine competitively inhibited NH$_2$OH oxidation to NO$^-$. Based on these and other investigations, Nicholas and Jones (1960) and Falcone et al. (1963) further suggested that the oxidation of NH$_2$OH involves the participation of the respiratory chain involving flavoproteins and cytochromes.

The hydroxylamine oxidase was located in the membranes and it was resolved into hydroxylamine cytochrome c reductase and cytochrome oxidase by Falcone et al. (1962, 1963). The role of the particulate hydroxylamine cytochrome c reductase enzyme system was subsequently confirmed by Aleem and Lees (1963) and Hooper and Nason (1965).

Hydroxylamine oxidoreductase (HAO) was purified and its properties determined (Hooper and Nason, 1965; Hooper et al., 1978; Tokuyama et al., 1979; Yamanaka et al., 1979; Yamanaka and Sakano, 1980; Miller and Wood, 1983b). Various electron transport components of HAO were then purified (Payne, 1978). Hydroxylamine oxidoreductase constitutes 5% of the soluble proteins and 40% of the c haem of Nitrosonomas (Hooper et al., 1978). Rees and Nason (1965) reported an unusual absorption maximum at 465nm from the dithionite reduction of extracts of Nitrosonomas. The addition of CO resulted in a shift of absorbancy to approximately 450nm suggesting a cytochrome P-450 like pigment. The 465nm haem protein was found to be soluble (Hooper et al., 1972) and was located in fraction containing HAO (Ritchie and Nicholas, 1974) but was considered of uncertain significance since it was not reduced by either NH$_2$OH or NH$_2$NH$_2$. Erickson and Hooper (1972) purified a small fraction of the total cellular pigment with absorption maxima at 435 (oxidised), 460 (dithionite reduced) and 450 (dithionite reduced CO complex). Based on its ligand binding properties it was identified as a haem and named P460. Hooper et al. (1978) subsequently reported that essentially all the cellular haem P460 was associated with HAO. Selective destruction of haem P460 of HAO with H$_2$O$_2$ resulted in a loss of both hydroxylamine dehydrogenase activity and the hydroxylamine reduction of c type haems indicating that haem P460 is a part of or near to the substrate binding site (Hooper and Terry, 1977; Hooper and Balny, 1982). Miller and Wood (1983c) recently reported that about 5% of total P460 was in 'free' form. The free trimeric cytochrome P460 had a native molecular weight of 52,000.
The purified HAO contained >20 moles Fe per mole enzyme (molecular weight 200,000) (Hooper et al., 1978; Terry and Hooper, 1981). The ratio of protein per heme was reported as 10,000 (Hooper et al., 1978) or 17,500 (Yamanaka et al., 1979b). Vickery and Hooper (1981) detected heme c centres in HAO by electron paramagnetic resonance (EPR).

Estimates of the ratio of P460: c heme range from 6 to 9 (Hooper et al., 1978; Hooper, 1982), 8 (Lipscomb and Hooper, 1982) and 7 (Lipscomb et al., 1982).

Some cellular cytochromes not associated with HAO have been purified and characterized (Rees, 1968; Tronson et al., 1973; Yamanaka and Shinra, 1974; Millar and Wood, 1982, 1983a, d). Cytochrome a was extracted from KCl washed membranes with Triton X-100 by Erickson et al. (1972) and purified with ammonium sulfate. This cytochrome contained non-covalently bound haem a identified as cytochrome a1 which oxidised cytochrome c-554 from Nitrosomonas (Yamanaka and Shinra, 1974). Hooper et al. (1972) identified the electron transfer components of the membrane envelope fractions, namely ubiquinone as well as cytochromes b, c and a1.

Aleem et al. (1962) suggested that nitrohydroxylamine is the intermediate formed by initial oxidative condensation between \( \text{NH}_2\text{OH} \) and \( \text{NO}_2^- \) as follows:

\[
\text{NH}_2\text{OH} \rightarrow (\text{NOH}) + 2(\text{H})
\]

\[
2(\text{H}) + 2 \text{Cyt. c Fe}^{3+} \rightarrow 2 \text{cyt. c Fe}^{2+} + 2\text{H}^+
\]

\[
(\text{NOH}) + \text{HNO}_2 \rightarrow \text{NO}_2^- \text{NHOH}.
\]

\[
\text{NO}_2^- \text{NH}OH + \frac{1}{2} \text{O}_2 \rightarrow 2\text{HNO}_2
\]

It was then proposed that dehydrogenation of the nitroxy1 to NO and \( \text{N}_2\text{O} \) occurs (Falcone et al., 1962, 1963; Anderson, 1964a, 1965a). It was observed that under anaerobic conditions when \( \text{NH}_2\text{OH} \) was added to extracts of Nitrosomonas equivalent amounts of both \( \text{N}_2\text{O} \) and NO were formed (Anderson, 1964a, 1965c; Hooper, 1968; Yoshida and Alexander 1970, 1971; Ritchie and Nicholas, 1972). Ritchie and Nicholas (1974) reported a nitrite reductase closely associated with HAO. Subsequently the ammonia oxidising chemolithotrophic bacteria have been shown to account for significant amounts of \( \text{N}_2\text{O} \) production (Bremner and Blackmer, 1978; Blackmer et al., 1980) or \( \text{N}_2\text{O} \) and NO (Goreau et al., 1980).
Recently Miller and Wood (1983b) purified a soluble cytochrome oxidase (molecular weight 120,000). These authors reported that the oxidase was a copper protein devoid of haem, and not a cytochrome o as was previously assumed (Rees and Nason, 1965). Soluble cytochrome oxidase activity co-purified with nitrite reductase activity and appeared to be associated with the same protein (Miller and Wood, 1983b).

The similarities in the mechanism of oxidation of ammonia by Nitrosomonas and methane by methanotrophic bacteria have been discussed (Higgins et al., 1981; Hooper, 1982). Recently, cells of Nitrosomonas europaea have been shown to oxidise methane (Jones and Morita, 1983; Hyman and Wood, 1983).

1.1.4 Nitrite oxidation by Nitrobacter agilis

The reduction of cytochromes 551 and 559 during nitrite oxidation by whole cells of Nitrobacter was first observed by Lees and Simpson (1957). Subsequently Butt and Lees (1958) proposed the following scheme for nitrite oxidation:

\[
\text{Cyt 551. } \text{Fe}^{3+} + \text{NO}_2^- \rightarrow \text{Cyt 551. } \text{Fe}^{2+} + \text{"NO}_2" \\
\text{Cyt 551. } \text{Fe}^{2+} + \text{"NO}_2" + \frac{1}{2} \text{O}_2 \rightarrow \text{Cyt 551. } \text{Fe}^{3+} + \text{NO}_3^- 
\]

According to the scheme proposed by Lees and Simpson (1957) and Butt and Lees (1958), NO\textsubscript{2}\textsuperscript{-} oxidation is mediated by cytochrome c and NO\textsubscript{3}\textsuperscript{-} is formed as a result of an O insertion from molecular O\textsubscript{2}. Aleem and Nason (1959) further characterized NO\textsubscript{2}\textsuperscript{-} oxidase that catalysed the following reaction:

\[
\text{NO}_2^- \rightarrow \text{cytochrome c reductase} \rightarrow \text{cytochrome c} \rightarrow \text{cytochrome a_1} \rightarrow \text{cytochrome oxidase} \rightarrow \text{O}_2.
\]

As this scheme involves the reduction of O\textsubscript{2} to H\textsubscript{2}O by the electrons supplied by NO\textsubscript{2}\textsuperscript{-}, it was proposed that molecular oxygen could not possibly supply an O atom to NO\textsubscript{2}\textsuperscript{-} for the formation of NO\textsubscript{3}\textsuperscript{-}. Subsequently Aleem et al. (1965) provided evidence from \textsuperscript{18}O isotope studies that H\textsubscript{2}O but not O\textsubscript{2} contributed O during NO\textsubscript{2}\textsuperscript{-} oxidation to NO\textsubscript{3}\textsuperscript{-}. They proposed the following mechanism to account for their results:
(i) \[ \text{NO}_2^- + H_2^18O \rightarrow \text{NO}_2^-H_2^18O \]

(ii) \[ \text{NO}_2^-H_2^18O + 2 \text{ cyt } a_1^1\text{Fe}^{3+} \rightarrow \text{N}_2^18O^- + 2 \text{ cyt } a_1^1\text{Fe}^{2+} + 2\text{H}^+ \]

(iii) \[ 2 \text{ cyt } a_1^1\text{Fe}^{2+} + 2 \text{ cyt } a_3\text{Fe}^{3+} \rightarrow 2 \text{ cyt } a_1^1\text{Fe}^{3+} + 2 \text{ cyt } a_3\text{Fe}^{2+} \]

(iv) \[ 2 \text{ cyt } a_3\text{Fe}^{2+} + 2\text{H}^+ + 1/2\text{O}_2 \rightarrow 2 \text{ cyt } a_3\text{Fe}^{3+} + \text{H}_2\text{O} \]

Sum: \[ \text{NO}_2^- + 1/2\text{O}_2 \rightarrow \text{NO}_3^- \]

The four steps in the scheme are as follows (Aleem, 1977): (i) hydration of the \text{NO}_2^- molecule prior to removal of electrons from \text{NO}_2^- and protons from \text{H}_2\text{O}; (ii) electrons are transferred from hydrated \text{NO}_2^- molecule to cytochrome \text{a}_1^1 and \text{NO}_3^- is formed in turn as a result of the incorporation of '0' from \text{H}_2\text{O}; (iii) a proton current is generated; (iv) finally electrons and protons are transferred to molecular 0 via a \text{cyt } a_3^1 type oxidase.

Aleem (1968) and Sewell and Aleem (1969) established that there is a second \text{NO}_2^- oxidising system in \text{Nitrobacter} which is coupled to the generation of reduced pyridine nucleotides and is highly endergonic:

\[ \text{NO}_2^- + \text{H}_2\text{O} + \text{NAD(P)}^+ \rightarrow \text{NO}_3^- + \text{NAD(P)}\text{H} + \text{H}^+ \]

\[ \Delta G^0 = +35 \text{ kcal.mol}^{-1} \]

Sewell et al. (1972) reported that there are five different types of cytochromes present in \text{Nitrobacter}: cytochrome \text{c} (Em = 274mV), cytochrome \text{a} (Em = 240mV), cytochrome \text{a}_3 (Em = 400mV) and two \text{a}_1 type cytochromes (Em = 352mV and Em = 100mV). Similar results were subsequently reported by Cobley (1973). The oxidation-reduction potentials of cytochromes \text{c}, \text{a} and \text{a}_3 showed no pH dependence but a high potential component of cytochrome \text{a}_1 had a gradient of approximately 25mV/pH unit and the lower potential component had a gradient of 50mV/pH unit (Ingledew et al., 1974). Similar results were reported by Aleem (1977) with mid point potential of \text{NO}_2^- / \text{NO}_3^- couple in extracts of \text{Nitrobacter}. The Em became more positive from pH 9.0 (Em = 328mV) to pH 6.8 (Em = 414mV). Because the Em for \text{NO}_2^- and of the high potential component of cytochrome \text{a}_1 at pH 8.0 (optimum for \text{NO}_2^- oxidation) are at 360mV and 327mV respectively, the reduction of high potential \text{a}_1 component by \text{NO}_2^- poses no thermodynamic barrier. Moreover since the Em of cytochrome \text{c} is not affected by pH changes, its reduction by \text{NO}_2^- and cytochrome \text{a}_1 would be endergonic.

It is well established now that the site of entry of \text{NO}_2^- in the electron
transport chain is cytochrome $a_1$ and the reduction of cytochrome $c$ by $\text{NO}_2^-$ is energy dependent (Kiesow, 1967; Aleem, 1967, 1968; Sewell and Aleem, 1969; Cobley, 1973; Ingledew et al., 1974; Cobley, 1976a,b). In recent years components of nitrite oxidase have been purified and studied in detail. Yamanaka et al. (1979a) purified a cytochrome $a$ type terminal oxidase from *Nitrobacter agilis* which consisted of two heterologous subunits of molecular weight 40,000 and 27,000 respectively. Chaudhry et al. (1980) purified the $a_3$ type terminal oxidase by hydrophobic interaction chromatography and reported that the enzyme had three subunits of molecular weight 37,000, 25,000 and 13,000 respectively. The absorption maxima were at 420 and 600 nm in the oxidised form and at 443 and 606 nm when reduced. Yamanaka et al. (1981) reported that this terminal oxidase contained 1 mole of haem $a$, 1.6 g-atom of Cu per 41,000 g. The enzyme rapidly oxidised ferrocytochrome of several eukaryotes as well as *Nitrobacter* cytochrome $c$-552.

Chaudhry et al. (1980) found a $b$ type cytochrome in *Nitrobacter agilis* which reacted with $\text{NO}_2^-$ and CO. Three $c$ type cytochromes have been purified (Chaudhry et al., 1981): $c$-553, $c$-550, $c$-559, 554 and their amino acid compositions studied. Yamanaka et al. (1982) independently isolated cytochrome $c$-550. The enzyme was composed of 108 amino acid residues, sixteen of which were lysine. The cytochrome rapidly reacted with *Nitrobacter* cytochrome $c$ oxidase. The complete amino acid sequence of cytochrome $c$-552 has been determined (Tanaka et al., 1982) and was found to be homologous with eukaryotic cytochrome $c$ and with cytochrome $c_2$ from photosynthetic bacteria.

Fukumori and Yamanaka (1982) studied the effects of cardiolipin on the reaction rates of cytochrome $c$ oxidase at various concentrations of phosphate buffer. In contrast to the oxidations of horse and ferrocytochrome $c$ which were stimulated by cardiolipin, the cytochrome $c$-550 oxidation by cytochrome oxidase was unaffected. They suggested that cardiolipin was not necessary for the reaction of cytochrome $c$ oxidase with cytochrome $c$-550 in *Nitrobacter.*

1.1.5 Energy coupling

1.1.5.1 *Nitrosomonas* species

Energy coupling in *Nitrosomonas* has been discussed by Peck (1968), Wallace and Nicholas (1969b), Aleem (1970, 1977), Suzuki (1974), and Hooper (1982). The oxidation of $\text{NH}_2\text{OH}$ to $\text{NO}_2^-$ is an energy yielding
The free energy changes during $\text{NH}_4^+$ oxidation are as follows:

(i) $\text{NH}_4^+ + \frac{3}{2} \text{O}_2 \rightarrow \text{NH}_2\text{OH} + \text{H}^+$

\[ \Delta G_1'' = -0.7 \quad \Delta G_2'' = +3.85 \]

(ii) $\text{NH}_2\text{OH} + \frac{1}{2} \text{O}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + \text{H}^+$

\[ \text{Sum: } \text{NH}_4^+ + 1.5 \text{O}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + 2\text{H}^+ \]

\[ \Delta G_1'' = -83.3 \quad \Delta G_2'' = -68.98 \]

\[ \Delta G_1'' = -84.0 \quad \Delta G_2'' = -65.04 \]

Where $\Delta G_1''$ and $\Delta G_2''$ denote the free energy values according to Gibbs and Schiff (1961) and Aleem (1970) respectively.

Burge et al. (1963) were unable to detect ATP synthesis during $\text{NH}_2\text{OH}$ oxidation, based on $^{32}\text{P}$ experiments. Ramaiah and Nicholas (1964) however demonstrated phosphorylation in particulate fractions with P/O ratio of around 0.20. Drozd (1976), reported $\frac{\text{H}^+/\text{O}}{\text{P}}$ ratio of about 2 for either $\text{NH}_4^+$ or $\text{NH}_2\text{OH}$ oxidation and suggested that substrate oxidation is closely associated with the reduction of cytochrome c so that there is only one active proton translocating loop (loop 3) composed of at least hydroxylamine cytochrome c reductase, cytochrome c and cytochrome oxidase of the a and/or 0 type (Aleem and Lees, 1963; Aleem, 1970).

The requirements for NADH and ATP for endergonic reduction reactions associated with $\text{CO}_2$ fixation in chemolithotrophic bacteria are well established (Wallace and Nicholas, 1969b). The generation of reduced nicotinamide adenine nucleotides coupled to oxidation of $\text{NH}_4^+$ or $\text{NH}_2\text{OH}$ would be thermodynamically unfavourable due to positive values of $\Delta G_2''$ for these coupled reactions (Gibbs and Schiff, 1961). The reduction of NAD$^+$ by energy dependent electron flow was proposed by Chance and Hollunger (1961) in animal mitochondria. Energy dependent reduction of NAD$^+$ by reversed electron transfer has been demonstrated in extracts of $\text{Nitrosomonas}$ and $\text{Nitrobacter}$ as well as in other chemolithotrophic bacteria (Aleem et al., 1963). This was subsequently confirmed by Aleem (1965, 1966).

The following scheme of NADH production was then proposed:
The oxidation of NO$^-$ by Nitrobacter is linked to the generation of ATP (Aleem and Nason, 1960; Malavolta et al., 1960; Fischer and Laudelout, 1965; Kiesow, 1964; Aleem, 1968; O'Kelly et al., 1970; Cobley and Chappell, 1974; Cobley, 1976a,b; Aleem, 1977). Aleem and Nason (1960) reported P/O ratios of 0.2, 0.1 and 0.03 in Nitrobacter particles for the oxidation of NO$^-$, succinate and NADH, and succinate respectively. Kiesow (1964) reported that NADH oxidation by Nitrobacter particles could yield P/O ratios of 3.0 and P/2e$^-$ ratios of 2 with O$_2$ and NO$_3^-$ as respective electron acceptors. Further, Kiesow (1964) suggested that NO$_2^-$ oxidation is an energy consuming process than energy generating one. However observations by Aleem (1968, 1977) ruled out the possibility that NAD$^+$ involvement is obligatory during NO$_2^-$ oxidation. Aleem (1968) reported P/O ratios of about 1 in the presence of an NADH trap or rotenone. Similar results were obtained for ascorbate as an electron donor indicating that ATP generation takes place in the terminal segment of electron transport chain involving coupling site 3. The NADH oxidation was also reported to be coupled to ATP biosynthesis with P/O ratios of about 2 (Aleem, 1968, 1977). It was found that rotenone, antimycin A and HOQNO all inhibited NADH oxidation as well as coupled phosphorylation. Sewell and Aleem (1979) observed P/O ratios of about 1.1 and P/NO$_3^-$ ratios of about 0.7 for NADH oxidation with O$_2$ or NO$_3^-$ as respective electron acceptors. They suggested that aerobic NADH oxidation and associated ATP formation involves all the three coupling sites and NADH-NO$_3^-$ reductase sites 1 and 2.

Cobley (1976a,b) proposed a chemiosmotic mechanism for ATP generation in Nitrobacter. Using membranes of Nitrobacter winogrdskyi
Cobley (1976a,b) found that uncouplers eg. CCCP inhibited NO$_2^-$ oxidation but stimulated aerobic NADH oxidation. He also reported that the compounds known to collapse membrane potential ($\Delta\Psi$) inhibited NO$_2^-$ oxidation and proposed that the flow of electrons from cytochrome $a_1$ to cyt c is facilitated by $\Delta\Psi$. Very low H$^+/O$ ratios ($\approx0.1$) were reported for NO$_2^-$ oxidation by membrane particles. The following mechanism of proton translocation was put forward by Cobley (1976b):

\[
\begin{align*}
\text{OUT} & \quad \text{IN} \\
1\text{H}^+ & \quad [\text{H}^+] \quad \text{cyt} a_1 \quad \text{NO}_2^- + \text{H}_2\text{O} \quad \text{NO}_3^- + \text{H}^+ \\
\text{cyt c} & \quad \text{cyt oxidase} \quad \text{2e} \quad \text{2e} \quad \text{H}_2\text{O} \\
\text{IN} & \quad \text{OUT} \\
\text{2H}^+ & \quad [2\text{H}^+] \quad \text{cyt} a_1 \quad \text{NO}_2^- + \text{H}_2\text{O} \quad \text{NO}_3^- \\
\text{cyt c} & \quad \text{cyt oxidase} \quad \text{2e} \quad \text{2e} \quad \text{H}_2\text{O}
\end{align*}
\]

An alternative mechanism proposed by Aleem (1977) predicts the movement of two protons and two net positive charges out of the cell for the flow of two electrons:

\[
\begin{align*}
\text{OUT} & \quad \text{IN} \\
2\text{H}^+ & \quad [2\text{H}^+] \quad \text{cyt} a_1 \quad \text{NO}_2^- + \text{H}_2\text{O} \quad \text{NO}_3^- \\
\text{cyt c} & \quad \text{cyt oxidase} \quad \text{2e} \quad \text{2e} \quad \text{H}_2\text{O} \\
\text{IN} & \quad \text{OUT} \\
\text{NO}_2^- & \quad \text{H}_2\text{O}
\end{align*}
\]

Aleem's scheme (Aleem, 1977) did not account for the stimulation of NO$_2^-$ oxidation by $\Delta\Psi$ whereas Cobley's scheme (Cobley, 1976b) predicted an unusual H$^-$ (hydride) transfer. Ferguson (1982) proposed that cytochrome oxidase ($aa_3$) can alternatively act as a proton pump in *Nitrobacter* to generate proton-motive force:
Interestingly, a recent report (Sone et al., 1983) indicates that unlike the cytochrome c oxidase of other bacteria (Solioz et al., 1982) and mitochondria (Wikstrom and Krab, 1980) the cytochrome c oxidase from Nitrobacter agilis lacked the proton-pump activity.

1.1.6 Nitrogen assimilation

Wallace and Nicholas (1968) reported that assimilatory nitrite and hydroxylamine reductases were present in both Nitrosomonas and Nitrobacter. In addition Nitrobacter also contained a nitrate reductase (Straat and Nason, 1965; Wallace and Nicholas, 1968; Faull et al., 1969; Herrera and Nicholas, 1974). The product of NO₂⁻ and NH₂OH reduction by either bacterium was found to be ammonia (Wallace and Nicholas, 1968). Both Nitrobacter and Nitrosomonas incorporated ¹⁵NH₄⁺, ¹⁵NH₂OH and ¹⁵NO₂⁻ into cell nitrogen and in addition Nitrobacter but not Nitrosomonas also incorporated ¹⁵NO₃⁻ (Wallace and Nicholas, 1968).

Glutamate dehydrogenase has been characterized and purified from Nitrosomonas (Hooper et al., 1967). The purified glutamata dehydrogenase from Nitrosomonas was specific for NADP⁺ and exhibited both amination and deamination reactions (Hooper et al., 1967). The specific activity of the enzyme was over 80 fold greater than the rate required to synthesize all the organic nitrogen by the bacterium. Wallace and Nicholas (1968) reported that the specific activity of glutamate dehydrogenase in Nitrobacter was about 80 times less than that of Nitrosomonas. They also showed that glutamate dehydrogenase from
*Nitrosomonas* is competitively inhibited by NH$_2$OH and oxime of α-ketoglutarate (Wallace and Nicholas, 1969a).

Glutamate was found to be major amino acid in the water soluble fraction of *Nitrobacter*, contributing about 25% (w/w) of total amino acid pool, while in *Nitrosomonas* alanine was the main amino acid constituting about 17.5% (w/w) of total pool (Wallace *et al.*, 1970).

Bhandari and Nicholas (1981) purified glutamine synthetase from *Nitrosomonas europaea* (molecular wt. 440,000) and studied some of its properties. Glutamate synthase was not detected in *Nitrosomonas europaea* (Bhandari and Nicholas, 1981). It was suggested that glutamine synthetase in *Nitrosomonas* is required to produce glutamine needed for the biosynthesis of various metabolic compounds. The purified enzyme was inhibited by a variety of amino acids and nucleotides.

### 1.2 AIMS OF THE STUDY

This thesis is concerned with the biochemical studies with two nitrifying bacteria; *Nitrosomonas europaea* and *Nitrobacter agilis*. The following lines of enquiry were pursued:

(i) Determination of the stoichiometry of NO$_2^-$ oxidation by washed cells, spheroplasts, and membrane vesicles of *Nitrobacter agilis* using electrode techniques to measure NO$_3^-$ production and O$_2$ uptake simultaneously and continuously. In addition the effects of various metabolic inhibitors on NO$_2^-$ oxidation were also investigated.

(ii) Investigation of possible pathways of nitrogen assimilation in *Nitrobacter agilis* using stable isotopes of $^{15}$N ($^{15}$NO$_2^-$, $^{15}$NO$_3^-$, $^{15}$NH$_2$OH and $^{15}$NH$_4^+$) and specific inhibitors of glutamine synthetase and glutamate synthase.

(iii) Purification, properties and regulation of glutamine synthetase from *Nitrobacter agilis* and *Nitrosomonas europaea* and the role of glutamate dehydrogenase in *Nitrobacter agilis*.

(iv) Studies on proton translocation during substrate oxidation by *Nitrosomonas europaea* and *Nitrobacter agilis* using oxygen pulse technique to determine $\Delta$H$^+$/O ratio for specific substrate.
Determination of proton-motive force (Δp) in *Nitrosomonas europaea* and *Nitrobacter agilis* and the role of Δp in mediating ATP biosynthesis.

Studies on Na\(^+\) and K\(^+\) transport in *Nitrosomonas europaea* and *Nitrobacter agilis* and their relationship to Δp.

Investigation of the source of oxygen in nitrate, produced from the oxidation of nitrite by *Nitrobacter agilis*, using stable isotopes of \(^{15}\)N (\(^{15}\)NO\(_2\) and \(^{15}\)NO\(_3\)) and \(^{18}\)O (\(^{18}\)O\(_2\), H\(^2\)\(^{18}\)O and P\(^{18}\)O\(_4\)) in GC/MS and \(^{15}\)N-NMR studies.
2. MATERIALS AND METHODS
2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and biochemicals

All nucleotides and amino acids, imidazole, γ glutamyl hydroxamate, valinomycin, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), sodium tetraphenyl boron (NaB(C₆H₅)₄), triphenyl methyl phosphonium bromide (TPMP⁺Br⁻) dimethyl sulfate, trimethyl phosphate, N-methyl-N-nitroso-p-toluene sulfonamide (for the preparation of diazomethane), α-ketoglutarate, hydroxylamine hydrochloride, azaserine, L-methionine-Dl-sulphoximine (l'1SX), N-N'-dicyclohexyl carbodiimide (DCCD), antimycin-A, 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO), quinacrine-hydrochloride, rotenone, pyruvic acid, succinic acid and Tris(hydroxymethyl)-aminoethane were from Sigma Chemical Co., St. Louis, U.S.A. 2-trichloromethyl pyridine (2-TlIP) was synthesized by Miss Tina Chambers and Dr. S.F. Lincoln of the Botany and Physical and Inorganic Chemistry Departments respectively, University of Adelaide. Nigericin was purchased from Eli Lilly & Co., Indiana, U.S.A. 2.5 diphenyloxazole (PPO) and 1,4 bis (2,(4 methyl-5-phenyl oxazolyl)) benzene(POPOP) were from Packard Instrument Co. Chicago, U.S.A. Phase combining system (PCS®) liquid scintillation fluid was from Amersham, Bucks, England.

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

2.1.2 Stable isotopes

¹⁵NH₄Cl (30 atom % excess), K¹⁵NO₃ and Na¹⁵NO₂ (both 32.5 atom % excess) were purchased from L'office National Industriel de '1' Azote (ONIA), Marseille, France. ¹⁵NH₂OH (=97 atom % excess) and H₂¹⁸O (97 atom % excess) were from Merck, Sharpe and Dohme, Montreal, Canada. H₂¹⁸O (98.3 atom % excess) was from Prochem, London, England and ¹⁸O₂ (99 atom % excess) from Yeda Research and Development, Israel. H¹⁵NO₃ (99 atom % excess) was obtained from Isomet
Corp. N.J., U.S.A. $^{15}$NO$_3^-$ (99 atom % excess) was prepared by neutralizing H$^{15}$NO$_3^-$ with KOH. $^{15}$NO$_2$ (99 atom % excess) was prepared through reduction of $^{15}$NO$_3^-$ (99 atom % excess) by lead (Pb) at 420$^\circ$C in silica crucible (Jolly, 1964). The $^{15}$NO$_2$ so prepared was dissolved in distilled water, filtered and dried. The stock solutions of $^{15}$NO$_2$ contained 10 and 30% $^{15}$NO$_3^-$ and rest $^{15}$NO$_2$ in two separate preparations used for GC/MS and NMR studies respectively.

H$_3^18$O$_4$ was prepared by the reaction of excess H$_2^18$O (97 atom excess) (0.5ml) with PCl$_5$ (1.3g): PCl$_5$ + 4H$_2^18$O $\rightarrow$ H$_3^18$O$_4$ + 5HCl. Hydrochloric acid and water were evaporated from the reaction mixture in a 100$^\circ$C oven and this step also served to hydrolyse traces of pyrophosphate present initially in the preparation. Pyrophosphate was detected by means of high voltage paper electrophoresis (Section 2.2.16.2). $^{15}$N, $^{18}$O-labelled nitrate standards (for NMR study) were prepared by the method of Bunton et al. (1953).

2.1.3 Radioisotopes

The radioisotopes [$^3$H] tetraphenyl phosphonium bromide (TPP$^+$/Br$^-$) (23.7 Ci.mmol$^{-1}$), acetyl [carboxyl-$^{14}$C] salicylic acid (20mCi.mmol$^{-1}$), and [$^{14}$C] inulin (5.6 Ci.mmol$^{-1}$) were from Amersham, Bucks, England. $^3$H$_2$O (1Ci.mol$^{-1}$), [7-$^{14}$C], benzoic acid (22.6 mCi.mmol$^{-1}$), [$^{14}$C] methylamine hydrochloride (51.8 mCi.mmol$^{-1}$), [$^{14}$C] sucrose (1 mCi.mmol$^{-1}$) and $^{22}$NaCl (293.4 mCi.mg$^{-1}$) were purchased from New England Nuclear (NEN), Boston, U.S.A.

2.1.4 Solutions, buffers and solvents

Unless stated otherwise, the aqueous solutions, buffers and reagents used in this study were prepared in double glass distilled water. All the organic solvents used were redistilled from glass before use.

2.1.5 Chromatographic materials

Affinity chromatographic media (Blue Sepharose CL-6B and 2',5'-ADP Sepharose-4B) and gel filtration media (Sepharose-4B and Sepharose-6B) were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Dowex-50 H$^+$ form, was from Sigma Chemical Co., St. Louis, U.S.A.
2.1.6 *Enzymes and marker proteins*

Phospholipase A₂, firefly lanterns, carbonic anhydrase, catalase and snake venom phosphodiesterase were purchased from Sigma Chemical Co., St. Louis, U.S.A. Molecular weight standards were from Pharmacia Fine Chemicals, Uppsala, Sweden.

2.1.7 *Bacterial cultures*

*Nitrobacter agilis* was kindly supplied by Prof. M.I.H. Aleem of University of Kentucky, U.S.A. and one strain (ATCC-14123) was also purchased from the American Type Culture Collection, U.S.A. The strain of *Nitrosomonas europaea* used was obtained from Dr. Jane Meiklejohn of Rothamsted Experimental Station, U.K.

2.2 *METHODS*

2.2.1 *Growth and harvesting the bacteria*

Cultures of *Nitrosomonas europaea* were grown in 40l batches at 28°C with vigorous aeration in the following growth medium (g l⁻¹): (NH₄)₂SO₄, 4; KH₂PO₄, 0.5; CaCl₂ · 2H₂O, 0.004; MgSO₄ · 7H₂O, 0.05; chelated iron (methyldiamine bis-orthohydroxy-phenylacetic acid), 0.0001 and CuSO₄ · 0.00002. The pH of the cultures were continuously adjusted to 7.8 with sterile 20% (w/v) K₂CO₃ using an automatic pH stat unit (Radiometer, Copenhagen).

*Nitrobacter agilis* was grown in batch cultures (8 or 40l) in a growth medium based on those of Aleem (1968) and Wallace and Nicholas (1968). The medium contained (g l⁻¹): NaNO₃, 1; KH₂PO₄, 1; NaCl, 0.3; MgSO₄ · 7H₂O, 0.5; MnSO₄, 0.002 and Fe₂(SO₄)₃, 0.005. After sterilization the pH of the medium was adjusted to 7.5 with sterile 20% (w/v) K₂CO₃.

Unless stated otherwise, the media were inoculated with 10% (v/v) of an exponentially growing culture of *Nitrosomonas europaea* or *Nitrobacter agilis* respectively.

Cells were harvested in a Sorvall RC-2B centrifuge fitted with a continuous flow rotor (Ivan Sorvall, Inc., Connecticut, U.S.A.), at 4°C at 30,000g with a
flow rate of 12.8 h⁻¹. Cells were washed several times with 50mM Tris-HCl buffer (pH 7.5 to 7.8) and either used immediately or stored at 4°C.

2.2.2 Preparation of spheroplasts and membrane vesicles

The spheroplasts and membrane vesicles of *Nitrobacter agilis* were prepared as follows: 1g wet weight of cells suspended in 50ml of 50mM Tris-HCl buffer (pH 8.0) containing 0.2M sucrose, 1mM Na₂EDTA and 80mg lysozyme in a 100ml Erlenmeyer flask were incubated at 30°C in a waterbath shaker (60 rev. min⁻¹) for 2h. Then 2 µg DNAse and 2ml of 2mM Mg-acetate were added and incubation continued for 1h. A pellet of spheroplasts was then obtained by centrifuging the suspension at 8000g for 15 min at 4°C. The pellet, washed twice with cold Tris-HCl-sucrose buffer was finally suspended in 5ml of the same buffer. The spheroplasts thus prepared remained active for 5 days. Membrane vesicles were prepared by incubating spheroplasts (1g wet weight) for 15 min in 25ml 50mM Tris-HCl buffer containing 10mM Na₂EDTA, 10mM MgSO₄ and 100ug DNAse. They were then subjected to ultrasonic treatment with an MSE probe unit at maximum output with short bursts of 1 min over a period of 20 min at 4°C in an icebath. After centrifuging at 5000g for 20 min to remove intact spheroplasts the supernatant fraction was further centrifuged at 100,000g for 2h. The pellet containing membrane vesicles was washed several times with cold 100mM Tris-HCl buffer (pH 7.5) containing 10mM Na₂EDTA and finally suspended in the same buffer. Both spheroplasts and membrane vesicles were stored at 2°C.

2.2.3 Preparation of cell-free extracts

Cell extracts of both *Nitrosomonas europaea* and *Nitrobacter agilis* were prepared by sonication of cell suspensions (approx.0.1g wet weight ml⁻¹) with an ultrasonic probe (20 kilocycles s⁻¹) with 1-2 min bursts over a period of 20-30 min at 4°C. Unless stated otherwise, the sonicated suspensions were centrifuged at 30,000g for 20 min at 4°C and the supernatant (S₃₀) used for study.

2.2.4 Incorporation of ¹⁵N labelled compounds into cell nitrogen

The incubation mixture contained: washed cell suspension (25mg protein), ¹⁵N-labelled substrate (either Na¹⁵NO₂, K¹⁵NO₃, ¹⁵NH₂OH.HCl or ¹⁵NH₄Cl, 1mg
equivalent $^{15}$N) and KHCO$_3$ (1.5mM final concentration) in a final volume of 6ml of 100mM-sodium phosphate buffer (pH 7.8). Unless stated otherwise, the incubation was for 2h in a 100ml Erlenmeyer flask in a waterbath shaker at 30°C. The reaction was terminated by adding 30ml chilled water and the contents were immediately centrifuged at 30,000g for 10 min at 4°C. The pellet thus obtained was washed several times with distilled water and finally suspended in a small volume (3-5ml) of water. The cell suspension was then transferred to a 100ml Kjeldahl flask containing 4ml 36-N H$_2$SO$_4$ and 2g of the digestion mixture (7gHgO + 93g Na$_2$SO$_3$) and digested by heating. The ammonia produced by NaOH treatment of digested samples was distilled into boric acid and concentrated to 2ml after adding 0.1ml of 1N H$_2$SO$_4$. The samples were then transferred into one limb of a Rittenberg tube and alkaline (1% w/v) hypobromite was added to the other (Sims and Cocking, 1958). The Rittenberg tube was evacuated to 133.22 x 10$^{-7}$ Pa and the contents mixed to generate N$_2$ gas from ammonia. The $^{15}$N$_2$ (+ N$_2$) was then introduced via an evacuated expansion flask into an AE-I-MS2 mass spectrometer (Nicholas and Fisher, 1960) for $^{15}$N enrichment analysis.

For experiments with cell extracts (S$_3$) the reaction mixture was as for washed cells, but the reaction was stopped by adding 10% (w/v) trichloroacetic acid (TCA) followed by centrifugation at 20,000g for 15 min. The pellet was washed several times in cold TCA and samples for $^{15}$N analysis were prepared as described above.

2.2.5 Enzyme purification

2.2.5.1 Glutamine synthetase

Cell extracts (S$_{30}$) were prepared in 10mM Tris-HCl, 1mM MnCl$_2$ buffer (pH 7.2). In *Nitrobacter agilis*, the S$_{30}$ fraction was heat-treated at 50°C for 15 min with constant stirring, then chilled in ice for 15 min and centrifuged at 30,000g for 15 min. The supernatant contained all the glutamine synthetase activity. Longer heat treatment or higher temperatures resulted in a loss of enzyme activity. The supernatant was loaded onto a Blue-Sepharose CL-6B column (1.5 x 9cm) preequilibrated with 10mM Tris-HCl, 1mM MnCl$_2$ buffer (pH 7.2). The column was then
washed with buffer (flow rate 50 ml h\(^{-1}\)) until the absorbance (A\(_{280}\)) was close to zero. Glutamine synthetase was eluted from the column with 2 mM ADP in the same buffer. Active fractions were pooled, dialysed against the buffer overnight, concentrated on an Amicon PM-10 membrane and loaded onto a Sepharose-4B column (2 x 70 cm) pre-equilibrated with the buffer. The enzyme was eluted with the same buffer (flow rate 12 ml h\(^{-1}\)) and the active fractions pooled and concentrated as before.

In *Nitrosomonas europaea*, the S\(_{30}\) was heat-treated at 65°C for 15 min with constant stirring, chilled on ice for 15 min and centrifuged as for *Nitrobacter agilis*. The pH precipitation step was carried out essentially as described by Bhandari and Nicholas (1981). The pH of the supernatant obtained after heat treatment adjusted to 5.2 with 1 M acetic acid, was allowed to stand on ice for 30 min and then centrifuged at 30,000 g for 30 min. The pellet was discarded and the pH of the supernatant fraction was adjusted to 4.2 with 1 M acetic acid. After standing on ice for 20 min, it was centrifuged at 30,000 g for 30 min and the pellet homogenised in 10 ml of 10 mM Tris-HCl, 1 mM MnCl\(_2\) buffer (pH 7.2). To this suspension was added half volume of 20% (w/v) polyethylene glycol (PEG) in 50 mM Tris-HCl buffer (pH 7.5), drop by drop with constant stirring and after standing on ice for 15 min it was centrifuged at 30,000 g for 15 min. The pellet resuspended in 4 M NaCl in 50 mM Tris-HCl buffer (pH 7.5) was added drop by drop with constant stirring to an equal volume of 20% (w/v) PEG in 50 mM Tris-HCl buffer (pH 7.5) and after standing on ice for 20 min it was centrifuged at 30,000 g for 20 min. Most of the glutamine synthetase was recovered in the supernatant fraction which was dialysed overnight against 10 mM Tris-HCl, 1 mM MnCl\(_2\) buffer (pH 7.2). To remove PEG, the second pH precipitation step was repeated to precipitate the enzyme which was then washed with buffer and resuspended in a small volume of the same buffer.

2.2.5.2 Glutamate dehydrogenase

A similar procedure was used to purify glutamate dehydrogenase from both *Nitrobacter agilis* and *Nitrosomonas europaea*. Crude extract (in 50 mM Tris-HCl, 1 mM β mercaptoethanol, pH 7.5) S\(_{30}\), was centrifuged at
at 110,000 x g for 1 h and the supernatant $S_{110}$ was loaded onto a 2'5' ADP Sepharose-4B column (0.8 x 9cm) preequilibrated with buffer (50mM Tris-HCl, 1mM β mercaptoethanol pH 7.5). The column was washed with buffer (flow rate 40ml.h$^{-1}$) until the absorbance ($A_{280}$) was close to zero. Enzyme was then eluted with 2mM NADPH in buffer and the fractions containing enzyme activity were pooled and dialysed overnight against the same buffer. All purified enzymes were stored at $-15^\circ$C.

2.2.6 Enzyme assays

2.2.6.1 Adenosine triphosphatase (ATPase)

ATPase activity in membrane fractions of Nitrobacter agilis was determined by measuring the release of inorganic phosphate (Pi) from ATP. The reaction mixture in a total volume of 1ml contained membrane proteins (1.0 to 2.0mg), Tris-HCl buffer (pH 7.3), 1.8 μmol of ATP (pH 7.3) and 3 μmol of MgSO$_4$. Incubation was at 30$^\circ$C in a water bath shaker. After appropriate incubation period, the reaction was terminated by adding 0.5ml 10% (w/v) trichloracetic acid (TCA) and then centrifuging at 10,000g for 15 min. The Pi release from ATP was then determined by the method of Fiske and SubbaRow (1925).

2.2.6.2 Glutamine synthetase

Both the $\gamma$ glutamyl transferase and biosynthetic activities of glutamine synthetase were determined by the method of Shapiro and Stadtman (1970a). For transferase assay, the reaction mixture in a final volume of 1ml contained (mM): imidazole-HCl (pH 7.2), 40; glutamine, 30; hydroxylamine hydrochloride (neutralized with 2M-NaOH), 30; MnCl$_2$.4H$_2$O, 0.5; sodium arsenate, 20; ADP, 0.4; and an appropriate aliquot of enzyme. For the in vivo assay in whole cells, the assay mixture also contained 20 μg ml$^{-1}$ cetyl trimethyl ammonium bromide (CTAB). Control tubes without glutamine and hydroxylamine respectively were always included. For biosynthetic activity the assay mixture in a final volume of 0.2ml contained (mM): imidazole-HCl (pH 7.0), 50; glutamate, 100; NH$_4$Cl, 50; ATP, 10; MgCl$_2$.5; and an appropriate aliquot of enzyme. Glutamate was omitted from control tubes and a correction was also made for non-enzymatic production of Pi from ATP. All incubations were at 37$^\circ$C, usually for 15-30 min.
2.2.6.3 Glutamate synthase

Glutamate synthase activity was determined spectrophotometrically as described by Meers et al. (1970) from the rate of oxidation of NADH at 340nm in a 1cm quartz cuvette, following the addition of aliquots of enzyme preparation to a solution containing (mM): α-ketoglutarate,5; NADH,0.35; glutamine 5 and 50mM Tris-HCl buffer (pH 7.6) in a final volume of 3ml.

2.2.6.4 Glutamate dehydrogenase

Activity of glutamate dehydrogenase was determined as described by Hooper et al. (1967) either from the rate of oxidation of NAD(P)H (amination reaction) or rate of reduction of NAD(P) (deamination reaction) at 340nm. For amination reaction (NAD(P)H+NAD(P)) the assay mixture in a total volume of 3ml contained (mM): α-ketoglutarate 20; NH₄Cl,240; NAD(P)H,0.33; Tris-HCl buffer (pH 7.8-8.0),50; and an appropriate aliquot of the enzyme preparation. For the deamination reaction (NAD(P)+NAD(P)H) the assay mixture in a final volume of 3ml contained (mM): sodium glutamate,17; NAD(P),0.33; Tris-HCl buffer (pH 9.0),50; and an appropriate aliquot of the enzyme preparation. The amination and deamination reactions were started by adding α-ketoglutarate and glutamate respectively. The reaction rates were corrected for endogenous oxidation/reduction of NAD(P)H/NAD(P).

2.2.7 Determination of $K_m$ and $K_i$ for glutamine synthetase and glutamate dehydrogenase

The $K_m$ values were determined as described by Lineweaver and Burk (1934) from double reciprocal plots of rate of the reaction against the initial substrate concentration. The $K_i$ values were determined by a double reciprocal plot of the reaction rate against the substrate concentration in the presence of an inhibitor as described by Lineweaver and Burk (1934).

2.2.8 Determination of molecular weight of glutamine synthetase by gel filtration

The molecular weight of glutamine synthetase was determined by the method of Andrews (1970) using Sepharose 6-B column. The column (1.6 x 100cm) prepared as described in Section 2.2.16.4 was equilibrated with 50mM Tris-HCl.
buffer (pH 7.5) and calibrated with aldolase (158,000), catalase (232,000), ferritin (440,000) and thyroglobulin (669,000). Blue dextran (=2,000,000) was used to determine the void volume. The distribution coefficient (Kd) was calculated by the formula,

\[ Kd = \frac{V_e}{V_o} \]

where Ve and Vo are elution and void volume respectively.

2.2.9 Calculation of cumulative inhibition for glutamine synthetase

Cumulative inhibition was calculated by the procedure of Stadtman et al. (1968):

\[ A + \frac{B}{100} (100-A) \]

where the percent inhibition due to each inhibitor is represented by A and B respectively.

2.2.10 Native and SDS polyacrylamide gel electrophoresis (PAGE and SDS-PAGE)

Discontinuous, non-denaturing PAGE was carried out in 5 and 7% (w/v) polyacrylamide tube gels (Davis, 1964). The stacking gel was 3% (w/v) polyacrylamide in 125mM Tris-HCl buffer (pH 6.8) and the running gel 5 or 7% (w/v) polyacrylamide in 375mM Tris-HCl buffer (pH 8.8). The electrode buffer contained 12.5mM Tris, 96mM glycine (pH 8.4). Electrophoresis was carried out at 2mA per gel at constant current. Gels were stained for protein either with coomassie brilliant blue R-250 (Chrambach et al., 1967) or by silver staining method of Wray et al. (1981). Glutamine synthetase activity was detected in the gels, washed once in cold 50mM Tris-HCl (pH 7.2) and then incubated at 37°C with transferase assay mixture (Section 2.2.6.2) for 15 to 20 min in a water bath shaker. Activity band for the enzyme was detected by adding FeCl₃ reagent (0.4g FeCl₃, 0.24g TCA and 0.25ml, 12N-HCl in a final volume of 10ml). The gels were immediately photographed. In another procedure the gels, after electrophoresis were cut into 2mm slices. Each slice was then individually checked for transferase activity using the standard assay mixture (Section 2.2.6.2).

Specific staining for glutamate dehydrogenase in the polyacrylamide gels was done following the deamination reaction according to Tally et al. (1972).
After electrophoresis the gels were incubated for 20 to 30 min in the dark at 37°C in a reaction mixture containing: 40ml 0.1M Tris-HCl buffer (pH 8.25), 3ml 0.1M sodium glutamate, 1ml 0.0065M phenazine methosulfate, 2ml 0.0057M nitro-blue tetrazolium and 1.3ml 0.022M NAD⁺ or NADP⁺ or both. After the bands had appeared the gels were rinsed in distilled water and stored in 7% (v/v) acetic acid until photographed.

The subunit molecular weight of glutamine synthetase was determined by discontinuous gel electrophoresis (10 to 12% w/v polyacrylamide) in the presence of 0.1% (w/v) sodium dodecyl sulfate (SDS) using Tris-glycine buffer (pH 8.3) according to the methods of Laemmli (1970) and Weber and Osborn (1975). The gels were calibrated with the following SDS treated protein standards: phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000) and trypsin inhibitor (20,100). Gels were stained by coomassie blue method of Chrambach et al. (1967).

2.2.11 Measurement of oxygen uptake with oxygen electrode

The aerobic oxidation of NH₄⁺, NH₂OH and N₂H₅⁺ by Nitrosomonas europaea and NO₂⁻ by Nitrobacter agilis was measured by means of oxygen electrodes (Rank Bros., U.K. and Department of Biochemistry., University of Bristol, U.K.) which take 4.5 and 5.5ml, respectively, of reaction mixture. The concentrations of dissolved O₂ in equilibrium with air were determined by the method of Chappell (1964). The relevant reactions at pH 7 to 8 for Nitrosomonas europaea are (Lees, 1952; Nicholas, 1963):

(i) \( \text{NH}_4^+ + 30 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + 2\text{H}^+ \)
(ii) \( \text{NH}_2\text{OH} + 20 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + \text{H}^+ \)
(iii) \( \text{N}_2\text{H}_5^+ + 20 \rightarrow \text{N}_2 + 2\text{H}_2\text{O} + \text{H}^+ \)

and for Nitrobacter agilis is (Nicholas, 1963; Aleem, 1965):
(iv) \( \text{NO}_2^- + 0 \rightarrow \text{NO}_3^- \)

Unless stated otherwise, for electrode measurements, the buffer was 50mM Tris-HCl buffer (pH 7.5).

2.2.12 Proton translocation

2.2.12.1 Fluorescence quenching method

The fluorescence emission was followed as described by Tuovinen
et al. (1977). Measurements were made in a 1 cm quartz cell in a Fluorispec (Model SF-1) fluorimeter (Baird Atomic, Cambridge, Massachusetts, U.S.A.) at 420nm excitation and 485 emission. A potentiometric unit (Servoscribe 1S) was used to record the data. Fluorescence of the reaction mixture was measured in arbitrary units.

2.2.12.2 Oxygen pulse experiments

These experiments measured the change in proton concentration in the extracellular medium of a dense anaerobic suspension of cells, spheroplasts or vesicles caused by a short (1-4s) burst of respiration initiated by injection of a small amount of O₂ (10 to 120ng atom O). 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 apparatus was limited by the injection and fluid mixing time which was about 1.5s. Typically, 112mg wet weight of cells in 1.5ml of 0.15M KCl was supplemented with carbonic anhydrase (80 μg ml⁻¹) and the salt of a permeant ion at sufficient concentration 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 N₂ for 10 min at 25°C, at which time the pH was adjusted as required by use of anaerobic 0.1M HCl or KOH. Once the pH had stabilized, a small volume of 0.15M KCl saturated under pure O₂ was injected to initiate respiration and the subsequent proton response was recorded. The response was calibrated by injecting an appropriate amount of anaerobic 5.00mM HCl (in 0.15M KCl), the concentration of which was determined titrimetrically with reference to accurately weighed Tris-base.

A positive response to O₂ by bacteria involved a rapid acidification of the medium, followed immediately thereafter by a slow (t½ = 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 perhaps 5 to 20%, depending on respiration rate and the permeability of the membrane to protons. To correct for
this effect the decay curve for passive proton diffusion was extrapolated back to a time, approximately 1 to 2 s after O₂ injection, at which the initial transient had reached half its final amplitude, as suggested by Scholes and Mitchell (1970b).

The solubility of O₂ in 0.15 M KCl was taken as 2.32 mg atom 0.1⁻¹ at 1 atm. and 25°C (Chappell, 1964).

Internal and external buffering capacities were estimated as described by Scholes and Mitchell (1970a) from the initial pH response of the system to acid pulses relative to the final equilibrium value after relaxation of protons across the membrane. With both Nitrosomonas europaea and Nitrobacter agilis about 2/3 of the total buffering capacity was external to the membrane and about 1/3 internal, under the conditions of experiment. A similar distribution applied in the case of spheroplasts and vesicles from Nitrobacter agilis.

2.2.12.3 Reductant pulse experiments

These experiments used the same apparatus as described above and are analogous to oxygen pulse experiments, except that small amounts (10 to 150 nmol) of NH₄⁺, NH₂OH or N₂H₅⁺ in the case of Nitrosomonas europaea were injected into a system under pure O₂. Because the initial concentration of reductant was typically well below its Kₘ value (e.g. 30 nmol NH₄⁺ in 1.5 ml = 20 μM; Kₘ for NH₄⁺ at pH 7.4 is approximately 1.0 mM) respiration rates were relatively slow. The response, especially in the absence of a permeant ion, was typically a progressive acidification from an initial value to a final value rather than one showing the sharp maxima observed for oxygen pulses.

2.2.12.4 Estimation of stoichiometric protons

The reactions considered in Equations i-iii (Section 2.2.11) involve the stoichiometric production of protons. In order to calculate the true + H⁺/O ratio in oxygen pulse studies for the protons translocated by the proton pump it was necessary to determine the yield of stoichiometric protons and subtract the value from the overall yield.
of protons. Stoichiometric protons were estimated by two techniques: (i) reductant pulse experiments in the absence of a permeant ion, and (ii) oxygen pulse experiments in which 3-5 \(\mu\)M CCCP was used to bring about the rapid equilibration of protons between internal and external buffer compartments. CCCP is known to be an effective inhibitor of respiration in *Nitrosomonas europaea* (Bhandari and Nicholas, 1979a) and *Nitrobacter* (Cobley, 1976a), but the extent of inhibition at 3 to 5 \(\mu\)M was insufficient to interfere seriously with the oxygen pulse experiments.

### 2.2.12.5 Permeant ions

In oxygen pulse studies, it is necessary to collapse membrane potential so that optimum proton ejection can be observed (Scholes and Mitchell, 1970b). The salts of all the permeant ions used in this study (Section 3.5.1.2) were dissolved in double distilled water. Where \(\text{ClO}_4^-\) was used, it was added as \(\text{NaClO}_4\) at a final concentration of 0.3M to a solution already 0.15M in \(\text{K}^+\). Inasmuch as \(\text{KClO}_4\) is relatively insoluble (\(K_{\text{sol}} = 10^{-2}\) at 25°C) much of the \(\text{K}^+\) and some of the \(\text{ClO}_4^-\) were precipitated to give actual concentrations of \(\text{K}^+, \text{Na}^+\) and \(\text{ClO}_4^-\) of about 0.05, 0.3 and 0.2M respectively.

### 2.2.13 Determination of membrane potential (\(\Delta\psi\)) and transmembrane pH gradient (\(\Delta p_{\text{H}}\)) in washed cells

#### 2.2.13.1 EDTA treatment of cells

Cells of both *Nitrosomonas europaea* and *Nitrobacter agilis* were suspended in 100mM Tris-HCl buffer, pH 8.0 (20mg . ml\(^{-1}\)) and treated with 5mM EDTA/KOH, pH 7.0 (for *Nitrosomonas europaea*) and 10mM (for *Nitrobacter agilis*) for 10 min at 37°C. The cells collected by centrifugation were washed once in the buffer and suspended in the appropriate buffer. The EDTA-treated cells were used within 2h.

#### 2.2.13.2 Intracellular water space

This was determined by using \(_3^3\text{H}_2\text{O}\) (for total pellet water), \(^{14}\text{C}\) sucrose (for total pellet water - intracellular water, which does not include periplasmic space) and \(^{14}\text{C}\) inulin (for extracellular water) according to the methods of Maloney *et al.* (1975) and Stock *et al.* (1977).
Thus for *Nitrosomonas europaea* and *Nitrobacter agilis* the intracellular water spaces were $1.7 \pm 0.2$ and $1.2 \pm 0.2 \mu l (mg\ dry\ weight)^{-1}$ respectively.

2.2.13.3 Uptake of radioactive probes

Untreated or EDTA-treated cells were incubated at $25^\circ C$ in either Na$^+$ or K$^+$ phosphate (100mM) or Tris-HCl (50mM) buffer at the appropriate pH. The cell suspensions were either vigorously oxygenated for 10 min with pure oxygen or mixed with catalase ($0.05mg\ ml^{-1}$) and H$_2$O$_2$ ($1\mu l\ ml^{-1}$). For *Nitrosomonas*, 5mM NH$_4$Cl and for *Nitrobacter* 5mM NaNO$_2$ was the substrate. Then the radioactive compound was added and incubation continued for a further 5-15 min. Aliquots (1 ml) were then centrifuged in Eppendorf microfuge at 13,000g for 1 min. Aliquots of the supernatant (100μl) and the cell pellet respectively were added to 1 ml, 3M perchloric acid in 15ml scintillation glass vials. After 30 min, when cell proteins were completely dissolved, 5ml of a scintillation counting fluid (PCS) (Amersham, England) was added to each vial, the contents mixed thoroughly and radioassayed in a Packard Tri-Carb 460 CD liquid scintillation spectrometer. In the standard protocol two consecutive experiments were carried out in which $\Delta \psi$ and $\Delta \rho$ were measured. For $\Delta \psi$ determination [${}^3H$] TPP$^+$ Br$^-$ (20-50 nCi.ml$^{-1}$) was added to a cell suspension (1-1.5mg dry weight. ml$^{-1}$). For $\Delta \rho$ determination [${}^{14}C$] benzoic acid (2 μCi.ml$^{-1}$), [${}^{14}C$] acetyl salicylic acid (2 μCi.ml$^{-1}$) or [${}^{14}C$] methylamine-hydrochloride (1 μCi.ml$^{-1}$) was added. $^3H_2O$ was used to determine total pellet water.

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

The calculations of $\Delta \psi$ and $\Delta \rho$ were made by using the Nernst equation as described by Mitchell (1966) after correcting the uptake data for non specifically bound [${}^3H$] TPP$^+$ and extracellular counts of [${}^{14}C$] benzoic acid, [${}^{14}C$] acetyl salicylic acid and [${}^{14}C$] methylamine respectively. Membrane potential ($\Delta \psi$) was calculated from the uptake of [${}^3H$] TPP$^+$ as follows:
\[ \Delta \psi = \frac{RT}{F} \ln \frac{[\text{TPP}^+]_{\text{in}}}{[\text{TPP}^+]_{\text{out}}} \]

or
\[ \Delta \psi = -2.303 \frac{RT}{F} \log \frac{[\text{TPP}^+]_{\text{in}}}{[\text{TPP}^+]_{\text{out}}} \]

At 27°C (T = 300°K) \( 2.303 \frac{RT}{F} \approx 60 \text{mV} \)

\[ \Delta \psi = 60 \times \log \frac{[\text{TPP}^+]_{\text{in}}}{[\text{TPP}^+]_{\text{out}}} \]

where \( \frac{[\text{TPP}^+]_{\text{in}}}{[\text{TPP}^+]_{\text{out}}} \) is the ratio of intracellular \(^{3}\text{H}\) TPP\(^+\) to extracellular \(^{3}\text{H}\) TPP\(^+\).

Intracellular pH (pHi) was calculated from the distribution of \(^{14}\text{C}\) weak acids (benzoic and acetyl salicylic);
\[ \text{pHi} = \text{pK} + \log \frac{[\text{Ain}]/[\text{Aout}]}{(10^{(\text{pHe} - \text{pK})} + 1)-1} \]

or by the distribution of weak base \(^{14}\text{C}\)-methylamine;
\[ \text{pHi} = \text{pK} - \log \frac{[\text{Bout}]}{[\text{Bin}]/(10^{(\text{pK} - \text{pHe})} + 1)-1} \]

\( \Delta \text{pH} \) was obtained from the difference of pHi and pHe (\( \Delta \text{pH} = \text{pHi} - \text{pHe} \)).

Proton-motive force (\( \Delta \rho \)) was calculated as:
\[ \Delta \rho = \Delta \psi - 2.303 \frac{RT}{F} \Delta \text{pH} \text{ or at 27°C } \Delta \rho = \Delta \psi - 60 \Delta \text{pH} \]

2.2.14 Na\(^+\) and K\(^+\) transport

2.2.14.1 K\(^+\) depletion of cells

The method used for K\(^+\) depletion of both Nitrosomonas europaea and Nitrobacter agilis was essentially the same as described by Nakamura et al. (1982). For routine use, freshly harvested cells (≈500mg wet weight) washed twice with either 100mM K-phosphate (pH 7.5) or 50mM Tris-HCl (pH 7.5) were suspended in about 50mL, 50mM diethanolamine-HCl, 150mM NaCl (pH 9.2) and incubated at 30°C for 30 min. The cell suspension was then centrifuged at 15,000g for 10 min. The pellet was re-suspended in 50mM diethanolamine-HCl, 150mM NaCl (pH 9.2) and treated similarly as described above but for 15 min instead of 30 min. The cell
suspension was then recentrifuged at 15,000g for 10 min and the pellet resuspended in the appropriate buffer. Cells prepared thus were washed once in an appropriate buffer and contained <5mM K⁺. Unless stated otherwise, these diethanolamine treated cells are referred as "K⁺ depleted cells" in the text.

2.2.14.2 Na⁺ and K⁺ determinations by atomic absorption spectroscopy

For cellular Na⁺ and K⁺ determination, cell suspensions were filtered through Millipore 0.22μm or 0.45μm filters (Type GS or HWA), washed twice with at least 2ml volume of either 50mM Tris -HCl (pH 7.5) or buffered choline chloride (0.2M choline chloride in 10mM Tris -HCl pH 7.25). The filters were then immersed in 5ml 5% (w/v) trichloroacetic acid (TCA) in acid washed plastic centrifuge tubes (10ml volume) and left overnight in a waterbath shaker at 30°C. The Na⁺ and K⁺ contents of the TCA extracts were determined in a Varian atomic absorption spectrometer after adjusting the volume to 10ml with deionized distilled water. The machine was calibrated with standard solutions of KCl and NaCl before and after each set of 6 determinations. Appropriate controls (including for K⁺ and Na⁺ contents in filters, plastic tubes, TCA and deionized distilled water) were always included. All solutions used in atomic absorption studies were prepared in deionized distilled water.

2.2.15 Stable isotope experiments with ¹⁵N and ¹⁸O labelled compounds to study NO₂⁻ oxidation by cells of Nitrobacter agilis

The aim of the experiments was to determine the ¹⁸O contents of NO₃⁻ produced during the aerobic oxidation of NO₂⁻ at pH 7.8 by Nitrobacter agilis in the presence of ¹⁸O₂, H₂¹⁸O or [¹⁸O] phosphate. Two techniques were used for isotopic analysis namely gas chromatography combined with mass spectrometry (GC/MS) and ¹⁵N-nuclear magnetic resonance (NMR) spectroscopy. The two sets of studies were carried out independently.

2.2.15.1 GC/MS studies

For experiments involving ¹⁸O₂ or H₂¹⁸O washed cells were suspended in either 0.1M Tris-HCl (pH 7.8) or 0.1M K-phosphate (pH 7.8). For experiments involving [¹⁸O] phosphate, cells were incubated in 0.1M [¹⁸O] K-phosphate (pH 7.8). The final volume of the reaction mixture
was 20ml except for experiments involving $H_2^{18}O$ in which case the final volume was 2ml and contained 0.5ml of $H_2^{18}O$. All experiments were carried out in 100ml Erlenmeyer flasks each closed with a Subaseal septum. For the $^{18}O_2$ experiment, flask was evacuated and back filled to latm with $^{18}O_2$. Each complete reaction mixture contained 100mg (wet weight) of cells and the reaction was started by the addition of 50 µmol of either NaNO₂ or K$^{15}$NO₂. Nitrite consumption was monitored colorimetrically (Nicholas and Nason, 1957) and upon its exhaustion another 50 µmol of nitrite was added. This was repeated until the cells oxidised a total of 250 µmol of NO₃⁻ (or $^{15}$NO₃⁻). All incubations were at 28°C in a waterbath shaker (120 rev.min⁻¹). After utilization of the final addition of nitrite, each reaction mixture was chilled to 0°C and centrifuged (15,000g for 10 min) to remove cells. Each supernatant was divided into two parts. The first part, if it contained phosphate was treated with stoichiometric amounts of NH₄OH and MgCl₂ in order to precipitate MgNH₄PO₄, which was removed by centrifugation. This step was omitted if Tris-HCl was the buffer. The phosphate depleted supernatant was then passed through a Dowex-50 H⁺ form column (10ml bed volume). The column was eluted with distilled water until the effluent reached a pH of about 6.5. The pooled effluent (pH 1.5 to 2) was neutralized to pH 7.0 with NH₄OH and lyophilized to a powder. The powder was pyrolysed in a 10ml Pyrex tube, closed with a Subaseal septum, evacuated and backfilled with Helium gas. The bottom of the tube was carefully heated on a burner flame and then 10-100µl sample of the resulting $N_2O$ gas mixture was analysed by GC/MS.

The second part was passed through a Dowex-50 column as above, but with omission of the precipitation step. In this case the acidic pooled effluent was lyophilized directly without neutralization to yield a small volume of liquid which contained mainly phosphoric and nitric acids. This liquid was methylated using diazomethane (prepared by the method of Fieser and Fieser, 1967). Excess of CH₂N₂ (in ether) was added to the sample at room temperature until the yellow colour of the distillate failed to fade away. A similar method was employed to methylate standard H₃PO₄ and H₃P¹⁸O₄ to yield trimethylphosphate ((CH₃)₃PO₄ or (CH₃)₃P¹⁸O₄). Methylation of HNO₃ was in the same manner.
but it was found necessary to add an approximately equal volume of concentrated H₂SO₄ to the HNO₃. This procedure generated a mixture of dimethyl sulfate and methyl nitrate which could be separated by GC/MS. Ether and excess CH₂N₂ were removed from the methylation mixtures by evaporation at 45°C. The methylated products were dissolved in CH₂Cl₂ for GC/MS analysis.

The yields of methyl nitrate produced by CH₂N₂ methylation of lyophilized liquid were low presumably because of the unstable nature of methyl-nitrate.

Isotopic analyses were carried out by use of a Hewlett-Packard GC/MS model 5992-B fitted with a glass column (1m x 2mm i.d.) packed with Tenax GC (60-80 mesh). The helium flow was 25ml.min⁻¹ and the electron multiplier at 2200 volts. Data were obtained by Peakfinder and Selected Ion Monitoring programmes. The latter programme allowed the monitoring of six selected amu values simultaneously. Trimethyl phosphate, dimethyl sulphate, methyl nitrate and N₂O had the following retention times respectively: 3.6 min at 160°C, 1.8 min at 160°C, 4.8 min at 50°C and 0.3 min at 25°C. The degree of ionization-induced fragmentation of N₂O⁺ into NO⁺ and N₂⁺ was similar to those reported previously, (Cady and Bartholomew, 1960; St. John and Hollocher, 1977). Trimethyl phosphate and dimethyl sulfate were dissolved in CH₂Cl₂ (1:1000) and usually 1μl was injected. Methyl nitrate was usually injected from the vapour phase without solvent (10-100μl) because it separated only poorly from common solvents such as CH₂Cl₂ on the column. The M⁺ peak for CH₃NO₃ (77 amu) was generally not observed; identification was based on the base (integral = 100) peak (NO₂⁺), NO⁺ and a weak (M-1)⁺ peak at 76 amu.

2.2.15.2 ¹⁵N-NMR studies

The incubation mixtures were similar to those described in Section 2.2.15.1 with minor changes. Washed cells of Nitrobacter agilis were suspended in 100mM K phosphate (pH 7.8) at a concentration of about 500 mg wet weight.ml⁻¹. The following experiments were done: (1) 1ml cell suspension was diluted to 10ml in 100mM phosphate, 5mM carbonate
buffer (pH 7.8); (ii) 1ml cell suspension was diluted to 10ml in the same buffer, and the flask closed with a Subaseal septum. The flask was evacuated and backfilled with $^{18}$O$_2$ to 1atm.; (iii) 1ml cell suspension was added with 1ml each of 200mM phosphate, 10mM carbonate (pH 7.8) and H$_2^{18}$O; (iv) 1ml cell suspension was centrifuged in an Eppendorf tube (1.5ml) at 13,000g for 5 min and the pellet resuspended in 10ml of $^{18}$O phosphate, 5mM carbonate buffer (pH 7.8). To all the cell suspensions in 50ml Erlenmeyer flasks was added, catalase (1mg) and 40% (v/v) H$_2$O$_2$ (5μl) (except for experiment ii) followed by incubation at 28°C in a water bath shaker. Then 50 μmol K$^{15}$NO$_2$ (97 atom %) was added to each flask to start the reaction. Aliquots (5-10μl) were withdrawn from the reaction mixtures to check NO$_2^-$ concentration by the method of Nicholas and Nason (1957). As soon as the nitrite was utilized completely another 50 μmol of $^{15}$NO$_2$ was added and the reaction continued until at least 200 μmol of total nitrite had been oxidised to nitrate. At the end of reaction, cell suspensions were centrifuged at 20,000g for 10 min at 4°C and the supernatant fractions were carefully dispensed with a Pasteur pipette. The volume of each supernatant fraction was made to 10ml with phosphate-carbonate buffer, the pH adjusted to 8.0 if needed and then immediately frozen in liquid N$_2$ until used in NMR studies.

30.42 MHz $^{15}$N-NMR spectra were obtained on a Bruker CXP 300 NMR spectrometer operating at a field strength of 7.05T. Spectra were acquired from 2 dm$^{-3}$ samples in 10mm NMR tubes as the result of approximately 200 scans into an 8K data table. A 15° (10μs) pulse was used with a 4.1s recycle time and no $^1$H decoupling. After acquisition, a line broadening of 0.1Hz was applied, together with apodisation. The data were zero filled to 16K before Fourier transformation.

2.2.16 General techniques

2.2.16.1 Electron-microscopy

Samples of spheroplasts and membrane vesicles were negatively stained with 2% (w/v) phosphotungstic acid (pH 6.6) on carbon coated
copper grids, dried in air prior to examination in an electron microscope JEOL (Model JEM.100 cx) at an acceleration voltage of 60kV.

2.2.16.2 High voltage paper electrophoresis (HVPE)

HVPE of phosphate and pyrophosphate was done according to the method of Tate (1968). Standard solutions or aliquots of reaction mixtures were spotted in the middle of a Whatman 3MM chromatographic paper (15 cm x 60 cm) near the cathode. The paper was moistened with 100 mM Na-tetraoxalate (pH 4.2) and then lightly blotted to remove surface moisture. It was then laid out on a polythene frame and placed in a ceramic tank filled with Perclene. The ends of the paper were connected by wicks to the buffer chambers containing 100 mM Na-tetraoxalate (pH 4.2). The Perclene was cooled by passing cold water through copper coils placed in the centre of the tank. A stabilized power pack (Paton Industries Ltd. Australia) was used to supply the current to the buffer chambers, (100 mA and 1500 volts for 30 min).

After electrophoresis the paper was dried by hot air and spots were detected either by silver nitrate or by molybdate staining method (Tate, 1968).

2.2.16.3 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 dried 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 460 CD).

2.2.16.4 Preparation of chromatographic columns

Affinity chromatography columns (Blue Sepharose CL-6B and 2'5'-ADP
Sepharose-4B) and gel filtration columns (Sepharose-4B and Sepharose-6B) were prepared according to the instructions given by the manufacturers (Pharmacia Fine Chemicals, Uppsala, Sweden). The columns were equilibration 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.

2.2.17 Chemical determinations

2.2.17.1 Nitrite

Nitrite was determined by the method of Nicholas and Nason (1957) and Hewitt and Nicholas (1964). An aliquot containing 30 to 500 nmol of nitrite was diluted to 1 ml with double distilled water. The red azodye was developed by adding 1 ml of 1% (w/v) sulphanilamide in 1N-HCl, followed by 1 ml of 0.01% (w/v) N-(1-naphthyl) ethylene diamine hydrochloride. After 15 min, the absorbance was read at 540 nm in 1 cm glass cuvettes, employing a Shimadzu (QV-50) spectrophotometer. The concentration of nitrite was determined from a standard curve.

2.2.17.2 Ammonia

Ammonia was determined by a modified Nessler's method (Ballentine, 1957). An aliquot containing 0.2 to 2 μmol NH₄⁺ was diluted to 0.5 ml with distilled water. Color was developed by adding 1 ml of reagent A (10% w/v, sodium potassium tartarate) and 2 ml of reagent B (2.272 g mercuric iodide, 1.826 g KI and 4 g NaOH in 100 ml distilled water). After 20 min the absorbance was read at 435 nm in 1 cm glass cuvettes employing a Shimadzu (QV-50) spectrophotometer. The concentration of NH₄⁺ was determined from a standard curve.

2.2.17.3 Protein

Protein was determined either by a microbiuret method (Itzhaki and Gill, 1964) or by the dye binding method of Bradford (1976), using bovine serum albumin as a standard. The absorbance was recorded in 1 cm quartz cuvettes in Shimadzu (QV-50) spectrophotometer.
2.2.17.4 Inorganic phosphate

Inorganic phosphate (Pi) was determined by the method of Fiske and Subba Row (1925). Samples containing Pi were diluted to 2m1 with distilled water and 0.5m1 of acid molybdate (2.5% w/v ammonium molybdate in 2.5M H2SO4) was then added followed by 0.1m1 of colour reagent. The colour reagent was prepared by mixing 1g l-amino 2-naphthol 4-sulphonic acid, 3g anhydrous sodium sulphite and 6g sodium metabisulphite and stored at 4°C in the dark. The colour reagent was prepared fresh before use by dissolving 0.25g of the mixture in 10m1 double distilled water. The colour was allowed to develop for 20 min and the absorbance read at 750nm.

2.2.17.5 ATP

ATP was determined by the firefly method of Stanley and Williams (1969). Aliquot (0.4m1) were dispensed from the reaction mixture into 0.1m1 3M perchloric acid in a test tube (1 x 5cm) kept in ice. After 15 min, 0.3m1 1M KOH was added and then 20u1 of this neutralized extract was assayed for ATP. The reaction mixture in a scintillation vial (15 x 45mm) contained 1m1 10mM sodium phosphate (pH 7.5), 0.9m1 distilled water, 0.1m1 5mM MgCl2 and 50u1 firefly extract. The vial was then placed in a liquid scintillation spectrometer (Packard Tri-Carb model 3375) set at maximum sensitivity with the two photomultipliers switched out of coincidence and assay continued for 6s. Standard solution of ATP (10-50 pmol) or the samples (20u1) were then added and after 30s assayed for 6s. The ATP concentration was calculated from a calibration of freshly prepared ATP standards.
3. RESULTS
3. RESULTS

3.1 NITRITE OXIDATION BY WASHED CELLS, SPHEROPLASTS AND MEMBRANE VESICLES OF NITROBACTER AGILIS

3.1.1 Electrode measurement of \( \text{NO}_3^- \) production and \( \text{O}_2 \) uptake

A technique has been developed to measure \( \text{O}_2 \) uptake and \( \text{NO}_3^- \) production simultaneously and continuously during \( \text{NO}_2^- \) oxidation by Nitrobacter agilis. The apparatus (Fig.1) consists of a double-walled perspex vessel (5ml volume) closed with a perspex lid. The port in the lid of the vessel accommodated an \( \text{O}_2 \) electrode (Department of Biochemistry, University of Bristol, U.K.), a \( \text{NO}_3^- \) sensitive electrode (Orion model 93-07-01) and a reference electrode (Orion model 90-02). The \( \text{NO}_3^- \) and reference electrodes were connected to a Beckman expanded scale pH meter (model 76). The electrode responses were recorded simultaneously and continuously using a two-channel Rikadenki (model B 181-H) potentiometric readout unit to convert the log response of the \( \text{NO}_3^- \) electrode to a linear scale. All the additions were made via a Hamilton microsyringe through a port in the lid of the vessel. The \( \text{NO}_3^- \) electrode was calibrated before each experiment using a standard \( \text{NO}_3^- \) solution, in 50mM Tris-HCl buffer (pH 8.0) for experiments with washed cells and in 50mM Tris-HCl buffer containing 0.2M sucrose for spheroplasts and membrane vesicles. Corrections were made for the response of electrode to \( \text{NO}_2^- \), after \( \text{NO}_2^- \) additions.

3.1.2 Stoichiometry of \( \text{NO}_2^- \) oxidation by washed cells

The addition of \( \text{NO}_2^- \) to a reaction mixture containing washed cells of Nitrobacter agilis in 50mM Tris-HCl buffer (pH 8.0) resulted in an immediate uptake of \( \text{O}_2 \) and extrusion of \( \text{NO}_3^- \) (Fig.2). Nitrite was determined in aliquots of the reaction mixture by the method of Nicholas and Nason (1957). The stoichiometry of \( \text{NO}_2^- \) oxidation by washed cells was \( 1\text{NO}_2^- : 0.5\text{O}_2 : 0.75\text{NO}_3^- \) (Fig.3). The maximum rates of \( \text{NO}_2^- \) and \( \text{O}_2 \) uptake and \( \text{NO}_3^- \) production were approximately 0.3, 0.15 and 0.26 \( \mu \text{mol min}^{-1} \cdot (\text{mg protein})^{-1} \) respectively and varied from one batch of cells to another. The uptake of \( \text{NO}_2^- \) and production of \( \text{NO}_3^- \) were dependent on \( \text{O}_2 \) and when all the \( \text{O}_2 \) from medium was consumed,
FIG. 1: ELECTRODE ASSEMBLY FOR MEASURING $\text{NO}_3^-$ PRODUCTION AND $\text{O}_2$ UPTAKE

A. $\text{NO}_3^-$ electrode (Orion model 93-07-01)
B. Reference electrode (Orion model 90-02)
C. Oxygen electrode (University of Bristol, U.K.)
D. Perspex reaction vessel (5 ml volume)
E. Port for additions (2 mm diameter)
FIG. 2: UPTAKE OF \( O_2 \) AND PRODUCTION OF \( NO_3^- \) BY WASHED CELLS

100\( \mu l \) of washed cell suspension (40\( \mu g \) wet weight) was added to a perspex vessel containing 5\( \mu l \) catalase (2\( mg.\ ml^{-1} \)) and 0.25 m mol Tris-HCl buffer (pH 8.0) in a final volume of 5\( ml \). The reaction mixture was maintained at 25\( ^{\circ} C \). Reaction was started by adding 10\( \mu mol \) of \( NO_2^- \) via a Hamilton microsyringe through a port in the lid of the vessel. \( O_2 \) was regenerated by injecting \( \approx 10\mu l \), 2\% (v/v) \( H_2O_2 \) into the reaction mixture. The reaction mixture was continuously stirred with a magnetic flea. The maximum rates alongside the traces are in \( \mu mol. min^{-1} \) for \( NO_3^- \) and \( O_2 \).

Broken line, \( NO_3^- \) production; continuous line, \( O_2 \) uptake.

FIG. 3: STOICHIOMETRY OF \( NO_2^- \) AND \( O_2 \) UPTAKE AND \( NO_3^- \) PRODUCTION BY WASHED CELLS

The reaction mixture used as given in Fig. 2. Nitrite was determined in aliquots withdrawn from the reaction mixture at 1 min intervals as described in Section 2.2.17.1.

\( NO_2^- \) utilization (●); \( NO_3^- \) production (○); \( O_2 \) uptake (□).
FIG. 2

CELLS NO$_2$

1 µMOL NO$_3$

0.9

0.63

0.1 µMOL O$_2$

1 MIN

FIG. 3

µMOL NO$_2$, NO$_3$ or O$_2$

0 2 4 6 8 10

min
both NO\textsubscript{2}\textsuperscript{−} uptake and NO\textsubscript{3}\textsuperscript{−} production ceased. Thus oxygen was regenerated in these experiments by means of catalase and H\textsubscript{2}O\textsubscript{2}.

3.1.3 Preparation of spheroplasts and membrane vesicles

Spheroplasts of Nitrobacter agilis were prepared by lysozyme-EDTA treatment of washed cells (Section 2.2.2). Under the electron microscope they appeared almost completely devoid of cell walls (Fig.4). The spheroplasts thus obtained were osmotically fragile and rapid lysis occurred when they were suspended in hypotonic solutions or distilled water, resulting in the release of DNA and a decrease in the absorbance of the spheroplast suspension. Ultrasonic treatment of spheroplasts resulted in vascicularization of membranes producing inside-out membrane vesicles. Electron microscopy of the membrane vesicles showed that they were bounded by a single membrane (Fig.5).

3.1.4 Stoichiometry of NO\textsubscript{2}\textsuperscript{−} oxidation by spheroplasts and membrane vesicles

Spheroplasts and membrane vesicles prepared as described in Section 2.2.2 were tested for NO\textsubscript{2}\textsuperscript{−} oxidising activity by the electrode method. Both preparations oxidised NO\textsubscript{2}\textsuperscript{−} at about 1/8th of the rate of washed cells. Stoichiometry of 1NO\textsubscript{2}\textsuperscript{−} : 0.5O\textsubscript{2} : 1NO\textsubscript{3}\textsuperscript{−} was recorded for both spheroplasts and membrane vesicles (Fig.6a and 6b respectively).

3.1.5 Optimum conditions for NO\textsubscript{2}\textsuperscript{−} oxidation by membrane vesicles

The optimum pH for NO\textsubscript{2}\textsuperscript{−} oxidation by membrane vesicles was 7.5 and the oxidation rate decreased rapidly above this pH. The optimum temperature was 23°C. The K\textsubscript{m} values of 0.8mM and 20µM were obtained for NO\textsubscript{2}\textsuperscript{−} and O\textsubscript{2}, respectively. Additions of any of the metal salts listed in Table 1, except for Ni\textsuperscript{2+} and Cu\textsuperscript{2+}, did not affect NO\textsubscript{2}\textsuperscript{−} oxidation while Ni\textsuperscript{2+} and Cu\textsuperscript{2+} inhibited NO\textsubscript{2}\textsuperscript{−} uptake by about 25 and 20% respectively at 1mM final concentration.

3.1.6 Effects of metabolic inhibitors on NO\textsubscript{2}\textsuperscript{−} oxidation

It is known that NO\textsubscript{2}\textsuperscript{−} oxidation by Nitrobacter is sensitive to a variety of metabolic inhibitors (O'Kelly et al., 1970; Aleem, 1977). The effects of
FIG. 4: ELECTRON MICROGRAPHS OF LYSOZYME-EDTA TREATED CELLS OF *NITROBACTER AGILIS*

Lysozyme-EDTA treatment of cells was carried out as described in Section 2.2.2. Samples were negatively stained with 2% (w/v) phosphotungstic acid (pH 6.6) on carbon coated copper grids and examined in an electron microscope JEOL (Model JEM-100cx) at an accelerating voltage of 60 kV (Section 2.2.16.1). From top:

A. Cells after 30 min lysozyme-EDTA treatment (x 20,000)
B. A single cell showing loose wall structure after a 45 min lysozyme-EDTA treatment (x 50,000)
C. Spheroplasts after washing twice with 50mM Tris-HCl, 0.2M sucrose (pH 8.0) (x 5,000).
FIG. 5: ELECTRON MICROGRAPHS OF *NITROBACTER AGILIS* MEMBRANE VESICLES

Membrane vesicles prepared as described in Section 2.2.2 were negatively stained with 2% (w/v) phosphotungstic acid (pH 6.6) on carbon coated copper grids and examined in an electron microscope JEOL (Model JEM-100 cx) at an accelerating voltage of 60 kV (Section 2.2.16.1).

From top:
A. *Nitrobacter* membranes (x 100,000)
B. A single membrane vesicle (x 150,000)
C. Sectioned membrane vesicle (x 150,000)
FIG. 5

A

B

C
FIG. 6: STOICHIOMETRY OF NO$_2^-$ AND O$_2$ UPTAKE AND NO$_3^-$ EXTRUSION BY SPHEROPLASTS (a) AND MEMBRANE VESICLES (b).

Experimental details as in Fig. 2 and 3 except that the buffer also contained 0.2M sucrose and washed cells were replaced by either spheroplasts (3.18 mg protein) or membrane vesicles (15 mg protein).

NO$_2^-$ utilization (●); NO$_3^-$ production (○); O$_2$ uptake (□).
TABLE 1: EFFECTS OF VARIOUS METALLIC IONS ON O, DEPENDENT NO\textsuperscript{2-} UPTAKE AND NO\textsuperscript{3-} PRODUCTION BY MEMBRANE VESICLES.

Experimental details as in Fig. 2 except that the reaction mixture also contained the indicated metal salt at 1mM final concentration.

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>( \text{NO}_2^- ) uptake</th>
<th>( \text{NO}_3^- ) production</th>
<th>( \text{O}_2 ) uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe\textsuperscript{2+}</td>
<td>0</td>
<td>3</td>
<td>Nd</td>
</tr>
<tr>
<td>Fe\textsuperscript{3+}</td>
<td>2</td>
<td>6</td>
<td>Nd</td>
</tr>
<tr>
<td>Co\textsuperscript{2+}</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Zn\textsuperscript{2+}</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mn\textsuperscript{2+}</td>
<td>3</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Mg\textsuperscript{2+}</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ni\textsuperscript{2+}</td>
<td>27</td>
<td>25</td>
<td>22</td>
</tr>
<tr>
<td>Cu\textsuperscript{2+}</td>
<td>17</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Nd - Not determined because of interference with determination.
some of these inhibitors on the utilization of both NO\textsuperscript{2} and O\textsubscript{2} and the production of NO\textsuperscript{3−} by washed cells, spheroplasts and membrane vesicles of *Nitrobacter agilis* were investigated. The effects of some metal inhibitors on NO\textsuperscript{2} oxidation in washed cells, spheroplasts and membrane vesicles are shown in Tables 2, 3 and 4 respectively. Thiourea, 8-hydroxy-quinoline, toluene dithiol and 2TMP all inhibited NO\textsuperscript{2} and O\textsubscript{2} uptake and NO\textsuperscript{3−} production by washed cells (Table 2). DIECA which inhibits NH\textsuperscript{4+} oxidation completely at very low concentrations (13µM) in *Nitrosomonas europaesa* (Bhandari and Nicholas, 1979a) restricted NO\textsuperscript{2} and O\textsubscript{2} uptake by only 33 and 30% respectively at a much higher concentration (0.2M) and this inhibition was not reversed by the addition of Cu\textsuperscript{2+}. Sodium azide at 40µM inhibited NO\textsuperscript{2−} production and O\textsubscript{2} uptake completely in washed cells. The overall pattern of inhibition of NO\textsuperscript{2−} oxidation in washed cells (Table 2), spheroplasts (Table 3) and membrane vesicles (Table 4) was similar but the extent of inhibitory effects varied. Thus azide was more effective in washed cell than in membrane vesicles. In washed cells inhibitors can affect many metabolic reactions and this would account for the different responses between washed cells, spheroplasts and membrane vesicles.

The effects of inhibitors of electron transport, oxidative phosphorylation (uncouplers) and ATPase on NO\textsuperscript{2−} and O\textsubscript{2} uptake and NO\textsuperscript{3−} production by washed cells, spheroplasts and membrane vesicles are shown in Tables 5, 6 and 7 respectively. The overall pattern of inhibition was very similar for the NO\textsuperscript{2−} oxidising systems but again the extent varied. The inhibitors of electron transport *viz.* rotenone, amytal and HOQNO, all inhibited NO\textsuperscript{2} and O\textsubscript{2} uptake and NO\textsuperscript{3−} production in washed cells and spheroplasts (Tables 5, 6) but had little or no effect in membrane vesicles (Table 7). Nitrite oxidation has been shown to be sensitive to uncouplers (Cobley, 1976a,b). The results of this study also indicate that all the uncouplers used, strongly inhibited NO\textsuperscript{2} and O\textsubscript{2} utilization and NO\textsuperscript{3−} production either by washed cells, spheroplasts or membrane vesicles. It is also evident from Tables 5, 6 and 7 that CCCP at low concentrations restricted NO\textsuperscript{2} and O\textsubscript{2} uptake and NO\textsuperscript{3−} production, whereas 2,4-DNP, DBP and PCP did so at higher levels. The inhibitors of ATPase, namely DCCD and NBD chloride, also affected the uptake and extrusion processes in all preparations.
TABLE 2: EFFECTS OF METAL INHIBITORS ON THE UPTAKE OF NO\textsuperscript{2−} AND O\textsubscript{2} AND PRODUCTION OF NO\textsubscript{3} BY WASHED CELLS.

The reaction mixture used is as in Fig. 2 except that it contained 25µl of washed cells (10mg wet wt.) and the reaction was started by adding 2µmol NaNO\textsubscript{2}. The following procedure was used for each compound: l-25µl of inhibitor was injected into the reaction vessel which was magnetically stirred and after 5 min, 2µmol NaNO\textsubscript{2} was added to start the reaction. In the absence of inhibitor the uptake values for NO\textsubscript{2} and O\textsubscript{2} were 0.41 and 0.20µmol min\textsuperscript{-1} respectively and the extrusion rate of NO\textsubscript{3} was 0.35µmol min\textsuperscript{-1}. The inhibitors KSCN, thiourea, DIECA and azide were dissolved in distilled water, whereas 8-hydroxy quinoline, toluene dithiol and 2TMP were dissolved in 95% (v/v) ethanol. Appropriate controls with equivalent volumes of ethanol were included for each inhibitor dissolved in ethanol.

<table>
<thead>
<tr>
<th>% Inhibition</th>
<th>KSCN (mM)</th>
<th>Thiourea (mM)</th>
<th>2TMP (mM)</th>
<th>8-hydroxy quinoline (mM)</th>
<th>DIECA (µM)</th>
<th>Azide (µM)</th>
<th>Toluene Dithiol (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO\textsubscript{2} uptake</td>
<td>0 27</td>
<td>42 50</td>
<td>21 50</td>
<td>47</td>
<td>12 33</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>NO\textsubscript{3} production</td>
<td>0 27</td>
<td>50 59</td>
<td>23 49</td>
<td>Nd</td>
<td>17 Nd</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>O\textsubscript{2} uptake</td>
<td>0 10</td>
<td>40 46</td>
<td>17 38</td>
<td>52</td>
<td>10 30</td>
<td>84</td>
<td>100</td>
</tr>
</tbody>
</table>

Nd - Not determined because of the interference with determination.
TABLE 3: EFFECTS OF METAL INHIBITORS ON THE UPTAKE OF NO$_2^-$ AND O$_2$ AND PRODUCTION OF NO$_3^-$ BY SPHEROPLATS.

Reaction mixture in a total volume of 5ml contained 50mM Tris-HCl, 0.2M sucrose (pH 7.5) and 2 to 5mg spheroplast protein. Experimental details as in Fig.2 and Table 2.

<table>
<thead>
<tr>
<th>% Inhibition</th>
<th>2TMP (mM)</th>
<th>8-Hydroxy quinoline (mM)</th>
<th>DIECA (μM)</th>
<th>Toluene dithiol (μM)</th>
<th>CO (15 lb for 15 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
<td>1.0</td>
<td>0.1</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>NO$_2^-$ uptake</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>100</td>
<td>45</td>
<td>50</td>
<td>15</td>
<td>35</td>
</tr>
<tr>
<td>NO$_3^-$ production</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td>Nd</td>
<td>Nd</td>
<td>17</td>
<td>Nd</td>
</tr>
<tr>
<td>O$_2$ uptake</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>100</td>
<td>50</td>
<td>56</td>
<td>15</td>
<td>35</td>
</tr>
</tbody>
</table>

Nd - Not determined because of interference with electrode.
### TABLE 4: EFFECTS OF METAL INHIBITORS ON THE UPTAKE OF $\text{NO}_2^-$ AND $\text{O}_2$ AND EXTRUSION OF $\text{NO}_3^-$ BY MEMBRANE VESICLES.

The reaction mixture in a final volume of 5ml contained 50mM Tris -HCl, 0.2M sucrose (pH 7.5) and 2 to 3mg vesicle protein. Experimental details as in Fig.2 and Table 2.

<table>
<thead>
<tr>
<th>% Inhibition</th>
<th>2TMP (mM)</th>
<th>8-hydroxy quinoline (mM)</th>
<th>Sodium azide (mM)</th>
<th>Toluene dithiol (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{NO}_2^-$ uptake</td>
<td>52 65 75 57</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{NO}_3^-$ production</td>
<td>50 62 Nd 55</td>
<td>40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{O}_2$ uptake</td>
<td>55 65 75 60</td>
<td>40</td>
<td></td>
<td></td>
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</tbody>
</table>

Nd – Not determined because of interference with electrode.
TABLE 5:  EFFECTS OF INHIBITORS OF ELECTRON TRANSPORT (a-c), OXIDATIVE PHOSPHORYLATION (d-f) AND ATPase (g,h) ON UPTAKE OF NO$_2^-$ AND O$_2$ AND THE PRODUCTION OF NO$_3^-$ BY WASHED CELLS.

All inhibitors were dissolved in 95% (v/v) ethanol. Experimental details as in Table 2 and Fig.2.

<table>
<thead>
<tr>
<th></th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>Rotenone (µM)</td>
</tr>
<tr>
<td>NO$_2^-$ uptake</td>
<td>7</td>
</tr>
<tr>
<td>NO$_3^-$ production</td>
<td>2</td>
</tr>
<tr>
<td>O$_2$ uptake</td>
<td>22</td>
</tr>
</tbody>
</table>
TABLE 6: EFFECTS OF INHIBITORS OF ELECTRON TRANSPORT (a-c) OXIDATIVE PHOSPHORYLATION (d-f) AND ATPase (g,h) ON THE UPTAKE OF NO\textsubscript{2} AND O\textsubscript{2} AND THE PRODUCTION OF NO\textsubscript{3} BY SPHEROPLASTS.

All inhibitors except KCN were dissolved in 95% (v/v) ethanol. KCN was dissolved in distilled water. Experimental details as in Fig.2 and Table 2.

% Inhibition

<table>
<thead>
<tr>
<th></th>
<th>a Amytal (µM)</th>
<th>b HOQNO (µM)</th>
<th>c KCN (µM)</th>
<th>d PCP (µM)</th>
<th>e CCCP (µM)</th>
<th>f 2,4-DNP (µM)</th>
<th>g NBD Chloride (µg)</th>
<th>h DCCD (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 20</td>
<td>20 50</td>
<td>50 150 200</td>
<td>20 40</td>
<td>5 10</td>
<td>50 100</td>
<td>50 100</td>
<td>50 100</td>
</tr>
</tbody>
</table>

\begin{align*}
\text{NO}_2^- & \text{uptake} \\
10 & 38 \ 16 \ 51 \ 2 \ 60 \ 100 \ 25 \ 75 \ 62 \ 71 \ 26 \ 42 \ 43 \ 50 \ 58 \ 74 \\
\text{NO}_3^- & \text{production} \\
10 & 39 \ 17 \ 56 \ 2 \ Nd \ Nd \ 26 \ 76 \ 62 \ 70 \ 30 \ 46 \ 46 \ 52 \ 60 \ 76 \\
\text{O}_2 & \text{uptake} \\
0 & 22 \ 11 \ 42 \ 12 \ 63 \ 100 \ 20 \ 72 \ 68 \ 73 \ 30 \ 45 \ 53 \ 72 \ 69 \ 78 \\
\end{align*}

Nd – Not determined.
TABLE 7: EFFECTS OF INHIBITORS OF ELECTRON TRANSPORT (a-c) OXIDATIVE PHOSPHORYLATION (d-g) AND ATPase (h, i) ON UPTAKE OF NO\textsubscript{2}\textsuperscript{-} AND O\textsubscript{2} AND THE PRODUCTION OF NO\textsubscript{3} BY MEMBRANE VESICLES.

The reaction mixture in a total volume of 5ml contained 50mM Tris-HCl, 0.2M sucrose (pH 7.5) and 2-3mg vesicle protein. Experimental details as in Fig.2 and Table 2.

<table>
<thead>
<tr>
<th>% Inhibition</th>
<th>a</th>
<th>Rotenone (mM)</th>
<th>b</th>
<th>Amytal (uM)</th>
<th>c</th>
<th>HOQNO (mM)</th>
<th>d</th>
<th>CCCP (uM)</th>
<th>e</th>
<th>2,4-DBP (uM)</th>
<th>f</th>
<th>2,4-DNP (uM)</th>
<th>g</th>
<th>PCP (uM)</th>
<th>h</th>
<th>NBD Chloride (ug)</th>
<th>i</th>
<th>DCCD (uM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO\textsubscript{2} uptake</td>
<td>24</td>
<td>36</td>
<td>10</td>
<td>62</td>
<td>76</td>
<td>85</td>
<td>100</td>
<td>27</td>
<td>43</td>
<td>51</td>
<td>85</td>
<td>80</td>
<td>41</td>
<td>46</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO\textsubscript{3} production</td>
<td>25</td>
<td>34</td>
<td>15</td>
<td>10</td>
<td>60</td>
<td>76</td>
<td>82</td>
<td>100</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>85</td>
<td>80</td>
<td>40</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O\textsubscript{2} uptake</td>
<td>20</td>
<td>30</td>
<td>10</td>
<td>7</td>
<td>65</td>
<td>75</td>
<td>80</td>
<td>100</td>
<td>31</td>
<td>42</td>
<td>52</td>
<td>87</td>
<td>79</td>
<td>42</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Sulfhydryl group inhibitors, NEM and pCMB which did not restrict NO\textsuperscript{−}_2 and O\textsubscript{2} uptake and NO\textsubscript{3}\textsuperscript{−} production in washed cells, inhibited these processes in spheroplasts and to a greater extent in membrane vesicles (Table 8). This would indicate that the bacterial outer membrane poses a barrier to the entry of NEM and pCMB into the cells. The inhibition indicates the involvement of \(-\text{SH}\) groups for O\textsubscript{2} dependent NO\textsubscript{2}\textsuperscript{−} utilization.

3.1.7 ATPase activity in membrane vesicles

A Mg\textsuperscript{2+} dependent ATPase activity was located in the membrane vesicles of *Nitrobacter agilis*. The time course of Pi production from ATP (ATP hydrolysis) by membrane vesicles is presented in Fig.7. The rate of ATP hydrolysis was approximately 4nmol min\textsuperscript{−1} (mg protein)\textsuperscript{−1}. Since the ATP hydrolysing F\textsubscript{1} subunit of ATPase is located on the cytosol side of the cell membrane, and ATP is impermeable to membranes, the results indicate that the vesicles are "inside out".

3.1.8 Effects of inhibitors on ATPase activity in membrane vesicles

Unlike the ATPase of *Nitrosomonas europaea* (Bhandari and Nicholas, 1980) the uncouplers (CCCP, 2.4.DNP and oligomycin) did not affect the ATPase activity in *Nitrobacter agilis* (Table 9). However *Nitrobacter* ATPase was inhibited by known ATPase inhibitors. Thus at 100\mu M, DCCD, the classical ATPase inhibitor, restricted the ATPase activity by 65\% (Table 9). Sodium vanadate (10\mu M), diethylstilbestrol (20\mu M) and NBD chloride (100\mu M) also inhibited ATPase in membrane vesicles by 50, 45 and 90\% respectively.

3.1.9 Effects of phospholipase A\textsubscript{2} on nitrite oxidase and ATPase activities in membrane vesicles

Phospholipase A\textsubscript{2} results in delipidation of membranes by breaking phospholipids into lysophosphatides and fatty acids:

\[
\text{membranes (phospholipids)} \xrightarrow{\text{PL-A}_2 + \text{Ca}^{2+}} \text{membranes (lysophosphatides + fatty acids)}
\]

Lysophosphatides and fatty acids can be removed by washing the membranes with serum albumin solution:
TABLE 8: EFFECTS OF NEM AND pCMB ON THE UPTAKE OF NO$_2^-$ AND O$_2$, AND THE PRODUCTION OF NO$_3^-$ BY SPHEROPLASTS AND MEMBRANE VESICLES.

Both NEM and pCMB were dissolved in 95% (v/v) ethanol. Experimental details as in Fig. 2 and Table 2.

% Inhibition

<table>
<thead>
<tr>
<th></th>
<th>NEM (mM)</th>
<th></th>
<th>pCMB (mM)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5</td>
<td>1.0</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Spheroplasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO$_2^-$ uptake</td>
<td>0</td>
<td>16</td>
<td>26</td>
<td>39</td>
</tr>
<tr>
<td>NO$_3^-$ production</td>
<td>11</td>
<td>13</td>
<td>24</td>
<td>31</td>
</tr>
<tr>
<td>O$_2$ uptake</td>
<td>7</td>
<td>14</td>
<td>20</td>
<td>36</td>
</tr>
<tr>
<td>Membrane vesicles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO$_2^-$ uptake</td>
<td>65</td>
<td>81</td>
<td>32</td>
<td>72</td>
</tr>
<tr>
<td>NO$_3^-$ production</td>
<td>58</td>
<td>80</td>
<td>30</td>
<td>71</td>
</tr>
<tr>
<td>O$_2$ uptake</td>
<td>69</td>
<td>72</td>
<td>24</td>
<td>70</td>
</tr>
</tbody>
</table>
FIG. 7: ATPase ACTIVITY IN MEMBRANE VESICLES.

Membrane vesicles were prepared as described in Section 2.2.2. The reaction mixture in a total volume of 1 ml contained 1.7 mg vesicle protein, 30 μmol Tris-HCl (pH 7.3), 1.8 μmol ATP (pH 7.3) and 3 μmol MgSO₄. Incubation was at 30°C in a waterbath shaker. At the indicated times the reaction was terminated by the addition of 0.5 ml 10% (w/v) TCA and then centrifuging at 10,000 x g for 15 min. Pi released from ATP was then determined in the supernatant fraction as described in Section 2.2.17.4.

FIG. 8: EFFECTS OF PHOSPHOLIPASE-A₂ TREATMENT ON ATPase AND NO₂ OXIDASE ACTIVITY IN MEMBRANE VESICLES.

The reaction mixture in a total volume of 1 ml contained 5 mg of vesicle protein, 25 μmol glycyl-glycine buffer (pH 8.9), 4 μmol CaCl₂, 0.1 mg bovine serum albumin and 20 units of phospholipase-A₂. Reaction mixture without phospholipase-A₂ was preincubated for 5 min at room temperature (25°C) then phospholipase-A₂ was added and incubation continued at 25°C in a waterbath shaker for the time period indicated. The reaction was terminated by adding 8 ml cold (4°C) 1% (w/v) bovine serum albumin in glycyl-glycine buffer followed by centrifugation at 144,000 g for 30 min. The pellet was resuspended in cold bovine serum albumin solution and washed 4 times with the same solution. The washed pellet was resuspended in 0.25M sucrose and activities of ATPase and NO₂ oxidase were determined as described in Sections 2.2.6.1 and 2.2.17.1 respectively. For the determination of lipid phosphate, aliquots of the reaction mixture were digested in 70% (v/v) perchloric acid and Pi determined as described in Section 2.2.17.4.

NO₂ oxidase (○); ATPase (●); residual lipid phosphate (□).
TABLE 9: EFFECTS OF SOME INHIBITORS ON ATPase ACTIVITY IN MEMBRANE VESICLES.

ATPase activity was determined as described in Section 2.2.6.1. Reaction mixture in a total volume of 1ml contained 1mg vesicle protein in 50mM Tris-HCl buffer (pH 7.3), 1.8μmol ATP and 3mM Mg^{2+}. Incubation was at 30°C. Inorganic phosphate was determined as described in Section 2.2.17.4. In the absence of inhibitors ATPase produced approx. 100 nmol Pi (30 min)^{-1} (mg protein)^{-1}. Except for sodium vanadate which was dissolved in H₂O, all the inhibitors were dissolved in 95% (v/v) ethanol.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (μM)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCCP</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>2,4-DNP</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>DCCD</td>
<td>100</td>
<td>65</td>
</tr>
<tr>
<td>Sodium vanadate</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>20</td>
<td>45</td>
</tr>
<tr>
<td>NBD chloride</td>
<td>100</td>
<td>92</td>
</tr>
</tbody>
</table>
5B

(lysophosphatides + fatty acids) membranes serum albumin lipid
deficient membranes + (lysophosphatides + fatty acids) serum albumin

This experiment was designed to determine the role of phospholipids and membrane conformation in maintaining the activity of NO\textsuperscript{2-} oxidase and ATPase enzymes. The effects of phospholipase A\textsubscript{2} treatment on the activities of two enzymes is shown in Fig.8. Phospholipase A\textsubscript{2} treatment of membrane vesicles resulted in loss of phospholipids from the membranes as shown by the release of Pi, and after 60 min incubation with the enzyme, membranes retained only about 10\% of residual lipid phosphate (Fig.8). During this incubation time, membranes lost about 30\% of their NO\textsuperscript{2-} oxidising activity and 55\% of their ATPase activity indicating a possible involvement of membrane structure in maintaining the activity of the two enzymes.

3.2 ASSIMILATION OF INORGANIC NITROGEN COMPOUNDS BY NITROBACTER AGILIS

3.2.1 Growth studies

To study the effects of NH\textsubscript{4}\textsuperscript{+} on the growth of Nitrobacter agilis cultures were grown in a nitrite medium supplemented with various concentrations of NH\textsubscript{4}Cl. The optimum concentration for growth was found to be 2mM (Fig.9). Exponentially growing cultures of the bacterium (in 100ml medium supplemented with 2mM NH\textsubscript{4}Cl) utilized 75 ± 5mg NaNO\textsubscript{3} (24h)\textsuperscript{-1} as compared with cultures without NH\textsubscript{4}Cl which utilized 50 ± 5mg (24h)\textsuperscript{-1}. The lag period of growth, with or without NH\textsubscript{4}Cl, was about 36h. After 6 days, cells from NH\textsubscript{4}Cl (2mM) supplemented cultures contained 0.55 ± 0.1mg protein ml\textsuperscript{-1} culture as compared with those without NH\textsubscript{4}Cl (0.29 ± 0.1mg) (Fig.10).

3.2.2 Inhibition of NO\textsubscript{2}\textsuperscript{-} oxidation by NH\textsubscript{4}\textsuperscript{+} in washed cells

Although the growth experiment indicated that Nitrobacter can utilize small amounts of NH\textsubscript{4}\textsuperscript{+} (≈2mM), high concentration resulted in an inhibition of growth. From O\textsubscript{2} electrode traces of NO\textsubscript{2} oxidation (Fig.11) it is clear that NH\textsubscript{4}\textsuperscript{+} inhibits NO\textsubscript{2}\textsuperscript{-} oxidation by washed cells of Nitrobacter agilis. The extent of inhibition increased with the increasing levels of NH\textsubscript{4}\textsuperscript{+} when NO\textsubscript{2} concentration was kept constant (Fig.11a-c,e,f). Thus NH\textsubscript{4}\textsuperscript{+} at concentrations equal to those of NO\textsubscript{2} inhibited NO\textsubscript{2} oxidation by 20\% (Fig.11b). The NH\textsubscript{4}\textsuperscript{+} inhibition could be reversed by increasing the NO\textsubscript{2} concentrations (Fig.11d,g).
FIG. 9: GROWTH OF *NITROBACTER AGILIS* IN NH$_4$Cl CONTAINING MEDIUM.

Cells were grown in 250 ml Erlenmeyer flasks containing 100 ml of culture medium (Section 2.2.1) and NH$_4$Cl at indicated concentration. The sterile medium was inoculated with 10 ml of an exponentially grown culture. Growth was monitored by following the rate of NO$\textsubscript{2}^-$ utilization (Section 2.2.17.1) which was found to be proportional to the increase in total cell nitrogen.

No NH$_4$Cl (○); 0.1mM NH$_4$Cl (●); 0.5mM NH$_4$Cl (□);
2mM NH$_4$Cl (■).

FIG. 10: CELL YIELDS FROM CULTURES OF *NITROBACTER AGILIS* GROWN WITH VARIOUS CONCENTRATIONS OF NH$_4$Cl.

After a 6 day incubation, cells grown with various concentrations of NH$_4$Cl, as described in Fig.9, were collected by centrifugation at 15,000 g for 15 min. Cell yields are expressed as mg protein ml$^{-1}$ culture, determined by micro-biuret method (Section 2.2.17.3).
FIG. 9

mg nitrite in the medium

incubation time (d)

FIG. 10

mg protein ml⁻¹ of 6 d old culture

NH₄⁺ (mM) in the medium
FIG. 11: INHIBITION OF NO$_2^-$ OXIDATION IN WASHED CELLS BY NH$_4$Cl.

100μl of washed cells (10 mg wet wt.) was added to a double walled glass vessel fitted with a Clark-type oxygen electrode, containing 0.25 mmol Tris-HCl buffer (pH 7.8) in a final volume of 5 ml. The reaction mixture was maintained at 25°C by circulating water through the outer jacket of the vessel. Either KNO$_2$ or NH$_4$Cl was added through a port in the lid of the vessel via a Hamilton microsyringe. The reaction mixture was continuously stirred with a magnetic flea. The response of the electrode was recorded on a potentiometric recorder. The rates alongside the traces are in μmol O$_2$ consumed min$^{-1}$.

Additions (♀): C, cells (100μl); N, KNO$_2$ (μmol); A, NH$_4$Cl (μmol).
If the data from O2 traces are plotted as % inhibition of NO2 oxidation vs molar ratio of NH4+: NO2 (Fig.12), it is clear that when NH4+ concentration was increased keeping NO2 concentration constant, the extent of inhibition of NO2 oxidation increased rapidly until NH4+: NO2 ratio was about 4 and increased slowly thereafter. To ascertain whether NH4+ at its saturating concentration resulted in a complete inhibition of NO2 oxidation activity, the data were plotted as double reciprocal plots of the fractional inhibition against the concentration of inhibitor (Wedler et al. 1976) (Fig.13). In this plot a complete inhibition at the saturating concentration of the modifier is indicated when the curve intersects the Y axis at a value of <1. Since the intercept for NH4+ was >1 (Fig.13), it only partially inhibited the oxidation of NO2.

3.2.3 Incorporation of 15N labelled compounds into cell nitrogen

The incorporation of K15NO3, Na15NO2, 15NH2OH and 15NH4Cl into washed cells of Nitrobacter agilis was studied as described in Section 2.2.4. All these compounds were readily incorporated into washed cells (Table 10). The incorporation of 15NH4+ was approximately 80 fold greater than that of 15NO2. The extent of incorporation was in the order 15NH4+ > 15NH2OH > 15NO2 > 15NO3. Time course for incorporation of 15N compounds (Fig.14) indicated that the further incorporation of 15NH4+ and 15NH2OH ceased after about 4h of incubation, indicating that either ATP or NAD(P)H or both become rate-limiting. When unlabelled NO2 was included as an energy source, in addition to 15N substrates, the extent of incorporation of 15NO3, 15NH2OH or 15NH4 was significantly increased (Table 10).

15N labelled compounds were incorporated far less rapidly into extracts of Nitrobacter than into washed cells (Table 11), perhaps because disruption of the cells may result in loss of some components of the protein synthesizing system. The inclusion of NADH or ATP increased the incorporation of 15NO2 into protein but not that of 15NH4+.

3.2.4 Enzymes of NH4+ assimilation

Enzymes of NH4+ assimilation viz glutamine synthetase, glutamate synthase and glutamate dehydrogenase were detected in cell extracts of Nitrobacter agilis. All the three enzymes were located in the cytosal fraction
FIG. 12: COMPETITIVE INHIBITION OF NO$_2^-$ OXIDATION BY NH$_4$Cl.

The data were taken from experiments similar to those described in Fig. 11.

FIG. 13: DOUBLE RECIPROCAL PLOT OF INHIBITION OF NO$_2^-$ OXIDATION AT VARIOUS CONCENTRATIONS OF NH$_4$Cl.

Rates of O$_2$ uptake by washed cells were determined as described in Fig. 11.  1/I is the reciprocal of NH$_4$Cl concentration and 1/i, reciprocal of fractional inhibition of NO$_2^-$ oxidation by NH$_4$Cl.
FIG. 12

% inhibition of O$_2$ uptake

molar ratio (NH$_4^+/NO_2^-$)

FIG. 13

1/i

1/i (mM NH$_4^+$/CD$_{-}$)
TABLE 10: INCORPORATION OF $^{15}$N-LABELLED$^a$ COMPOUNDS INTO WASHED CELLS.

Washed cells (25mg protein), suspended in 6ml 0.1M phosphate buffer (pH 7.5), supplemented with 1.5mM KHC$_{10}$O$_3$ and 1mg equivalent of $^{15}$N substrate were incubated at 30°C for 2h in a 50ml Erlenmeyer flask. Where indicated, the reaction mixture also contained 10mM NaNO$_2$. The reaction was stopped by adding 30ml cold distilled water and samples for mass spectrometry were prepared as described in Section 2.2.4. Results are the mean values from three experiments from the same batch of cells, ± S.E.M.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$^{15}$N incorporated [µg (mg protein)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^{15}$NO$_2$</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>K$^{15}$NO$_3$</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>K$^{15}$NO$_3$ + NaNO$_2$</td>
<td>0.60 ± 0.02</td>
</tr>
<tr>
<td>$^{15}$NH$_2$OH</td>
<td>4.00 ± 0.50</td>
</tr>
<tr>
<td>$^{15}$NH$_2$OH + NaNO$_2$</td>
<td>5.20 ± 0.50</td>
</tr>
<tr>
<td>$^{15}$NH$_4$Cl</td>
<td>8.30 ± 0.80</td>
</tr>
<tr>
<td>$^{15}$NH$_4$Cl + NaNO$_2$</td>
<td>14.50 ± 0.80</td>
</tr>
</tbody>
</table>

$^a$Initial enrichment of the $^{15}$N compounds was as follows:
$^{15}$NH$_4$Cl (30 atom % excess), Na$^{15}$NO$_2$ and K$^{15}$NO$_3$ (both 32.5 atom % excess) and $^{15}$NH$_2$OH (97 atom % excess).
FIG. 14: TIME COURSE FOR INCORPORATION OF $^{15}\text{NO}_2^-$, $^{15}\text{NO}_3^-$, $^{15}\text{NH}_2\text{OH}$ AND $^{15}\text{NH}_4^+$ RESPECTIVELY INTO WASHED CELLS.

The reaction mixture was as described in Table 10. At the indicated times the reaction was stopped by adding 30 ml cold distilled water to the reaction mixture and samples for $^{15}$N analysis were prepared as described in Section 2.2.4.

$^{15}\text{NH}_4\text{Cl}$ (○); $^{15}\text{NH}_2\text{OH}$ (●); $^{15}\text{NO}_2^-$ (□); $^{15}\text{NO}_3^-$ (■).
TABLE 11: INCORPORATION OF $^{15}$N-LABELLED COMPOUNDS INTO PROTEIN OF CELL EXTRACTS ($S_5$).

Samples (6ml) of cell extracts (4mg protein ml$^{-1}$) in 0.1M phosphate buffer pH 7.8, supplemented with KHCO$_3$ (1.5mM) and 1mg equivalent of $^{15}$N substrate, were incubated for 2h at 30°C in 50ml Erlenmeyer flasks. ATP or NADH was added as indicated and ATP was regenerated by adding creatine phosphate (100mg) and creatine kinase (50 µg). The reaction was stopped by adding 5ml 10% (w/v) trichloro acetic acid and after standing overnight the contents were centrifuged and the protein pellet was washed once with 10% (w/v) trichloro acetic acid. Samples for $^{15}$N enrichment analysis were prepared and analysed as described in Section 2.2.4. Results are the mean values from three determinations with $S_5$ from the same batch of cells, ± S.E.M.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$^{15}$N incorporated [µg (mg protein)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{15}$NO$_2^-$</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>$^{15}$NO$_2^- + 0.5$ mM NADH</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>$^{15}$NO$_2^- + 1.0$ mM NADH</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>$^{15}$NO$_2^- + 1.0$ mM ATP</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>$^{15}$NO$_2^- + 1.0$ mM ATP + 1.0mM NADH</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>$^{15}$NH$_4^+$</td>
<td>0.49 ± 0.02</td>
</tr>
<tr>
<td>$^{15}$NH$_4^+$ + 0.5mM NADH</td>
<td>0.46 ± 0.02</td>
</tr>
<tr>
<td>$^{15}$NH$_4^+$ + 1.0mM NADH</td>
<td>0.47 ± 0.03</td>
</tr>
<tr>
<td>$^{15}$NH$_4^+$ + 1.0mM ATP</td>
<td>0.49 ± 0.03</td>
</tr>
<tr>
<td>$^{15}$NH$_4^+$ + 1.0mM NADH + 1.0mM ATP</td>
<td>0.50 ± 0.02</td>
</tr>
</tbody>
</table>
of the bacterium (Table 12). Glutamine synthetase (GS) required Mn\(^{2+}\) for its transferase activity and Mg\(^{2+}\) for biosynthetic activity. Glutamate synthase (GOGAT) was NADH dependent, while glutamate dehydrogenase (GDH) required either NADH or NADPH for its amination reaction, but the latter was more effective. Since the specific activity of these enzymes may vary from one batch of cells to another, for experiments described in Table 12 two bottles of medium with and without 2mM NH\(_4\)Cl were inoculated with a single culture (divided into two equal volumes) and incubated for the same length of time under similar incubation conditions. The experiment was repeated with different batches of cells; although the absolute enzyme activities varied, the overall pattern was as shown in Table 12. Glutamate synthase (GOGAT) was not detected in cells grown in a medium supplemented with 2mM NH\(_4\)Cl. However the GS activity was unaffected by NH\(_4^+\) and there was a substantial increase in GDH activity compared with cells grown with NO\(_2^-\) alone (Table 12).

3.2.5 Effects of L-methionine-DL-sulphoximine (MSX) and azaserine on the activities of GS, GOGAT and GDH

MSX and azaserine are inhibitors of GS and GOGAT respectively (Stewart et al., 1981). The effects of the two inhibitors on GS, GOGAT and GDH are shown in Fig.15. Neither MSX nor azaserine inhibited GDH but GS activity was depressed by about 95% in cells incubated with 250\(\mu\)M-MSX for 3h. Higher concentrations of MSX (up to 1mM) inhibited GS completely. The preincubation of cells with 200\(\mu\)M azaserine for 2h or 1mM azaserine for 30 min completely inactivated GOGAT. This indicates that both MSX and azaserine were taken up by the cells. In control experiments with untreated cells (Fig.15), the activities of GS, GOGAT and GDH remained constant over the period of incubation indicating that there was no apparent enzyme synthesis during preincubation. This would also suggest that MSX and azaserine inhibited the activities of pre-existing GS and GOGAT respectively rather than repressing their synthesis.

3.2.6 Effects of MSX and azaserine pretreatment of cells on the incorporation of \(^{15}\)NO\(_2^-\) and \(^{15}\)NH\(_4^+\) into cell nitrogen

Cells pretreated with MSX (250\(\mu\)M) or azaserine (200\(\mu\)M) or both were used for \(^{15}\)N incorporation studies described in Table 10. The results in Table 13 indicate that there was no effect of preincubation of cells with either MSX or azaserine or both together on the incorporation of \(^{15}\)NH\(_4^+\) and
TABLE 12: SPECIFIC ACTIVITIES OF GLUTAMINE SYNTHETASE (GS), GLUTAMATE SYNTHASE (GOGAT) AND GLUTAMATE DEHYDROGENASE (GDH) IN CELL EXTRACTS (S144).

Cell extracts (S144) were prepared by centrifuging crude S5 fraction at 144,000g for 1h at 4°C. Enzyme activities for GS, GOGAT and GDH were assayed in supernatant (S144) as described in Section 2.2.6. Results are the mean values from 5 determinations with S144 from the same batch of cells ± SEM.

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Specific activity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GS(^a)</td>
<td>GOGAT(^b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal medium without NH(_4)Cl</td>
<td>121±6.0</td>
<td>8±0.5</td>
</tr>
<tr>
<td>Basal medium with 2mM NH(_4)Cl</td>
<td>110±5.0</td>
<td>0±0.5</td>
</tr>
</tbody>
</table>

\(^a\) nmol γ glutamyl hydroxamate produced min\(^{-1}\) (mg protein)\(^{-1}\)  
\(^b\) nmol NADH oxidised min\(^{-1}\) (mg protein)\(^{-1}\)  
\(^c\) nmol NAD(P)H oxidised min\(^{-1}\) (mg protein)\(^{-1}\).
FIG. 15: EFFECTS OF MSX AND AZASERINE ON GLUTAMINE SYNTHETASE, GLUTAMATE SYNTHASE AND GLUTAMATE DEHYDROGENASE.

Washed cells (1g wet weight in 10ml sodium phosphate buffer, pH 7.8) were incubated with 250µM-MSX and 200µM azaserine at 30°C in a reciprocating water bath. At times indicated, cells were harvested by centrifugation (15,000g for 15 min), washed with buffer and disrupted by sonication as described in Section 2.2.3. The cell extract was centrifuged at 144,000g and the supernatant was used for enzyme assays as described in Section 2.2.6.

Glutamine synthetase in treated (■) and untreated (□) cells; glutamate synthase in treated (♦) and untreated (○) cells; glutamate dehydrogenase in treated (●) and untreated (○) cells.
FIG. 15

![Graph showing multiple curves representing nmol γ-GH formed min⁻¹ (mg protein)⁻¹ and nmol NAD(P)H oxidised min⁻¹ (mg protein)⁻¹ vs time (h).]
TABLE 13: INCORPORATION OF $^{15}\text{NO}_2^-$ and $^{15}\text{NH}_4^+$ INTO CELLS PRETREATED WITH MSX AND AZASERINE.

A suspension of washed cells (1g wet wt. in 10ml, 0.1M phosphate buffer, pH 7.8) was incubated at 30°C with either 200µM azaserine or 250µM-MSX or both. Treated cells were harvested by centrifugation at 10,000g for 15 min. For $^{15}\text{NH}_4^+$ incorporation experiments, reaction mixture was as described in Table 10. For $^{15}\text{NO}_2^-$ incorporation studies, cells (25mg protein) were suspended in 75ml culture medium (Section 2.2.1) in 125ml Erlenmeyer flasks and unlabelled $^{15}\text{NO}_2^-$ was replaced with Na$^{15}\text{NO}_2^-$ (32.5 atom % excess). The incubation was continued for 6h at 30°C in a water bath shaker. The cells collected by centrifugation were prepared and analysed for $^{15}\text{N}$ enrichment as described in Section 2.2.4. Results are the mean values of three determinations with the same batch of cells ± SEM.

<table>
<thead>
<tr>
<th>Additions to cells</th>
<th>$^{15}\text{N}$ incorporated [µg (mg protein)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{15}\text{NO}_2^-$</td>
<td>3.40 ± 0.20</td>
</tr>
<tr>
<td>Azaserine (200µM) + $^{15}\text{NO}_2^-$</td>
<td>3.00 ± 0.20</td>
</tr>
<tr>
<td>MSX (250µM) + $^{15}\text{NO}_2^-$</td>
<td>2.80 ± 0.20</td>
</tr>
<tr>
<td>Azaserine (200µM) + MSX (250µM) + $^{15}\text{NO}_2^-$</td>
<td>3.10 ± 0.30</td>
</tr>
<tr>
<td>$^{15}\text{NH}_4^+$</td>
<td>8.90 ± 0.50</td>
</tr>
<tr>
<td>Azaserine (200µM) + $^{15}\text{NH}_4^+$</td>
<td>8.80 ± 0.40</td>
</tr>
<tr>
<td>MSX (250µM) + $^{15}\text{NH}_4^+$</td>
<td>9.00 ± 0.20</td>
</tr>
<tr>
<td>Azaserine (200µM) + MSX (250µM) + $^{15}\text{NH}_4^+$</td>
<td>8.50 ± 0.30</td>
</tr>
</tbody>
</table>
$^{15}\text{NO}_2$ respectively. This would mean that GS-GOGAT pathway is not the sole NH$_4^+$ assimilating system in the bacterium.

3.3 PURIFICATION, PROPERTIES AND REGULATION OF GLUTAMINE SYNTHETASE (GS) FROM NITROBACTER AGILIS AND NITROSONOMAS EUROPAEA

3.3.1 Purification of GS

The purification procedures for GS from *Nitrobacter agilis* and *Nitrosonomas europaea* are summarized in Tables 14 and 15 respectively. The purified enzymes from *Nitrobacter agilis* and *Nitrosonomas europaea* had specific activities of 220 and 4.26 $\mu$mol $\gamma$GHH produced min$^{-1}$ (mg protein)$^{-1}$ respectively. The details of purification procedures are described in Section 2.2.5.1. *Nitrobacter* enzyme was purified by Blue-Sepharose CL-6B affinity chromatography since this column binds enzymes which require nucleotides as cofactors. Interestingly, *Nitrosonomas* enzyme did not bind to Blue-Sepharose CL-6B so that more conventional techniques were used for its purification. The elution profiles for *Nitrobacter* enzyme from Blue-Sepharose CL-6B column and Sepharose-4B column are shown in Fig.16 and 17. *Nitrosonomas europaea* grown with NH$_4^+$ had very little GS. Heat treatment of crude extracts at 65°C for 10 min resulted in the precipitation of about 20% of the total protein without any loss of GS activity. The pH precipitation step was essentially as described by Bhandari and Nicholas (1981) which resulted in 70 fold purified enzyme. Polyethylene glycol precipitation step was carried out as described by Stericher and Tyler (1980) and resulted in 710 fold purification. Purified GS from both *Nitrobacter agilis* and *Nitrosonomas europaea* appeared as one single protein band in polyacrylamide gels under non-denaturing electrophoretic conditions (Fig. 18 and 19 respectively).

3.3.2 Properties of GS

Since the kinetic properties of the enzyme from *Nitrosonomas europaea* have been extensively studied by Bhandari and Nicholas (1981) this section will largely deal with the properties of the *Nitrobacter* enzyme.

3.3.2.1 Molecular weight

Under denaturing conditions in SDS-polyacrylamide electrophoresis the purified enzyme from *Nitrobacter agilis* moved as a single protein band.
TABLE 14: PURIFICATION OF GS FROM NITROBACTER AGILIS.

All purification steps, except for heat treatment, were performed at 4°C as described in Section 2.2.5.1. Enzyme activity was determined by following the production of \( \gamma \) GH from L-glutamine and NH\(_2\)OH at pH 7.2 as described in Section 2.2.6.2. One unit is defined as \( \mu \)mol \( \gamma \) GH produced min\(^{-1}\). Specific activity is defined as number of units (mg proteins\(^{-1}\)).

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total Protein (mg)</th>
<th>Total units</th>
<th>Specific activity</th>
<th>Fold purification</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract (S(_{30}))</td>
<td>148.56</td>
<td>75.81</td>
<td>0.51</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>100.81</td>
<td>69.92</td>
<td>0.69</td>
<td>1.4</td>
<td>94</td>
</tr>
<tr>
<td>Pooled Blue Sepharose CL-6B fractions</td>
<td>2.10</td>
<td>45.33</td>
<td>21.59</td>
<td>42.3</td>
<td>60</td>
</tr>
<tr>
<td>Pooled Sepharose-4B fractions</td>
<td>0.20</td>
<td>44.0</td>
<td>220.0</td>
<td>431.0</td>
<td>58</td>
</tr>
</tbody>
</table>
TABLE 15: PURIFICATION OF GS FROM NITROSOMONAS EUROPAEA.

All purification steps were performed as described in Section 2.2.5.1. Enzyme activity was determined by following the production of γGH from L-glutamine and NH₂OH at pH 7.2 (Section 2.2.6.2). One enzyme unit is defined as μmol γGH produced min⁻¹ and specific activity as number of units (mg protein)⁻¹.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total Protein (mg)</th>
<th>Total units</th>
<th>Specific activity</th>
<th>Fold purification</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract (S₃₀)</td>
<td>500.0</td>
<td>83.6</td>
<td>0.006</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>399.0</td>
<td>91.2</td>
<td>0.008</td>
<td>1.3</td>
<td>109</td>
</tr>
<tr>
<td>pH precipitation</td>
<td>12.50</td>
<td>156.4</td>
<td>0.420</td>
<td>70.0</td>
<td>187</td>
</tr>
<tr>
<td>PEG precipitation</td>
<td>0.20</td>
<td>25.6</td>
<td>4.26</td>
<td>710.0</td>
<td>31</td>
</tr>
</tbody>
</table>
FIG. 16: ELUTION PROFILE FOR GS OF NITROBACTER AGILIS FROM BLUE SEPHAROSE CL-6B COLUMN.

Heat treated cell extract (Section 2.2.5.1) was passed through a Blue Sepharose CL-6B column (1.5 x 9 cm) pre-equilibrated with 10 mM Tris-HCl, 1 mM MnCl₂ buffer (pH 7.2). The column was then washed with buffer (flow rate 50 ml h⁻¹) until the absorbance (A₂₈₀) was close to zero. The enzyme (GS) was eluted from the column with 2 mM ADP in the same buffer. Enzyme activity was determined by following the production of YGH from glutamine and NH₂OH as described in Section 2.2.6.2.

A₂₈₀ (○); enzyme activity (●).

FIG. 17: ELUTION PROFILE FOR GS OF NITROBACTER AGILIS FROM SEPHAROSE-4B COLUMN.

Pooled Blue-Sepharose CL-6B fractions (Section 2.2.5.1) were dialysed against 10 mM Tris-HCl, 1 mM MnCl₂ buffer (pH 7.2), concentrated on an Amicon PM-10 membrane filter and loaded onto a Sepharose-4B column (2 x 70 cm) pre-equilibrated with 10 mM Tris-HCl, 1 mM MnCl₂ buffer (pH 7.2). The enzyme was eluted with the same buffer (flow rate 12 ml h⁻¹). Transferase activity was determined as described in Section 2.2.6.2.

A₂₈₀ (○); enzyme activity (●).
FIG. 18: POLYACRYLAMIDE GEL ELECTROPHORESIS OF PURIFIED GS FROM NITROBACTER AGILIS.

PAGE was carried out in 7% (w/v) gels as described in Section 2.2.10. Gel A was stained for enzyme activity and gel B for protein by coomassie blue method (Section 2.2.10).

GS, glutamine synthetase; BPB, bromophenol blue.

FIG. 19: POLYACRYLAMIDE GEL ELECTROPHORESIS OF PURIFIED GS FROM NITROSOMONAS EUROPEA.

PAGE was carried out in 5% (w/v) gels as described in Section 2.2.10. Protein band was stained using silver staining method (Section 2.2.10).

GS, glutamine synthetase; BPB, bromophenol blue.
The molecular weight of enzyme subunit was estimated to be 58,000 using standard SDS-treated protein markers (Fig.21). The molecular weight of the native enzyme determined by gel filtration using a Sepharose 6-B column (1.6 x 100cm) was estimated to be 700,000 (Fig.22). The results indicate that the enzyme molecule (700,000) is composed of 12 homologous subunits of approximately 58,000 each.

3.3.2.2 Substrate requirements for enzyme activity

Both \( \gamma \) glutamyl transferase and biosynthetic activities were recorded for the Nitrobacter GS. The substrate requirements for transferase activity are shown in Table 16. The results indicate that the transferase activity required a divalent cation (Mn\(^{2+}\)). Little or no activity was recorded when either Mn\(^{2+}\), glutamine, NH\(_2\)OH of arsenate was omitted from the reaction mixture. When ADP was omitted, 20% of the activity of the complete reaction mixture was recorded.

The results in Table 17 indicate that the biosynthetic activity of the enzyme also required a divalent cation (Mg\(^{2+}\)), glutamate, NH\(_4\)Cl and ATP. No activity was recorded when either of these compounds was omitted from the reaction mixture.

3.3.2.3 Effects of metal ions

The effects of various metal ions on transferase and biosynthetic activity of purified glutamine synthetase from Nitrobacter agilis are shown in Tables 18 and 19. Optimum transferase activity was obtained by using either Mn\(^{2+}\) or Cu\(^{2+}\) between 0.1 and 10mM final concentration. Higher concentrations of either of the metal ions resulted in a decrease in transferase activity (Table 18). The order of effectiveness of the divalent cations was Mn\(^{2+}\) > Cu\(^{2+}\) > Mg\(^{2+}\) > Co\(^{2+}\) > Ni\(^{2+}\). Optimum biosynthetic activity was recorded with Mg\(^{2+}\) and the order of effectiveness was Mg\(^{2+}\) > Mn\(^{2+}\) > Zn\(^{2+}\) > Co\(^{2+}\) > Cu\(^{2+}\) > Ni\(^{2+}\) > Ca\(^{2+}\) > Fe\(^{2+}\) (Table 19).

3.3.2.4 \( K_m \) for substrates of transferase and biosynthetic reactions

The effects of various concentrations of substrates of Mn\(^{2+}\) dependent transferase activity and Mg\(^{2+}\) dependent biosynthetic activity
FIG. 20: SDS-PAGE OF PURIFIED GS FROM *NITROBACTER AGILIS*

SDS-PAGE of enzyme was carried out in 10% (w/v) polyacrylamide gels as described in Section 2.2.10. Left hand lane contained 15μg purified GS (SDS treated) and the right hand lane approximately 150μg of a mixture of SDS-treated protein markers.

From top to bottom - phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and bromophenol blue.

FIG. 21: DETERMINATION OF SUBUNIT MOLECULAR WEIGHT OF PURIFIED GS FROM *NITROBACTER AGILIS*.

Subunit molecular weight of GS was determined in SDS-PAGE as shown in Fig. 20 and described in Section 2.2.10. K\_av was calculated from electrophoretic mobilities of standard proteins.

A. phosphorylase b (94,000)
B. albumin (67,000)
C. ovalbumin (43,000)
D. carbonic anhydrase (30,000)
E. trypsin inhibitor (20,100)
GS. glutamine synthetase.
FIG. 22: DETERMINATION OF NATIVE MOLECULAR WEIGHT OF PURIFIED GS FROM *NITROBACTER AGILIS*.

The molecular weight of the native enzyme was determined by gel-filtration in a Sepharose-6B column (1.6 x 100cm) equilibrated with 50mM Tris-HCl buffer (pH 7.5) as described in Section 2.2.8. The column was calibrated with the following standard proteins:

A. aldolase (158,000)
B. catalase (232,000)
C. ferritin (440,000)
D. thyroglobulin (669,000)
GS. glutamine synthetase.
FIG. 22

- Log molecular weight vs. $\frac{V_e}{V_0}$
- Points marked with letters A, B, C, D, G5
- Line of best fit
TABLE 16: SUBSTRATE REQUIREMENTS FOR THE γ GLUTAMYL TRANSFERASE REACTION OF PURIFIED GS FROM NITROBACTER AGILIS.

The complete assay mixture in a final volume of 1ml contained purified enzyme (5µg protein), L-glutamine (30mM), NH₂OH.HCl (neutralized with 2M NaOH) (30mM), MnCl₂.4H₂O (0.5mM), sodium arsenate (20mM), ADP (0.4mM) and imidazole-HCl buffer (40mM) pH 7.2. In other test-tubes Mn²⁺, L-glutamine, NH₂OH, sodium arsenate and ADP were omitted in turn from the reaction mixture. Enzyme activity was measured after a 15 min incubation at 37°C as described in Section 2.2.6.2.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>Omit Mn²⁺</td>
<td>0</td>
</tr>
<tr>
<td>Omit L-glutamine</td>
<td>0</td>
</tr>
<tr>
<td>Omit hydroxylamine</td>
<td>0</td>
</tr>
<tr>
<td>Omit sodium arsenate</td>
<td>5</td>
</tr>
<tr>
<td>Omit ADP</td>
<td>20</td>
</tr>
</tbody>
</table>
TABLE 17: SUBSTRATE REQUIREMENTS FOR THE BIOSYNTHETIC ACTIVITY OF PURIFIED GS FROM *NITROBACTER AGILIS*.

The complete assay mixture in a final volume of 0.2ml contained purified enzyme (5µg protein), L-glutamate (100mM), NH₄Cl (50mM), ATP (10mM), Mg²⁺ (5mM) and imidazole-HCl buffer (50mM, pH 7.0). In other test-tubes Mg²⁺, L-glutamate, NH₄Cl and ATP were omitted in turn from the reaction mixture. The Pi produced after a 15 min incubation period at 37°C was determined as described in Section 2.2.17.4.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100</td>
</tr>
<tr>
<td>Omit Mg²⁺</td>
<td>5</td>
</tr>
<tr>
<td>Omit glutamate</td>
<td>0</td>
</tr>
<tr>
<td>Omit NH₄Cl</td>
<td>0</td>
</tr>
<tr>
<td>Omit ATP</td>
<td>0</td>
</tr>
</tbody>
</table>
TABLE 18: EFFECTS OF VARIOUS DIVALENT CATIONS ON THE γ GLUTAMYL TRANSFERASE ACTIVITY OF PURIFIED GS FROM NITROBACTER AGILIS.

An aliquot of purified enzyme was desalted by passing through a Sephadex G-10 column (2.5 x 20cm) which had been previously equilibrated with 50mM Tris-HCl buffer (pH 7.2). Desalted enzyme (5-10μg) was used in the reaction mixture described in Section 2.2.6.2 except that Mn$^{2+}$ was replaced by either of the cations listed below. Results are expressed as % of the Mn$^{2+}$ dependent transferase activity.

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>% activity at cation concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>100</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>23</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>0</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>30</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>0</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>100</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>0</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>5</td>
</tr>
</tbody>
</table>

Nd - Not determined.
TABLE 19: EFFECTS OF VARIOUS DIVALENT CATIONS ON THE BIOSYNTHETIC ACTIVITY OF PURIFIED GS FROM NITROBACTER AGILIS.

Purified enzyme was desalted as described in Table 18. Biosynthetic activity was determined as described in Section 2.2.6.2 except that Mg$^{2+}$ was replaced by either of the cations listed below. Results are expressed as % of Mg$^{2+}$ dependent biosynthetic activity.

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>% activity of cation concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>73</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>95</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Nd</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>Nd</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>Nd</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>Nd</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>Nd</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>Nd</td>
</tr>
</tbody>
</table>

Nd - Not determined.
were considered. The effects of glutamine concentrations over a range of 0-50mM are illustrated in Fig.23a. The $K_m$ value of 14.6 ± 1.5 was calculated from the double reciprocal plot (Fig.23b) of glutamine and γ-glutamyl-hydroxamate produced.

The effects of various concentrations of NH$_2$OH (0-40mM) on transferase activity are shown in Fig.24a. The enzyme activity increased up to 20mM NH$_2$OH. The $K_m$ value for NH$_2$OH was 2.6 ± 0.8 calculated from double reciprocal plot (Fig.24b), as described in Section 2.2.7.

The effects of various concentrations of L-glutamate (0-50mM) on Mg$_2^+$ dependent biosynthetic activity are shown in Fig.25a. The enzyme activity increased up to 40mM glutamate. The double reciprocal plot (Fig.25b) of glutamate concentration against Pi produced gave a $K_m$ value of 6.3 ± 1.6mM. The effects of NH$_4$Cl over a range of 0-5mM are shown in Fig.26a. The biosynthetic activity increased up to about 2mM NH$_4$Cl. The $K_m$ value for NH$_4$Cl was 0.2 ± 0.1mM as calculated from double reciprocal plot (Fig.26b).

A summary of general properties of purified GS from *Nitrobacter agilis* is given in Table 20.

### 3.3.2.5 Heat stability and effects of denaturing agents

The purified GS from *Nitrobacter agilis* remained active after incubating at 50°C for 15 min. At 60°C, the enzyme was inactivated by 60% within 10 min and at 70°C it was completely inactivated. The addition of glutamine (30mM) and Mn$_2^+$ (3mM) protected the enzyme by about 20% at 60°C, while NH$_2$OH (30mM) accelerated its deactivation. The effects of NH$_2$OH were further investigated on both biosynthetic and transferase activities of the enzyme. The enzyme was preincubated for various periods with 10mM NH$_2$OH. The data indicate that both transferase and biosynthetic activities decreased; thus after 20 min preincubation with NH$_2$OH, the enzyme lost about 75% and 85% of its biosynthetic and transferase activities respectively (Fig.27). The incubation of purified enzyme with 4M urea caused about 85% loss of transferase activity within 20 min.
FIG. 23: THE EFFECTS OF VARIOUS CONCENTRATIONS OF L-GLUTAMINE ON \( \gamma \)-GLUTAMYL TRANSFERASE ACTIVITY OF PURIFIED GS FROM \textit{NITROBACTER AGILIS}.

Aliquots of purified enzyme were added to the reaction mixture (final volume 1ml) containing L-glutamine (0-50mM), \( \text{NH}_2\text{OH-HCl} \) (neutralized with 2N NaOH) (30mM), Mn\(^{2+}\) (3mM), sodium arsenate (20mM), ADP (0.4mM) and imidazole-HCl buffer, pH 7.2 (40mM). Control tubes without glutamine were included. \( \gamma \)GH produced was determined as described in Section 2.2.6.2.

FIG. 24: THE EFFECTS OF VARIOUS CONCENTRATIONS OF \( \text{NH}_2\text{OH} \) ON \( \gamma \) GLUTAMYL TRANSFERASE ACTIVITY OF PURIFIED GS FROM \textit{NITROBACTER AGILIS}.

Experimental details as in Fig. 23, except that \( \text{NH}_2\text{OH} \) was varied from 0-40mM at a fixed concentration of glutamine (30mM).
FIG. 25: THE EFFECTS OF VARIOUS CONCENTRATIONS OF L-GLUTAMATE ON THE BIOSYNTHETIC ACTIVITY OF PURIFIED GS FROM NITROBACTER AGILIS.

Aliquots of enzyme were added to the reaction mixture (0.2ml) containing L-glutamate (0-50 mM), NH₄Cl (50mM), ATP (10mM), Mg²⁺ (5mM) and imidazole-HCl buffer pH 7.0 (50mM). L-glutamate was omitted from the control tubes. After a 30 min incubation period at 37°C the Pi formed was determined as described in Section 2.2.17.4.

FIG. 26: THE EFFECTS OF VARIOUS CONCENTRATIONS OF NH₄Cl ON THE BIOSYNTHETIC ACTIVITY OF PURIFIED GS FROM NITROBACTER AGILIS.

Experimental details as in Fig. 25, except that NH₄Cl was varied from 0-5mM at fixed glutamate concentration (100mM).
FIG. 25

(a) [Graph showing enzymatic activity vs. substrate concentration (mM GLUTAMATE)]

(b) [Graph showing reciprocal of V vs. reciprocal of S]

FIG. 26

(a) [Graph showing enzymatic activity vs. substrate concentration (mM NH₄Cl)]

(b) [Graph showing reciprocal of V vs. reciprocal of S]
TABLE 20: PROPERTIES OF PURIFIED GS FROM *NITROBACTER AGILIS*.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight of native enzyme</td>
<td>700,000</td>
</tr>
<tr>
<td>Subunit molecular weight in SDS gels</td>
<td>58,000</td>
</tr>
<tr>
<td>Number of subunits</td>
<td>12</td>
</tr>
<tr>
<td>$K_m$ for glutamine</td>
<td>$14.6 \pm 1.5 \text{mM}$</td>
</tr>
<tr>
<td>$K_m$ for $\text{NH}_2\text{OH}$</td>
<td>$2.6 \pm 0.8 \text{mM}$</td>
</tr>
<tr>
<td>$K_m$ for glutamate</td>
<td>$6.3 \pm 1.6 \text{mM}$</td>
</tr>
<tr>
<td>$K_m$ for $\text{NH}_4^+$</td>
<td>$0.2 \pm 0.1 \text{mM}$</td>
</tr>
</tbody>
</table>
FIG. 27: THE COURSE FOR INACTIVATION BY NH₂OH OF γ GLUTAMYL TRANSFERASE AND BIOSYNTHETIC ACTIVITIES OF PURIFIED GS FROM NITROBACTER AGILIS.

Purified enzyme was preincubated with 10mM NH₂OH.HCl (neutralized with 2M NaOH) for various times (0-20 min). Biosynthetic and transferase activities were determined as described in Section 2.2.6.2 except that the reaction mixture for latter activity contained 10mM NH₂OH. The results are expressed as percent of activities without preincubation with hydroxylamine.

Transferase activity (○), Biosynthetic activity (●).

FIG. 28: NH₄⁺ INHIBITION OF γ GLUTAMYL TRANSFERASE ACTIVITY OF PURIFIED GS₄ FROM NITROBACTER AGILIS.

Transferase activity of the purified enzyme was determined as described in Section 2.2.6.2 except that NH₄Cl (0-50mM) was also included in the reaction mixture.
3.3.2.6 Inhibition of transferase activity by NH$_4$Cl

The inhibition of transferase activity by NH$_4$Cl (0-50mM) is shown in Fig.28; maximum inhibition (40%) was at 40mM NH$_4$Cl. The type of inhibition was further investigated. The inhibitory effects of NH$_4$Cl at various concentrations of glutamine and NH$_2$OH are shown in Fig.29 and 30 respectively. The kinetic data for double reciprocal plots indicate that NH$_4$Cl was a competitive inhibitor of glutamine (Fig.29) and a mixed type inhibitor of NH$_2$OH (Fig.30). Ammonium chloride reduced the affinity of glutamine and NH$_2$OH for the enzyme resulting in increase in $K_m$ values (Table 21).

3.3.3 Regulation of GS

3.3.3.1 Feed back inhibition

Glutamine synthetase from *Nitrosomonas europaea* is inhibited by a variety of feed back inhibitors including various amino acids and nucleotides (Bhandari and Nicholas, 1981). The purified enzyme from *Nitrobacter agilis* was also inhibited by amino acids and nucleotides. Thus at 10mM final concentration, alanine, serine, glycine and tryptophan inhibited transferase activity by 65, 45, 40 and 33% and biosynthetic activity by 60, 30, 35 and 5% respectively (Table 22). The enzyme activity was little affected by the other amino acids studied (Table 22). The effects of various concentrations of alanine, glycine and serine, on transferase activity are illustrated in Fig.31a. The extent of inhibition by these amino acids increased with an increase in concentration. The results in Fig.31b are expressed as double reciprocal plots of the fractional inhibition (calculated as described by Wedler et al., 1976) against the concentrations of the inhibitor. The data indicate that glycine, serine as well as alanine only partially inhibited the enzyme activity, because the curves intersect the Y axis at a value of >1. A complete inhibition at saturating concentrations of inhibitor is indicated when the plot intersects the Y axis at a value of <1.
FIG. 29: COMPETITIVE INHIBITION BY NH₄Cl OF γ-GLUTAMYL TRANSFERASE ACTIVITY OF PURIFIED GS FROM NITROBACTER AGILIS.

Experimental details as in Fig. 23 except that NH₄Cl (0-30mM) was also included in the assay mixture.

No NH₄Cl (●); 10mM NH₄Cl (○); 20mM NH₄Cl (■); 30mM NH₄Cl (□).

FIG. 30: MIXED TYPE INHIBITION BY NH₄Cl OF γ-GLUTAMYL TRANSFERASE ACTIVITY OF PURIFIED GS FROM NITROBACTER AGILIS.

Experimental details as in Fig. 24 except that NH₄Cl (0-30mM) was also included in the assay mixture.

No NH₄Cl (●); 10mM NH₄Cl (○); 20mM NH₄Cl (■); 30mM NH₄Cl (□).
FIG. 29

\[ \frac{1}{V} \text{ (\text{mol} \text{L}^{-1} \text{min}^{-1})} = \frac{1}{S} \text{ (mM glutamine) }^{-1} \]

FIG. 30

\[ \frac{1}{V} \text{ (\text{mol} \text{L}^{-1} \text{min}^{-1})} = \frac{1}{S} \text{ (mM } \text{NH}_2\text{OH) }^{-1} \]
TABLE 21: EFFECT OF NH₄⁺ ON Kₘ OF THE SUBSTRATES OF γ GLUTAMYL TRANSFERASE ACTIVITY OF PURIFIED GS FROM NITROBACTER AGILIS

Transferase activity of the enzyme was measured as described in Section 2.2.6.2 except that the amounts of glutamine and NH₂OH were varied between 0 to 50mM and NH₄Cl was included in the assay mixture as indicated. Kₘ values were calculated from double reciprocal plots (Section 2.2.7).

<table>
<thead>
<tr>
<th>NH₄⁺ concentration (mM)</th>
<th>Kₘ (mM)</th>
<th>Glutamine</th>
<th>NH₂OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.6</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>18.2</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>23.8</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>27.8</td>
<td>5.6</td>
<td></td>
</tr>
</tbody>
</table>
TABLE 22: EFFECTS OF VARIOUS AMINO ACIDS ON γ GLUTAMYL TRANSFERASE AND BIOSYNTHETIC ACTIVITIES OF PURIFIED GS FROM NITROBACTER AGILIS.

Transferase and biosynthetic activities of GS were determined as described in Section 2.2.6.2 except that reaction mixture also contained 10mM of the appropriate amino acid as indicated.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>% Inhibition</th>
<th>Transferase activity</th>
<th>Biosynthetic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Glutamate</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Glycine</td>
<td>40</td>
<td></td>
<td>36</td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>14</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>4</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>4</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>14</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>L-Serine</td>
<td>45</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>L-Valine</td>
<td>0</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>L-Proline</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>10</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>33</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>0</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>65</td>
<td></td>
<td>60</td>
</tr>
</tbody>
</table>
FIG. 31: INHIBITION OF γ-GLUTAMYL TRANSFERASE ACTIVITY OF PURIFIED GS FROM NITROBACTER AGILIS BY VARIOUS CONCENTRATIONS OF ALANINE (□), GLYCINE (●) AND SERINE (○).

Transferase activity of the purified enzyme was determined as described in Section 2.2.6.2 except that the reaction mixture also contained either alanine, glycine or serine (0–10 mM). In Fig. 31b, results of Fig. 31a are plotted as double reciprocal plots of inhibitor (amino acid) concentration (I) against fractional inhibition (i).
The combined effects of various amino acids on the transferase activity are shown in Table 23. The inhibition due to various combinations of amino acids was lower than the sum of the inhibitions resulting from an individual amino acid. Thus the inhibitory effects due to alanine and glycine were 40 and 27% respectively, but their combined inhibitory effect was 52% instead of 67% (40 + 27%). The effects due to alanine, glycine, and serine alone were 40, 27 and 36% respectively but their observed combined inhibitory effect was only 69% instead of 103, the sum of the effects of the individual amino acids alone, i.e., 40 + 27 + 36%. The values for the cumulative inhibitions were then calculated as described in Section 2.2.9. For all the pairs of amino acids studied, the observed inhibitions were in close agreement with the values calculated for their cumulative inhibitory effects. Thus the combined inhibitory effect of 52% for the pair of alanine and glycine was close to 56% calculated for cumulative effect, whereas the additive effect was 67%.

The effects of various mono-, di- and triphosphate nucleotides on transferase activity was examined. The effects of various nucleotides presented in Table 24 indicate that the enzyme was inhibited to a greater extent by di- and tri-phosphates nucleotides (except GDP and GTP) than by their mono-phosphate nucleotides (except AMP). Thus IDP, CDP and UDP inhibited the enzyme by 45, 50 and 53% respectively and the inhibitory effects of ITP, CTP, TTP and ATP were 63, 55, 57 and 56% respectively. AMP restricted the enzyme activity by 65% but other mono-phosphate nucleotides viz. IMP, CMP, TMP and UMP had little or no effect.

3.3.3.2 Adenylylation and deadenylylation of GS.

In enteric bacteria (Stadtman et al., 1970), photosynthetic bacteria (Johansson and Gest, 1977) and rhizobia (Darrow and Knotts, 1977) the extent of transferase activity in the presence of 60mM MgCl₂ has been used as an indication of the degree of adenylylation of GS. The fully adenylylated enzyme is inactive in the presence of Mg²⁺ whereas the deadenylylated one is not affected. Since the adenylylation state of the enzyme can change during harvesting of the bacteria (Bender et al., 1977) the addition of cetyl trimethyl ammonium bromide (CTAB) to cultures stabilizes the adenylylation state of the enzyme.
TABLE 23: COMBINED EFFECTS OF AMINO ACIDS ON γ GLUTAMYL TRANSFERASE ACTIVITY OF PURIFIED GS FROM NITROBACTER AGILIS.

Glutamine synthetase activity was determined as described in Table 22 and Section 2.2.6.2 except that each amino acid was at 5mM. Values for cumulative inhibition were calculated as described in Section 2.2.9.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
</tr>
<tr>
<td>Ala</td>
<td>40</td>
</tr>
<tr>
<td>Gly</td>
<td>27</td>
</tr>
<tr>
<td>Ser</td>
<td>36</td>
</tr>
<tr>
<td>Ala + Gly</td>
<td>52</td>
</tr>
<tr>
<td>Ala + Ser</td>
<td>62</td>
</tr>
<tr>
<td>Ser + Gly</td>
<td>52</td>
</tr>
<tr>
<td>Ala + Gly + Ser</td>
<td>69</td>
</tr>
</tbody>
</table>
TABLE 24: EFFECTS OF NUCLEOTIDES ON γ GLUTAMYL TRANSFERASE ACTIVITY OF PURIFIED GS FROM NITROBACTER AGILIS.

Transferase activity was determined as described in Section 2.2.6.2 except that the reaction mixture also contained 20mM of the appropriate nucleotide as indicated.

<table>
<thead>
<tr>
<th>Nucleotides</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDP</td>
<td>45</td>
</tr>
<tr>
<td>ITP</td>
<td>63</td>
</tr>
<tr>
<td>CDP</td>
<td>50</td>
</tr>
<tr>
<td>CTP</td>
<td>55</td>
</tr>
<tr>
<td>TTP</td>
<td>57</td>
</tr>
<tr>
<td>UDP</td>
<td>53</td>
</tr>
<tr>
<td>AMP</td>
<td>65</td>
</tr>
<tr>
<td>ATP</td>
<td>56</td>
</tr>
</tbody>
</table>
The results shown in Table 25 indicate that the enzyme from normally grown *Nitrobacter* cells was severely inhibited by Mg$^{2+}$. The CTAB treatment of *Nitrobacter* cells prior to harvest had little effect on the extent of Mg$^{2+}$ inhibition of transferase activity. Similar results were observed when cells harvested with or without CTAB were assayed for *in vivo* transferase activity. Based on a 12 subunit enzyme and applying the Shapiro & Stadtman (1970) formula (En = 12 - 12. + Mg$^{2+}$ - Mg$^{2+}$), an adenylylation state of 9 can be calculated for the *Nitrobacter* enzyme, and it varied between 8 and 11 in twenty separately grown batches of cells.

In *Nitrosomonas europaea*, for cultures harvested without added CTAB, an adenylylation state of 9 was recorded (Table 25), however when the cells were harvested with CTAB (2.5 mg ml$^{-1}$) the value decreased to 4. When cells grown at a low ammonia concentration (20 mM) were harvested after all the NH$_4^+$ in the medium had been utilized, those harvested with and without CTAB gave adenylylation states of 8 and −5 respectively. Although the Mg$^{2+}$ effect on transferase activity (Table 25) may be related to the adenylylation state of the enzyme in *Nitrosomonas*, the Shapiro & Stadtman formula does not appear to apply to crude extracts. If CTAB serves the same function as reported for other bacteria (Bender et al., 1977; Johansson & Gest, 1977; Michalski et al., 1983), its addition to cultures prior to harvest should prevent the adenylylation of the *Nitrosomonas* enzyme. The Mg$^{2+}$ effect on GS in crude preparations of *Nitrosomonas europaea* varied from batch to batch and was dependent on the incubation period of the extracts. Freshly prepared extract gave results shown in Table 25 but after a few hours incubation, either at room temperature or at 4°C, the transferase activity of the enzyme in extracts from cells harvested without CTAB was stimulated by Mg$^{2+}$.

*Nitrosomonas europaea* is usually grown with high concentrations of ammonia (about 120 mM) (see Bhandari & Nicholas, 1979a) so that at the end of exponential phase, the residual NH$_4^+$ is about 40 mM. Thus in these cells, glutamine synthetase would be highly adenylylated. The results of an experiment in which cells were grown with 20 mM NH$_4^+$, harvested in the presence of CTAB and the effect of Mg$^{2+}$ on the transferase activity of crude preparations determined are shown in Fig.32.
TABLE 25: EFFECTS OF CTAB TREATMENT ON Y-GLUTAMYL TRANSFERASE ACTIVITY IN CELL EXTRACTS (S₂₅) FROM NITROBACTER AGILIS AND NITROSOMONAS EUROPAEA.

Exponentially growing cultures (1 l) of Nitrobacter agilis and Nitrosomonas europaea were harvested in polycarbonate bottles (250ml volume) in Sorvall GSA rotor at 10,000g for 20 min at 4°C. As indicated, CTAB was added to cultures (2.5µg ml⁻¹) before harvesting. Cells washed once with cold 10mM Tris-HCl, 1mM MnCl₂ buffer (pH 7.2) and finally suspended in a small volume (approx. 5ml) of the buffer. Cell suspensions were sonicated for 20 min and then centrifuged at 25,000g for 15 min. The supernatant (S₂₅) was used to determine the transferase activity with and without 60mM MgCl₂, as described in Section 2.2.6.2. Transferase activity is defined as µmol γGlu produced (30 min)⁻¹ (mg protein)⁻¹.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>CTAB treatment</th>
<th>Transferase activity</th>
<th>+Mg²⁺/-Mg²⁺ Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goldenberg</td>
<td></td>
<td>-Mg²⁺</td>
<td>+60mM Mg²⁺</td>
</tr>
<tr>
<td>Nitrobacter agilis growing on 40mM NO⁻₂</td>
<td>-</td>
<td>10.50</td>
<td>2.10</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>9.30</td>
<td>1.50</td>
</tr>
<tr>
<td>Nitrosomonas europaea growing on 30mM NH₄⁺</td>
<td>-</td>
<td>0.16</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.16</td>
<td>0.11</td>
</tr>
<tr>
<td>NH₄⁺ depleted cultures</td>
<td>-</td>
<td>0.13</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.08</td>
<td>0.11</td>
</tr>
</tbody>
</table>
FIG. 32: EFFECTS OF NH$_4^+$ CONCENTRATION IN CULTURE MEDIUM ON $\gamma$-GLUTAMYL TRANSFERASE ACTIVITY OF GS IN CRUDE EXTRACTS OF NITROSOMONAS EUROPAEA AT VARIOUS STAGES OF GROWTH.

Cultures were grown in 10 1 batches (2 1 inoculum from a culture completely depleted of NH$_4^+$) at 28°C with vigorous aeration. Aliquots (0.5-1 1) dispensed aseptically at the times indicated were harvested in polycarbonate tubes (250ml volume) in Sorvall-GSA rotor at 10,000g for 20 min at 4°C in the presence of CTAB (2.5g ml$^{-1}$). Cell extracts were prepared as described in Section 2.2.3. Transferase activity was determined with or without 60mM MgCl$_2$ at pH 7.4. Growth of bacterium was monitored by determining cell protein ml$^{-1}$ culture and was found to be directly proportional to the rate of NH$_4^+$ disappearance from the medium. Aliquots (1-2ml) were withdrawn aseptically from growing cultures, centrifuged at 15,000g for 10 min to remove cells and supernatant used to determine NH$_4^+$ as described in Section 2.2.17.2.

NH$_4^+$ utilization (○); glutamine synthetase specific activity (●); +Mg$^{2+}$/-Mg$^{2+}$ ratio (□).
FIG. 32

NH₄⁺ conc. in growth medium (mM)

+Mg²⁺ ratio

nmol/min (mg protein)⁻¹

incubation time (h)

20
16
12
8
4
0

20
40
60

0
2
4
6
8
10
12
The medium (8 l) was inoculated with 2 l of a culture which had been depleted of NH₄⁺ (the ratio of transferase activity with and without Mg²⁺ was 1.6). The oxidation of ammonia started immediately and after 55 h all the NH₄⁺ was utilized. The specific activity of GS decreased from about 0.011 μmol min⁻¹ (mg protein)⁻¹ at 15 h to 0.004 μmol min⁻¹ (mg protein)⁻¹ at 40 h and then increased thereafter. The +Mg²⁺/-Mg²⁺ ratio increased from 0.1 at 15 h to about 2.0 at 48 h when the cultures contained only 1 mM residual ammonia indicating that Mg²⁺ effect reflected the adenylylation state of the enzyme.

The effects of snake venom phosphodiesterase treatment on transferase activity of GS with and without 60 mM MgCl₂, are shown in Fig. 33. In these experiments transferase assays were carried out at pH 7.4 in the presence of 0.3 mM MnCl₂. The Mg²⁺ inhibition of transferase activity of purified enzyme from *Nitrobacter agilis* was completely reversed after a 30 min treatment with snake venom phosphodiesterase (Fig. 33a), however phosphodiesterase had no effect on Mg²⁺-inhibited transferase activity of *Nitrosomonas* enzyme (Fig. 33b). Moreover, longer incubation times (up to 4 h) and higher phosphodiesterase concentrations (up to 200 μg ml⁻¹) did not affect the extent of Mg²⁺ inhibition.

The adenylylated and deadenylyted forms of GS have different pH optima for transferase activity; thus the adenylylated form has a lower pH optimum (Bender et al., 1977). By treating purified enzyme from *Nitrobacter* with phosphodiesterase for defined periods, it was possible to prepare the enzyme at various stages of adenylylation as shown in Fig. 34. The native enzyme (without phosphodiesterase treatment) had a pH optimum of around 7.0 and the deadenylylated form (phosphodiesterase treated for 60 min) at 7.8. The isoactivity pH of two activities of the enzyme was about 7.4 and this explains why the transferase activity of enzyme was not affected by phosphodiesterase treatment when assayed in the absence of Mg²⁺ (Fig. 33a).

The two forms of enzyme have been shown to be inhibited differentially by feed back inhibitors (Ginsburg, 1969; Bender et al., 1977). Similar results were obtained with the *Nitrobacter* enzyme (Table 26). The deadenylylated form of enzyme appeared to be inhibited to a greater extent by alanine, glycine and serine and deadenylated enzyme by 5'AMP (Table 26).
FIG. 33: EFFECTS OF PHOSPHODIESTERASE TREATMENT ON PURIFIED GS FROM NITROBACTER AGILIS (a) AND NITROSOMONAS EUROPAEA (b).

The pH of an aliquot of purified enzyme was adjusted to 8.8 with M Tris. This was then divided into two equal volumes, one treated with phosphodiesterase (50µg ml⁻¹) at 37°C and the other taken as control. At the times indicated, aliquots of untreated and phosphodiesterase-treated enzyme were withdrawn and assayed for transferase activity with and without 60mM MgCl₂ at pH 7.4. Enzyme activity is expressed as A₅₄₀. The enzyme preparation was diluted initially so that 50µl of enzyme produced enough γGH in 15 min to give an absorbance of approx. 0.15 (without added Mg²⁺).

Untreated enzyme with (●) and without (○) Mg²⁺, treated enzyme with (□) and without (■) Mg²⁺.
If the transferase activity (Gs) is plotted against incubation time (min), the following patterns emerge:

(a) shows a general increase in transferase activity with time, especially for one of the experimental conditions.

(b) displays a more stable activity profile, with slight fluctuations over time.

The graph depicts the transferase activity (A_{540}) on the y-axis and incubation time (min) on the x-axis.
FIG. 34: DETERMINATION OF ISOACTIVITY pH FOR PURIFIED GS FROM 
NITROBACTER AGILIS.

Phosphodiesterase treatment (20µg ml⁻¹) of purified glutamine synthetase was as described in Fig. 33. The transferase activity of treated and untreated preparations was determined at various pH values as described in Section 2.2.6.2. Untreated glutamine synthetase (○); phosphodiesterase treated for 20 min (●); 40 min (□) and 60 min (■) respectively.
Transferase activity ($A_{540}$) vs pH

FIG. 34
TABLE 26: EFFECTS OF SOME AMINO ACIDS AND 5'-AMP ON ADENYLYLATED AND DEADENYLYLATED FORMS OF THE PURIFIES GS FROM NITROBACTER AGILIS.

Deadenylylated form of glutamine synthetase was prepared by phosphodiesterase treatment as described in Fig. 33 except that the incubation was for 1h. The adenylylation state of the enzyme was checked by determining the Mg$^{2+}$ effect on transferase activity at pH 7.4 as described in Table 25. All amino acids were at a final concentration of 10mM and AMP at 20mM. The activities of untreated (GS-AMP) and phosphodiesterase treated (GS) enzyme were 215 and 190 µmol YGH produced min$^{-1}$ (mg protein)$^{-1}$ respectively.

<table>
<thead>
<tr>
<th>Additions</th>
<th>% Inhibition of transferase activity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GS-AMP</td>
<td>GS</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>70</td>
<td>85</td>
</tr>
<tr>
<td>L-Glycine</td>
<td>35</td>
<td>60</td>
</tr>
<tr>
<td>L-Serine</td>
<td>40</td>
<td>55</td>
</tr>
<tr>
<td>5'-AMP</td>
<td>65</td>
<td>36</td>
</tr>
</tbody>
</table>
3.4 GLUTAMATE DEHYDROGENASES OF NITROBACTER AGILIS

3.4.1 Evidence for two isozymes of GDH in Nitrobacter agilis

The crude preparations of Nitrobacter agilis contain GDH which utilizes either NADH or NADPH for its amination reaction (Wallace and Nicholas, 1968; Section 3.2.4). To check whether the two activities were associated with two distinct proteins, electrophoresis of aliquots of the crude extract (S_{30}) was carried out in 5% (w/v) polyacrylamide gels (Section 2.2.10). The gels were stained for GDH activity using either NADH or NADPH or both (Fig. 35). The activity bands stained in gels A (NADH) and B (NADPH) had different electrophoretic mobilities and those in gel C (NADH + NADPH) also had two distinct bands. It is known that 2'-5' ADP Sepharose-4B binds enzymes that require NADPH. When crude extracts of Nitrobacter agilis were loaded onto a 2'-5' ADP Sepharose-4B column and washed with buffer (Section 2.2.5.2), the effluent contained NADH-GDH only (gel D). When the affinity column was eluted with 2mM NADPH, the eluate did not contain any NADH-GDH (gel E) but NADPH activity was detected (gels F and G). There are also two minor bands in gels F and G which may be either aggregates or active dissociated subunits of NADPH-GDH (see Smith et al., 1975). Only the NADPH specific enzyme was detected in crude extracts (S_{30}) of Nitrosomonas europaea analysed in polyacrylamide gels. The results indicate that Nitrobacter agilis has two distinct isozymes of GDH, one dependent on NADH and the other on NADPH while in Nitrosomonas europaea one single NADPH dependent GDH is present.

3.4.2 Purification of NADPH-GDH

The NADPH-GDH from Nitrobacter agilis was partially purified by affinity chromatography (Table 27). The purified enzyme from Nitrosomonas europaea reported to be dependent on NADPH only (Hooper et al., 1967) was also prepared (Table 28). The crude extracts (S_{30}) of Nitrobacter agilis contained both NADH and NADPH dependent activities (Table 27) while those of Nitrosomonas europaea had NADPH-GDH with only negligible NADPH-dependent activity (Table 28). In S_{110} fractions the specific activity of NADPH-GDH of Nitrosomonas europaea was about 13 fold greater than that of the NADPH enzyme from Nitrobacter agilis. The NADPH enzymes from Nitrobacter agilis and Nitrosomonas europaea were purified 52- and 142-fold by affinity chromatography on 2'-5' ADP Sepharose-4B. Both the purified preparations were free from the NADH-GDH.
FIG. 35: DETECTION OF NAD$^+$ AND NADP$^+$ DEPENDENT GDH IN *NITROBACTER AGILIS*

$S_{30}$ fraction was loaded onto a $2'5'$ADP Sepharose-4B column (0.8 x 11cm) and washed with 50mM Tris/HCl (pH 7.5), then eluted with 2mM NADPH in the buffer. Aliquots of $S_{30}$,$2'5'$ADP Sepharose-4B buffer washings and NADPH eluted proteins were dialysed overnight against the same buffer and then loaded onto 5% (w/v) polyacrylamide tubes. Electrophoresis and GDH specific staining was carried out as described in Section 2.2.10. Gels A to D were loaded with approx. 250µg protein and gels E to G, approx. 5µg protein. Gel A, ($S_{30}$ + NAD$^+$); gel B, ($S_{30}$ + NADP$^+$); gel C, ($S_{30}$ + NAD$^+$ + NADP$^+$); gel D, ($2'5'$ADP Sepharose-4B buffer washings + NAD$^+$ + NADP$^+$); gel E, ($2'5'$ADP Sepharose-4B NADPH eluate + NAD$^+$); gel F, ($2'5'$ADP Sepharose-4B NADPH eluate + NADP$^+$); gel G, ($2'5'$ADP Sepharose-4B eluate + NAD$^+$ + NADP$^+$).
TABLE 27: PURIFICATION OF NADP⁺-GDH FROM *NITROBACTER AGILIS*.

Enzyme purification and assay of enzyme activity as described in Section 2.2.5.2 and 2.2.6.4. Activity was determined by following the oxidation of either NADH or NADPH at 340nm. One enzyme unit is defined as nmol NAD(P)H oxidised min⁻¹ and specific activity as units (mg protein)⁻¹.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total Protein (mg)</th>
<th>Total Units</th>
<th>Specific Activity</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NADP⁺ NAD⁺</td>
<td>NADP⁺ NAD⁺</td>
<td>NADP⁺ NAD⁺</td>
</tr>
<tr>
<td><strong>S₁₅₀</strong></td>
<td>121</td>
<td>3111 2133</td>
<td>26 18</td>
<td>1 1</td>
</tr>
<tr>
<td><strong>S₁₁₀</strong></td>
<td>59.5</td>
<td>2609 1649</td>
<td>44 28</td>
<td>1.7 1.5</td>
</tr>
<tr>
<td>Pooled 2'5'ADP Sepharose-4B fractions</td>
<td>1</td>
<td>1353 3</td>
<td>1353</td>
<td>52</td>
</tr>
</tbody>
</table>
All purification steps and assay of enzyme activity as described in Sections 2.2.5.2 and 2.2.6.4. Activity was determined by following the oxidation of NAD(P)H at 340nm. One enzyme unit is defined as nmol NAD(P)H oxidised min⁻¹ and specific activity as units (mg protein)⁻¹.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total Protein (mg)</th>
<th>Total Units</th>
<th>Specific Activity</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NAD⁺ NAD⁺</td>
<td>NAD⁺ NAD⁺</td>
<td>NAD⁺ NAD⁺</td>
</tr>
<tr>
<td>S₁₀₀</td>
<td>1360</td>
<td>448000 131</td>
<td>330 0.1</td>
<td>1 1</td>
</tr>
<tr>
<td>S₁₁₀</td>
<td>975</td>
<td>402000 0</td>
<td>412 0</td>
<td>1.3 -</td>
</tr>
<tr>
<td>Pooled 2'5'-ADP</td>
<td>4.95</td>
<td>231000 0</td>
<td>46670 0</td>
<td>142 -</td>
</tr>
<tr>
<td>Sepharose-4B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.4.3 Amination and deamination reactions in $S_{110}$ fraction

The NADP$^+$-GDH from Nitrosomonas europaea functions in either direction, i.e., amination of α-ketoglutarate to form glutamate and deamination of glutamate to α-ketoglutarate (Hooper et al., 1967). It is clear from Table 27 that the amination activity of NADP$^+$-GDH from Nitrobacter agilis was about twice that of NAD$^+$-GDH. Since the gels were stained following deamination reaction, it is clear that the NADP$^+$-GDH of Nitrobacter agilis predominantly operates in the direction of glutamate production. That this is indeed the case is supported by the data in Table 29. In the $S_{110}$ fraction of Nitrobacter agilis the amination and deamination activities of the NAD$^+$ enzyme were approximately equal, however the deamination activity of the NADP$^+$-GDH was only about 4% of the amination activity (Table 29).

3.4.4 Properties of NADP$^+$-GDH from Nitrobacter agilis

Since the NADP$^+$-GDH from Nitrosomonas europaea has been studied in detail (Hooper et al., 1967) this section will deal with some properties of partially purified (52-fold) NADP$^+$-GDH from Nitrobacter agilis. The pH optima for amination and deamination reactions were 8 and 9 respectively (Fig.36). The rate of NADPH oxidation (amination reaction) was maximal with 10mM α-ketoglutarate (Fig.37a). A double reciprocal plot of the rate of NADPH oxidation against substrate concentration gave a $K_m$ value of 3.57 for α-ketoglutarate (Fig.37b). The NADPH oxidation rate for NADPH increased rapidly up to 20mM NH$_4$Cl and slowly thereafter so that the system appeared to be biphasic. Double reciprocal plots of the data (Fig.38b) produced two distinct $K_m$ values viz. 33mM for >20mM NH$_4^+$ and 6.3mM for <20mM NH$_4^+$. A similar type of substrate stimulation of enzyme activity was observed with the NADPH (Fig.39). Two $K_m$ values of 100μM and 7μM were recorded for NADPH concentrations >50μM and <50μM respectively.

The amination reaction of NADP$^+$-GDH from Nitrosomonas europaea is known to be inhibited by carboxylic acids and nicotinamide adenine nucleotides (Hooper et al., 1967). The effects of some of these compounds on the amination reaction of NADP$^+$-GDH of Nitrobacter agilis were studied (Table 30). Thus fumaric acid inhibited the amination reaction appreciably (45% at 20mM) while malic and pyruvic acids (20mM) and α-ketoglutarate (100mM) were without effect. Cis-oxaloacetic acid (OAA) slightly stimulated the reaction (18% at 5mM), but this effect was not enhanced by increasing the concentrations
TABLE 29: AMINATION AND DEAMINATION REACTION RATES IN EXTRACTS (S\textsubscript{110}) OF NITROBACTER AGILIS.

Crude extract of Nitrobacter agilis prepared as described in Section 2.2.3 was further centrifuged at 110,000g and supernatant (S\textsubscript{110}) used for enzyme assays. Amination and deamination reactions of glutamate dehydrogenase were determined at 25°C according to the methods described in Section 2.2.6.4. Specific activity is expressed as nmol NAD(P)H oxidised or NAD(P) reduced min\textsuperscript{-1} (mg protein)\textsuperscript{-1}.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amination:</strong></td>
<td></td>
</tr>
<tr>
<td>NADPH → NADPH\textsuperscript{+}</td>
<td>48.6</td>
</tr>
<tr>
<td>NADH → NAD\textsuperscript{+}</td>
<td>25.0</td>
</tr>
<tr>
<td><strong>Deamination:</strong></td>
<td></td>
</tr>
<tr>
<td>NADPH\textsuperscript{+} → NADPH</td>
<td>≤2.0</td>
</tr>
<tr>
<td>NAD\textsuperscript{+} → NADH</td>
<td>22.5</td>
</tr>
</tbody>
</table>
FIG. 36: DETERMINATION OF pH OPTIMA FOR AMINATION AND DEAMINATION ACTIVITIES OF PARTIALLY PURIFIED NADP⁺-GDH FROM NITROBACTER AGILIS.

Amination (●) and deamination (O) reactions of glutamate dehydrogenase were determined at 30°C as described in Section 2.2.6.4 except that 50mM Tris-HCl buffer was replaced by 200mM Tris-HCl at the pH indicated. For amination and deamination reactions, each assay mixture received approximately 10 and 50μg partially purified enzyme protein respectively.

FIG. 37: THE EFFECTS OF VARIOUS CONCENTRATIONS OF α-KETOGLUTARATE ON THE AMINATION REACTION OF PARTIALLY PURIFIED NADP⁺-GDH FROM NITROBACTER AGILIS.

Amination reaction of partially purified NADP⁺-GDH (Table 27) was determined as described in Section 2.2.6.4, except that α-ketoglutarate concentration was varied over the range of 0-30mM. The data in Fig. 37b are plotted as reciprocal of reaction velocity (1/V) against reciprocal of α-ketoglutarate concentration.
FIG. 38: THE EFFECTS OF VARIOUS CONCENTRATIONS OF NH$_4$Cl ON THE AMINATION REACTION OF PARTIALLY PURIFIED NADP$^+$-GDH FROM *NITROBACTER AGILIS*.

Amination reaction of partially purified NADP$^+$-GDH (Table 27) was determined as described in Section 2.2.6.4 except that NH$_4$Cl concentration was varied over the range 0-200mM. The data in Fig.38b are plotted as reciprocal of reaction velocity (1/V) against reciprocal of NH$_4$Cl concentration (1/S).

FIG. 39: THE EFFECTS OF VARIOUS CONCENTRATIONS OF NADPH ON THE AMINATION REACTION OF THE PARTIALLY PURIFIED NADP$^+$-GDH FROM *NITROBACTER AGILIS*.

Amination reaction of partially purified NADP$^+$-GDH (Table 27) was determined as described in Section 2.2.6.4 except that NADPH concentration was varied over the range of 0-200µM. The data in Fig.39b are plotted as reciprocal of reaction velocity (1/V) against reciprocal of NADPH concentration (1/S).
TABLE 30: EFFECTS OF ORGANIC ACIDS ON THE AMINATION ACTIVITY OF PARTIALLY PURIFIED NADP⁺-GDH FROM NITROBACTER AGILIS.

Amination reaction of the NADP⁺ enzyme was determined at pH 8.0 as described in Section 2.2.6.4, except that the assay mixture also contained an organic acid as indicated. The enzyme activity of the control was 1.32μmol NADPH oxidised min⁻¹ (mg protein)⁻¹.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Final concentration (mM)</th>
<th>% Inhibition (-) or stimulation (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fumaric acid</td>
<td>10</td>
<td>-28</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>-45</td>
</tr>
<tr>
<td>Cis oxaloacetic acid</td>
<td>5</td>
<td>+18</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>+13</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>+13</td>
</tr>
<tr>
<td>α-ketoglutaric acid</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>DL-malic acid</td>
<td>20</td>
<td>-13</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>10</td>
<td>-5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>-10</td>
</tr>
</tbody>
</table>

*aAll the acids were either neutralized to pH 7.5 before use or used as Na⁺ salt.*
of OAA to 20mM. None of the nucleotides (NAD\(^+\), NADP\(^+\), NADPH, and ATP) affected enzyme activity substantially (Table 31).

3.5 ENERGY CONSERVATION IN *NITROSOMONAS EUROPAEA* AND *NITROBACTER AGILIS*

3.5.1 *Proton translocation and oxygen pulse experiments*

Proton translocation during NH\(_4\)\(^+\) and NH\(_2\)OH oxidation by *Nitrosomonas europaea* has been studied by a fluorescence quenching technique (Bhandari and Nicholas, 1979a,b) and oxygen pulse technique (Drozd, 1976). Cobley (1976a,b) has shown by the oxygen pulse technique that the membrane particles of *Nitrobacter winogradskyi* translocated protons during NO\(_2\)\(^-\) oxidation.

Using fluorescence quenching technique (Bhandari and Nicholas, 1979a) in the present study, *Nitrosomonas europaea* translocated protons during either NH\(_4\)\(^+\) or NH\(_2\)OH oxidations (Fig.40a-c) and the results were similar to those reported earlier (Bhandari and Nicholas, 1979a). The addition of NO\(_2\)\(^-\) to a cell suspension of *Nitrobacter agilis* resulted in a very slight change in quinacrine fluorescence (Fig.40d,e). Varying the concentration of either substrate (0.2 to 20mM), cells (40 to 100mg) or quinacrine (0.05 to 0.2 \(\mu\)mol) had no effect on the magnitude of response. Similar results were obtained with the spheroplasts of *Nitrobacter agilis*. Thus attempts to study proton translocation in washed cells and spheroplasts of *Nitrobacter agilis* using fluorescence technique were largely unsuccessful under the experimental conditions described above.

For the quantitative estimation of proton translocation, experiments were done using the oxygen-pulse technique. The methodology and the details of apparatus used are described in Section 2.2.12.2. This subsection will deal with the results of oxygen pulse experiments with *Nitrosomonas europaea* and *Nitrobacter agilis*.

3.5.1.1 *Kinetic parameters of respiration in Nitrosomonas europaea and Nitrobacter agilis*

As the respiration activities and kinetic parameters of a particular biological system play an important role in overall energy metabolism these were considered for *Nitrosomonas europaea* and *Nitrobacter agilis* before carrying out the oxygen pulse experiment. The kinetic data are summarized in Tables 32 and 33.
TABLE 31: EFFECTS OF NUCLEOTIDES ON THE AMINATION ACTIVITY OF PARTIALLY PURIFIED NADP⁺-GDH FROM NITROBACTER AGILIS.

Amination reaction of the NADP⁺ enzyme was determined at pH 8.0 as described in Section 2.2.6.4, except that the assay mixture also contained a nucleotide as indicated. The enzyme activity of the control was 1.32 μmol NADPH oxidised min⁻¹ (mg protein)⁻¹.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Final concentration (mM)</th>
<th>% Inhibition (-) or stimulation (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH</td>
<td>0.50</td>
<td>+5</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>0.17</td>
<td>-5</td>
</tr>
<tr>
<td></td>
<td>0.35</td>
<td>-10</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>0.25</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>-10</td>
</tr>
<tr>
<td>ATP</td>
<td>0.50</td>
<td>0</td>
</tr>
</tbody>
</table>
Cell suspensions (20mg wet wt.) were preincubated for 5 min with 0.1μmol quinacrine and 25μmol Tris-HCl buffer (pH 7.5) in a final volume of 2.6ml in a series of 1cm cuvettes. Either NH₄Cl (0.5mM) or NH₂OH (0.5mM) were the substrates for Nitrosomonas europaea and NaNO₂ (1mM) for Nitrobacter agilis. The increase in fluorescence emission indicates an outwardly directed movement of protons measured in a fluorimeter at 420nm excitation and 485nm emission wavelength as described in Section 2.2.12.1.
FIG. 40

NITROSOMONAS

(a) NH₄Cl

(b) NH₂OH

(c) NH₄Cl + 2 µM CCCP

NITROBACTER

(d) NO₂⁻

(e) NO₂⁻ + 5 µM CCCP

2 MIN

FLUORESCENCE INCREASE
TABLE 32: KINETIC PARAMETERS OF RESPIRATION IN WASHED CELLS OF NITROSO MONAS EUROPAEA.

The respiration rates were determined at pH 7.5 by using a Clark-type oxygen electrode as described in Section 2.2.11. Oxygen uptake values for a specific substrate were corrected for the endogenous rate. The $K_m$ for $O_2$ was determined from electrode traces at low $O_2$ concentrations. Respiration rates are expressed as ng atom $O$ min$^{-1}$ (mg protein)$^{-1}$.

<table>
<thead>
<tr>
<th>Substrate$^a$</th>
<th>Respiration rate</th>
<th>$K_m$ Substrate (mM)</th>
<th>$O_2$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous</td>
<td>20–60</td>
<td></td>
<td>10–20</td>
</tr>
<tr>
<td>$NH_4Cl$</td>
<td>800–1510</td>
<td>1.0</td>
<td>5–10</td>
</tr>
<tr>
<td>$NH_2OH.HCl$</td>
<td>400–690</td>
<td>0.3</td>
<td>15–20</td>
</tr>
<tr>
<td>$N_2H_4.H_2SO_4$</td>
<td>130–250</td>
<td>1.0</td>
<td>15–20</td>
</tr>
</tbody>
</table>

$^a$The given respiration rates are for 2mM concentration of each substrate.
Table 33: Kinetic Parameters of Respiration in Washed Cells, Spheroplasts and Membrane Vesicles of *Nitrobacter Agilis*.

Experimental details as in Table 32. Spheroplasts and vesicles were prepared as described in Section 2.2.2. Respiration rates (at pH 7.8) corrected for endogenous respiration are expressed as ng atom O min⁻¹ (mg protein)⁻¹.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Substrate</th>
<th>Respiration rate</th>
<th>(K_m) NO₂ (mM)</th>
<th>(K_m) O₂ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Washed cells</td>
<td>Endogenous</td>
<td>20-40</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaNO₂</td>
<td>400-800</td>
<td>0.8</td>
<td>15-20</td>
</tr>
<tr>
<td>Spheroplasts</td>
<td>Endogenous</td>
<td>20-30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaNO₂</td>
<td>350-700</td>
<td>0.8</td>
<td>15-20</td>
</tr>
<tr>
<td>Vesicles</td>
<td>Endogenous</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaNO₂</td>
<td>30-40</td>
<td>0.8</td>
<td>15-20</td>
</tr>
</tbody>
</table>

*a* The given respiration rates are for 4mM NaNO₂.
There were considerable changes in specific activities among several batches of bacteria used (Tables 32 and 33). The difference arose partly as the result of the length of storage of cells. It was observed for example that *Nitrosomonas europaea* progressively lost its NH$_4^+$ oxidising activity, but gained in NH$_2$OH oxidising activity over three days of storage (the maximum storage time) at 0-4°C. The $K_m$ values for specific substrates and for O$_2$ were in reasonable agreement with the reported values (Drozd, 1976; Bhandari and Nicholas, 1979a).

### 3.5.1.2 Permeant ion requirement

For a number of bacteria, eg. denitrifiers (Kristjansson et al., 1978) valinomycin/K$^+$ serves as a permeant ion in oxygen pulse experiments to collapse membrane potential ($\Delta\psi$) and allow proton ejection, but in the case of *Nitrosomonas europaea* it was totally ineffective in the range of 50 to 150µg valinomycin ml$^{-1}$ even after incubation periods of up to 24h at 0-4°C. It appears that valinomycin (molecular weight 1111.4) does not penetrate the outer membrane of this Gram negative bacterium. Valinomycin produced a small oxygen-pulse response after incubation periods of about 1h at 0°C in the case of *Nitrosomonas europaea* cells, pretreated with 1mM EDTA, but these results were not reproducible. Several salts of permeant ions were studied for their ability to promote oxygen pulse responses. These included KSCN, which has been used widely in oxidant pulse experiments (Scholes and Mitchell, 1970b; Drozd, 1976), NaClO$_4$, sodium trichloroacetate (NaCl$_3$C$_2$O$_2$), KI, tetraphenyl boron (NaB(C$_6$H$_5$)$_4$) and triphenyl-methyl phosphonium bromide (TFMP$^+$Br$^-$) which has been recently employed to collapse $\Delta\psi$ in oxidant pulse studies with denitrifiers (Boogerd et al., 1981). To be useful, the permeant ions must not inhibit respiration so strongly that the oxygen pulse response would be lost and they must be sufficiently permeant as to permit efficient ejection of protons. Tetraphenyl boron (NaB(C$_6$H$_5$)$_4$) proved to be insufficiently soluble and so was effectively impermeable. Iodide (KI) allowed proton ejection, but inhibited substrate oxidation in *Nitrosomonas europaea*. Thiocyanate (KSCN), NaClO$_4$ and NaCl$_3$C$_2$O$_2$ produced similar and maximal -H$^+$/O ratios over the range 0.15 to 0.20M. Data for inhibition of oxidation of substrates by these anions are summarized in Table 34. The complete inhibition of NH$_4^+$ oxidation by 0.15M KSCN ruled out its use in oxygen pulse experiments with NH$_4^+$ as substrate, but it was otherwise acceptable and in fact enhanced the rate
TABLE 34: EFFECTS OF SALTS OF THREE PERMEANT ANIONS ON AEROBIC OXIDATION OF SUBSTRATES BY WASHED CELLS OF NITROSONOMAS EUROPaea AND NITROBACTER AGilis.

Respiration rates were determined by oxygen electrode as described in Table 32. The results for Nitrosomonas europaea were recorded at pH 7.5 and for Nitrobacter agilis at pH 7.8. The results for spheroplasts and vesicles prepared from Nitrobacter agilis were similar to those for cells.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Substrate</th>
<th>KSCN 0.15M</th>
<th>NaClO₄ 0.15M</th>
<th>NaClO₄ 0.30M</th>
<th>Na-trichloroacetate 0.1M</th>
<th>Na-trichloroacetate 0.2M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrosomonas europaea</td>
<td>Endogenous</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2mM NH₄Cl</td>
<td>100</td>
<td>34</td>
<td>59</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2mM NH₂OH-HCl</td>
<td>75</td>
<td>23</td>
<td>39</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2mM N₂H₄-H₂SO₄</td>
<td>+240a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrobacter agilis</td>
<td>Endogenous</td>
<td>75</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4mM NaNO₂</td>
<td>41</td>
<td>36</td>
<td>35</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

aStimulation of respiration.
of \( \text{N}_2\text{H}_5^+ \) oxidation. \( \text{NaClO}_4 \) was an effective inhibitor of endogenous respiration in both bacteria but otherwise was a weak inhibitor. \( \text{NaClO}_4 \) was used only in oxygen pulse studies with \( \text{Nitrobacter agilis} \) and so inhibition studies were carried out only with this organism. Effects of lipophilic cation TPMP\(^+\) on \( \text{NH}_4^+ \) and \( \text{NH}_2\text{OH} \) oxidation by \( \text{Nitrosomonas europaea} \) and \( \text{NO}_2^- \) oxidation by \( \text{Nitrobacter agilis} \) are shown in Table 35. TPMP\(^+\) was a potent inhibitor of oxidation of endogenous substrates and of added \( \text{NH}_4^+ \) in \( \text{Nitrosomonas europaea} \) at low concentrations (5mM), but was much less effective for \( \text{NH}_2\text{OH} \) oxidation. However TPMP\(^+\) even at very high concentrations (100 to 300mM) only partially inhibited \( \text{NO}_2^- \) oxidation (15 to 20\%) by \( \text{Nitrobacter agilis} \). Thus TPMP\(^+\) was suitable for \( \text{O}_2 \) pulse experiments with \( \text{Nitrosomonas europaea} \) when \( \text{NH}_2\text{OH} \) was the oxidizable substrate and with \( \text{Nitrobacter agilis} \) utilizing nitrite.

3.5.1.3 Stoichiometric proton production

Stoichiometric protons were determined as described in Section 2.2.12.4 in reductant pulse experiments in the absence of a permeant ion and in oxygen pulse experiments in which 3 to 5\( \mu \text{M} \) CCCP was used to un-couple the system by rapid equilibration of protons between internal and external cell compartments (Fig.41d). From the following reactions the theoretical stoichiometric proton values for \( \text{NH}_4^+ \), \( \text{NH}_2\text{OH} \) and \( \text{N}_2\text{H}_5^+ \) oxidation by \( \text{Nitrosomonas europaea} \) and \( \text{NO}_2^- \) oxidation by \( \text{Nitrobacter agilis} \) are 2,1,1 and 0 respectively for each mole of substrate oxidised:

(i) \( \text{NH}_4^+ + 30 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + 2\text{H}^+ \)
(ii) \( \text{NH}_2\text{OH} + 20 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + \text{H}^+ \)
(iii) \( \text{N}_2\text{H}_5^+ + 20 \rightarrow \text{N}_2 + 2\text{H}_2\text{O} + \text{H}^+ \)
(iv) \( \text{NO}_2^- + 0 \rightarrow \text{N}_2\text{O}_2^- \)

The results shown in Table 36 for the oxidation of \( \text{NH}_4^+ \), \( \text{NH}_2\text{OH} \) and \( \text{N}_2\text{H}_5^+ \) by \( \text{Nitrosomonas europaea} \) at pH 7.4 and of \( \text{NO}_2^- \) by \( \text{Nitrobacter agilis} \) at pH 7.8 are in reasonable agreement with the values expected from equations (i) to (iv) and the following pK values (Jencks and Rogenstein, 1968):

\( \text{NH}_4^+ \), 9.25; \( \text{NH}_3\text{OH}^+ \), 5.96; \( \text{N}_2\text{H}_5^+ \), 8.1; \( \text{HNO}_2 \), 3.29.
TABLE 35: EFFECTS OF LIPOPHILIC CATION TPMP$^+$ ON AEROBIC OXIDATION OF SUBSTRATES BY WASHED CELLS OF *NITROSOMONAS EUROPAEA* AND *NITROBACTER AGILIS*.

Respiration rates were determined by oxygen electrode as described in Table 32. The results for *Nitrosomonas europaea* were recorded at pH 7.5 and for *Nitrobacter agilis* at pH 7.8. The results for spheroplasts and vesicles prepared from *Nitrobacter agilis* were similar to those of whole cells.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>TPMP$^+$ concentration (mM)</th>
<th>Substrate$^a$</th>
<th>% Inhibition of respiration</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nitrosomonas europaea</em></td>
<td>10</td>
<td>Endogenous</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>NH$_4$Cl</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>NH$_4$Cl</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>NH$_2$OH.HCl</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>NH$_2$OH.HCl</td>
<td>50</td>
</tr>
<tr>
<td><em>Nitrobacter agilis</em></td>
<td>50</td>
<td>KNO$_2$</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>KNO$_2$</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>KNO$_2$</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>KNO$_2$</td>
<td>50</td>
</tr>
</tbody>
</table>

$^a$ Concentration of substrates, NH$_4$Cl and NH$_2$OH were 5mM and for KNO$_2$ 2mM.
The $\rightarrow H^+/O$ ratios in presence of CCCP were determined as described in Section 2.2.12.4. The substrate concentrations were over the range 2 to 4mM; CCCP, 4 to 5µM; amount of O$_2$, 10 to 40 ng atom O per pulse; pH for *Nitrosomonas europaea* at 7.4 and for *Nitrobacter agilis* at 7.8. The permeant ion was 0.15M SCN$^-$ except for NH$_4^+$ as substrate, when 0.2M ClO$_4^-$ was used. The $\rightarrow H^+/substrate$ ratios were determined in reductant pulse experiments (Section 2.2.12.3). The amount of substrate per pulse was 6 to 50 nmol and the system was vigorously stirred under pure O$_2$. The values in parenthesis represent expected values for *Nitrosomonas europaea* at pH 7.4 and for *Nitrobacter agilis* at pH 7.8.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Substrate</th>
<th>$\rightarrow H^+/O$ ratio in presence of CCCP</th>
<th>$\rightarrow H^+/substrate$ ratio in reductant pulse experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nitrosomonas europaea</em></td>
<td>Endogenous</td>
<td>0 - 0.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NH$_4$Cl</td>
<td>0.68 ± 0.04 (0.66)</td>
<td>1.9 ± 0.1 (1.99)</td>
</tr>
<tr>
<td></td>
<td>NH$_2$OH.HCl</td>
<td>0.48 ± 0.05 (0.52)</td>
<td>1.02 ± 0.06 (1.04)</td>
</tr>
<tr>
<td></td>
<td>N$_2$H$_4$-H$_2$SO$_4$</td>
<td>0.46 ± 0.05 (0.42)</td>
<td>0.89 ± 0.07 (0.83)</td>
</tr>
<tr>
<td><em>Nitrobacter agilis</em></td>
<td>Endogenous</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaNO$_2$</td>
<td>0.0 ± 0.06 (0.0)</td>
<td>undetermined</td>
</tr>
</tbody>
</table>
3.5.1.4 Oxygen pulse experiments with *Nitrosomonas europaea*

Freshly harvested cells of *Nitrosomonas europaea* contained endogenous substrate which promoted proton translocation. The oxygen responses were typically as reported earlier (Drozd, 1976) and shown in Fig. 41. Endogenous substrate exhibited $\Delta H^+/O$ ratios of 4.6 to 5.6 typically for the first few pulses of $O_2$ (Fig. 41b) at pH 7.0 to 7.4 but decreased to 3.5 to 4.5 during subsequent pulses. It was frequently possible however to exhaust endogenous substrate for periods of 20 to 30 min by means of a series of large (100 to 200 ng atom) pulses of $O_2$. In one batch of cells out of 13 the endogenous substrate became permanently exhausted following oxygen pulses. The $\Delta H^+/O$ ratios for endogenous substrate oxidation were independent of the amount of $O_2$ per pulse from 5 to 40 ng atom $O$. The use of perchlorate (Table 34) and TPMP$^+$ (Table 35), which totally inhibited oxidation of endogenous substrates, and use of cells lacking endogenous substrates permitted a clear distinction between proton-translocation due to three amine-like substrates and that due to oxidation of endogenous substrates. It was found that the $\Delta H^+/O$ ratios obtained with the amine-like substrates at concentrations $>2$ mM referred largely or entirely to the oxidation of these substrates and not to endogenous substrates. This result was consistent with the data in Table 32 which show that the rates of oxidation of endogenous substrates were small compared to those for specific substrates and with the results in Table 36 which indicate that the yield of stoichiometric protons correlated well with the oxidative reaction of the specific substrate.

The $\Delta H^+/O$ ratio was essentially independent of the size of the oxygen pulse up to about 30 ng atom $O$ in the case of $NH_4^+$ oxidation, but dependent on pulse size in the case of $NH_2OH$ (Fig. 42) and $N_2H_5^+$ oxidations. In part this effect may be a consequence of a moderately high $K_m$ ($O_2$) for the oxidation of $NH_2OH$ and $N_2H_5^+$ (Table 32). For small pulses of oxygen, a larger fraction of the total reaction lies in the kinetically slow phase encountered at $O_2$ concentration $<K_m$ ($O_2$). The relevant $\Delta H^+/O$ ratio in the case of $NH_2OH$ and $N_2H_5^+$ was taken to be the asymptotic value obtained with larger oxygen pulses.

The asymptotic $\Delta H^+/O$ ratios in turn depended on the concentrations of $NH_4^+$, $NH_2OH$ and $N_2H_5^+$ (Fig. 43). At high concentrations of these substrates (20-40 mM) the $\Delta H^+/O$ ratios approached the stoichiometric value (Table 36) for the reaction, e.g. 0.7 for $NH_4^+$. The effect was not due to substrate inhibition.
FIG. 41: OXYGEN PULSE RESPONSES IN WASHED CELLS OF *NITROSMONAS EUROPaea*.

Freshly harvested cells were washed twice in 150mM KCl and finally suspended in the same solution (approximately 75mg wet weight ml⁻¹). Oxygen pulse experiments were carried out as described in Secton 2.2.12.2. Reaction mixture in a final volume of 1.5ml contained approximately 105mg wet weight cells in 150mM KCl, 100μg carbonic anhydrase and 150mM KSCN (where indicated). The initial pH of the cell suspension was adjusted to 7.4 with 50mM HCl or 50mM NaOH. Fig. 41a illustrates the O₂ pulse response in cells containing endogenous substrates, without any permeant ion in the reaction mixture; 41b shows the proton translocation associated with endogenous respiration with 150mM KSCN as permeant ion and 41c,d show proton translocation associated with NH₂OH oxidation in cells depleted of endogenous substrates and with 150mM KSCN as permeant ion. Fig. 41d illustrates the fast equilibration of protons after addition of 4μM CCCP to the reaction mixture.
FIG. 42: DEPENDENCE OF \( \rightarrow H^+ / O \) RATIO ON THE SIZE OF THE OXYGEN PULSE FOR OXIDATION OF \( NH_2OH \) BY \textit{NITROSOMONAS EUROPAEA}.

The oxygen pulse experiments were carried out at 25°C and pH 7.4 as described in Section 2.2.12.2. KSCN (150mM) was used as a permeant ion. The \( NH_2OH \) concentrations were (mM):

- 1.2 (○);
- 3.1 (□) and 10.2 (Δ).

FIG. 43: DEPENDENCE OF \( \rightarrow H^+ / O \) RATIO ON THE SUBSTRATE CONCENTRATION FOR OXIDATION OF AMINE-LIKE SUBSTRATES BY \textit{NITROSOMONAS EUROPAEA}.

The oxygen pulse experiments were carried out at 25°C and pH 7.4 as described in Section 2.2.12.2. The system contained 0.2M perchlorate as permeant ion with \( NH_4^+ \) (○) and 0.15M KSCN as that with \( NH_2OH \) (□) and \( N_2H_5^+ \) (Δ). Each point on the curves represents the asymptotic limit or plateau inferred from an experiment of the type illustrated in Fig. 42.
**FIG. 42**

![Graph showing observed $\mathrm{H^+O}$ ratio against ng atom O per pulse.](image)

**FIG. 43**

![Graph showing $\mathrm{H^+O}$ ratio against substrate conc. (mM).](image)
of respiration. The respiratory proton pump would appear to be inhibited at high concentrations of these amine-like substrates. In this regard methylammonium (CH$_3$NH$_3^+$) which is not oxidised by Nitrosomonas europaea mimicked the effect of the amine-like substrate in diminishing H$^+/O$ ratios. For example, 20mM CH$_3$NH$_3^+$ lowered the H$^+/O$ ratios from 3-4 for 2mM concentrations of these substrates to 0.9-1.5. Similar effects were also noted for the endogenous substrate oxidation. Thus, the H$^+/O$ ratios are taken to be the values extrapolated to zero substrate concentration (Fig.43), namely 3.4 for NH$_4^+$ and 4.4 for NH$_2$OH and N$_2$H$_5^+$. The data for NH$_2$OH and N$_2$H$_5^+$ (Fig.43) were similar to those obtained with perchlorate as the permeant anion or TPMP$^+$ as the permeant cation (Table 37).

For the oxidation of NH$_4^+$ and NH$_2$OH respectively, the possibility existed (Ritchie and Nicholas, 1972) that the product NO$_2^-$ might itself serve as an oxidant as in the following dismutation reaction:

\[
\text{NH}_2\text{OH} + \text{NO}_2^- \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O} + \text{OH}^-
\]

Such a reaction could, if rapid, provide a different overall reaction and possibly a different H$^+/O$ ratio:

\[
\text{NH}_2\text{OH} + 0 \rightarrow \frac{1}{2} \text{N}_2\text{O} + 1 \frac{1}{2} \text{H}_2\text{O}
\]

Pulse experiments carried out with NO$_2^-$ instead of O$_2$ with NH$_4^+$ and NH$_2$OH as substrates indicated that no proton translocation occurred. Moreover, the rate of anaerobic reduction of NO$_2^-$ by NH$_2$OH in Nitrosomonas europaea was very low. Thus these reactions were not considered to be kinetically relevant.

3.5.1.5 Oxygen pulse experiments with *Nitrobacter agilis*

Attempts to evoke respiration dependent proton translocation in *Nitrobacter agilis* cells were unsuccessful. The permeant ions employed in the study were valinomycin/K$^+$ (50 to 100μg ml$^{-1}$), thiocyanate (0.15 to 0.38M), perchlorate (0.2M), trichloroacetate (0.1 to 0.25M) and triphenyl methyl phosphonium cation (0.005 to 0.3M). Oxygen pulses were varied from 10 to 120ng atom O per pulse, NO$_2^-$ concentration from 0 to 8mM, pH from 7.0 to 8.0 and the amount of cells from 100 to 150mg wet wt. Experiments were also done in the absence of a permeant ion. A rapid transient acidification occurring in the 1 to 5s range was not observed and in fact the most consistent rapid response was a slight
TABLE 37: APPARENT $\rightarrow H^+/O$ RATIOS IN WASHED CELLS OF NITROSONOMAS EUROPAEA AND NITROBACTER AGILIS DURING SUBSTRATE OXIDATION USING TPMP$^+$ AS PERMEANT ION.

$\rightarrow H^+/O$ ratios were determined as described in Section 2.2.12.2. The data are from approx. 10 experiments. The resting pH in the oxygen pulse experiments was 7.4 for both bacteria and the amount per pulse was 40 ng atom.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>TPMP$^+$ concentration (mM)</th>
<th>Substrate$^a$</th>
<th>Apparent $\rightarrow H^+/O$ ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrosomonas europaea</td>
<td>5</td>
<td>endogenous</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>NH$_4$Cl</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>NH$_2$OH</td>
<td>3.5-4.0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>NH$_2$OH</td>
<td>3.5-4.0</td>
</tr>
<tr>
<td>Nitrobacter agilis</td>
<td>50</td>
<td>KNO$_2$</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td>100-300</td>
<td>KNO$_2$</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

$^a$NH$_4$Cl and NH$_2$OH concentrations were 2-5mM and that of KNO$_2$, 2mM.

$^b$Difficult to estimate due to very slow reduction of O$_2$. Apparent ratios were about 1.0.
alkalinization with $\Delta H^+/0$ ratio of about -0.15. This was usually followed by a slow but variable acidification that peaked within 20 to 35s, and had an apparent $\Delta H^+/0$ ratio of 0.1 to 0.2. Neither the extent of acidification nor the time required for its completion was proportional to the amount of $O_2$ injected. The response was not clearly dependent on the prior addition of $NO_2^-$. The responses were largely abolished by 4$\mu$M CCCP and so probably represented differences in proton concentration between inner and outer buffer compartments of cell.

A study was made of spheroplasts of Nitrobacter agilis (prepared as described in Section 2.2.2) in a system containing valinomycin (50$\mu$g ml$^{-1}$), 0.15M thiocyanate or 0.2M perchlorate, 10mg of spheroplast protein and 0 to 8mM $NO_2^-$ at pH 7.0 and 7.5. The only rapid response observed was a slight alkalinization with $\Delta H^+/0$ ratio of about -0.06 (Fig.44). Lysis of spheroplasts was slight as determined by DNA release.

The study also included membrane vesicles of Nitrobacter agilis in a system containing valinomycin (50$\mu$g ml$^{-1}$)/$K^+$ or 0.15M KSCN,≈17mg of vesicle protein and 0 to 4mM $NO_2^-$ at pH 7.5. In the absence of $NO_2^-$ a transient alkalinization was observed with peak at 4 to 5s and an $\Delta H^+/0$ ratio of about -0.2 (Fig.45a). With 2 and 4mM $NO_2^-$ the initial alkalinization was smaller ($\Delta H^+/0 = 0.1$) and followed by a slow acidification in 30 to 50s range (Fig.45b,c). Both the amount of vesicle protein and the internal buffering capacity were similar to those found in systems containing intact cells. Thus vesicles were able, in principle, to translocate at least 100 nmol of proton before the $\Delta pH$ might prohibit further proton translocation. A null result could have accrued if the vesicles had been half inverted ('inside out') and half right side out, but this was not the case. Vesicles bounded apparently by a single membrane were studied to test the possibility that the null result with cells and spheroplasts occurred because the proton pump in Nitrobacter agilis translocated protons from one intracellular compartment to another as is the case in eukaryotic cells.
FIG. 44: OXYGEN PULSE RESPONSES IN SPHEROPLASTS OF NITROBACTER AGILIS.

Spheroplasts were prepared as described in Section 2.2.2 except that lysozyme/EDTA treated cells were washed and suspended in 150mM KCl. Oxygen pulse experiments were carried out as described in Section 2.2.12.2 with 150mM KSCN as a permeant ion. The reaction mixture in a final volume of 1.5ml contained spheroplasts (70mg wet weight ml⁻¹) in 150mM KCl, carbonic anhydrase (80μg ml⁻¹) and 150mM KSCN. The resting pH of the suspension was 7.55. The system was calibrated with 5mM HCl.
FIG. 45: OXYGEN PULSE RESPONSES IN MEMBRANE VESICLES OF *NITROBACTER AGILIS*.

Membrane vesicles, prepared as described in Section 2.2.2, washed twice in 150mM KCl and finally suspended in the same solution (11.4mg protein ml⁻¹). The reaction mixture in a final volume of 1.5ml contained vesicles (∼17 mg protein), carbonic anhydrase (100μg) and either valinomycin (75μg) or KSCN (150mM). The initial pH of the suspension at 8.2 was titrated to 7.5 with 50mM HCl. The system was calibrated with 5mM HCl.
Figure 45

(a) endogenous substrate val/K^+

10 µl HCl (5 mM)

10 µl KCl/0₂

(b) NO₂⁻ substrate -KSCN

60 nmol NO₂⁻

10 µl KCl/0₂

170 µl 1.5 M KCl/0₂

(c) NO₂⁻ substrate +KSCN

30 µl KCl/0₂

1 min.
3.5.1.6 Duration of respiration after an oxygen pulse

The slowest rates of respiration were those supported by endogenous substrates in cells and by NO$_2^-$ in vesicles from Nitrobacter agilis (Table 33). These rates were taken as 15ng atom 0 min$^{-1}$ (mg protein)$^{-1}$ to account for partial inhibition of respiration by permeant ions. Because the amount of proteins was about 15mg, the overall rate was about 4ng atom 0 s$^{-1}$. The duration of 20ng atom pulse of O$_2$ would have therefore been 5s. In all other systems reported in Tables 32 and 33 the respiration time would have been so short as to lie always within the mixing time.

3.5.2 Proton electrochemical-gradients in washed cells of *Nitrosomonas europaea* and *Nitrobacter agilis*

3.5.2.1 Uptake of radioactive probes

All probes, listed in Table 38, used to determine membrane potential ($\Delta\psi$) and transmembrane pH gradient ($\Delta pH$) were readily taken up by the cells of *Nitrosomonas europaea* and *Nitrobacter agilis* and an equilibrium state was reached within 5 min. The EDTA treatment of the cells was necessary in order to make them permeable to radioactive compounds, indeed without this treatment the results were variable and erratic. High concentrations of EDTA (5mM for *Nitrosomonas* and 10mM for *Nitrobacter*) relative to those used for *E. coli* (Padan et al., 1976) were employed because of the complex cell membrane structures of these bacteria (Murray and Watson, 1965). EDTA treated cells were metabolically active since oxygen uptake values were similar to those of untreated cells.

3.5.2.2 Measurement of $\Delta pH$ as a function of external pH (pHe)

The uptake of [$^{14}$C] benzoic acid, [$^{14}$C] acetylsalicylic acid and [$^{14}$C] methylamine respectively by *Nitrosomonas europaea* and *Nitrobacter agilis* has been plotted against pHe in Fig.46a and b. The uptake of all three compounds was pHe dependent. *Nitrosomonas europaea* accumulated the two weak acids only when the pHe was below 7.0 and the weak base methylamine when the pHe was above 7.0 (Fig.46a), indicating that the internal pH (pHi) of bacterium was maintained around neutrality. The $\Delta pH$ was almost zero at pHi of 7.0, but when pHe was $>$7.0 the pHi became acidic in relation to pHe (inside acidic). For *Nitrobacter agilis* (Fig.46b),
TABLE 38: PROBES FOR DETERMINING $\Delta \psi$ AND $\Delta \text{pH}$ IN WASHED CELLS OF NITROSOMONAS EUROPAEA AND NITROBACTER AGILIS.

Washed cell suspensions in 50mM Na-phosphate buffer (pH 6.0) were used to determine $\Delta \text{pH}$ and $\Delta \psi$ as described Section 2.2.13. EDTA treatment of the cells was carried out at 37°C for 10 min (Section 2.2.13.1). $\Delta \text{pH}$ and $\Delta \psi$ are the mean values of 10 determinations with the same batch of cells.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Probe</th>
<th>$\Delta \text{pH} \pm \text{SEM}$</th>
<th>$\Delta \psi \pm \text{SEM (mV)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Untreated cells</td>
<td>EDTA-treated cells</td>
</tr>
<tr>
<td>Nitrosomonas europaea</td>
<td>[14C] Benzoic Acid</td>
<td>0.22 ± 0.12</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>[14C] Acetyl salicylic</td>
<td>0.20 ± 0.12</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[14C] Methylamine-HCl</td>
<td>0.0 ± 0.01</td>
<td>0.0 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>[3H] TPP †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrobacter agilis</td>
<td>[14C] Benzoic Acid</td>
<td>0.80 ± 0.10</td>
<td>1.36 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>[14C] Acetyl salicylic</td>
<td>0.72 ± 0.12</td>
<td>1.22 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[14C] Methylamine-HCl</td>
<td>0.00 ± 0.00</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>[3H] TPP †</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIG. 46: UPTAKE OF $^{14}\text{C}$ BENZOIC ACID, $^{14}\text{C}$ ACETYLSALICYLIC ACID AND $^{14}\text{C}$ METHYLAMINE AS A FUNCTION OF pH BY EDTA TREATED CELLS OF (a) NITROSOMONAS EUROPAEA AND (b) NITROBACTER AGILIS.

EDTA treated cells of *Nitrosomonas europaea* and *Nitrobacter agilis* were prepared as described in Section 2.2.13.1. Treated cells were suspended in 50mM Na-phosphate buffer at pH values indicated. Uptake studies were carried out as described in Section 2.2.13.3.

$^{14}\text{C}$ benzoic acid (○); $^{14}\text{C}$ acetyl salicylic acid (●); and $^{14}\text{C}$ methylamine (□).
the pH at which neither of the two weak acids nor weak base was
taken up by the cells, was about 7.5. Benzoic and acetyl salicylic
acids were not metabolized by either bacterium. *Nitrosomonas europaea*
however slowly utilized the weak base methylvamine when the external pH
was greater than 7.5. Because methylvamine was utilized slowly by
*Nitrosomonas europaea*, the uptake studies which were completed within
5 min were unaffected by this metabolism.

*Nitrosomonas europaea* had a limited capacity to maintain a constant
pH and thus it increased from 6.3 to 7.8 when the pH was varied over
the range 6.0 to 8.5 (Fig.47a). On the other hand in *Nitrobacter agilis* (Fig.47b) pH increased from 7.3 to 7.8 when the pH was in-
creased from 6.0 to 8.5. Thus at pH 6.0 *Nitrosomonas europaea* and
*Nitrobacter agilis* had a ΔpH of 0.3 and 1.3 pH units respectively
(Table 38). As these bacteria respire optimally between pH 7.5 to 8.0,
it appears that they do not have a ΔpH (inside alkaline) but instead
the pH are either similar to pH or more acidic (inside acid). Over
a range of pH (6.0 to 8.5), the pH in *Nitrobacter agilis* increased
only by about 0.5 units, whereas in *Nitrosomonas europaea* it increased
by 1.5 units.

3.5.2.3 Measurement of Δψ as a function of pH

The variations in Δψ as determined by [3H]TPP⁺ uptake over a pH
range 6 to 8.5 are shown in Fig.47. TPP⁺ tends to bind to cellular
components so that data were corrected for this non-specific binding
(Section 2.2.13.3). In both bacteria Δψ increased with increasing pH. Thus in *Nitrosomonas europaea* Δψ increased from 125mV at pH 6.0 to 178
at pH 8.5. In *Nitrobacter agilis* the effect of pH on Δψ was less
pronounced than in *Nitrosomonas europaea*, thus it increased from 105mV
at pH 6.0 to 135mV at pH 8.5, an increase of approximately 10mV for
each pH unit. The increase in Δψ in *Nitrosomonas europaea* was non-
linear and approached a plateau at pH 8.0, while in *Nitrobacter agilis*
the increase was almost linear.

3.5.2.4 Total proton-motive force (Δp)

Since Δp is a function of Δψ and ΔpH, it is clear from Fig.47a
that Δp remained almost constant (135-145mV) in *Nitrosomonas europaea*
FIG. 47: EFFECTS OF pH ON pH, ΔpH, Δψ AND Δp IN EDTA-TREATED CELLS OF (a) NITROSOMONAS EUROPAEA AND (b) NITROBACTER AGILIS.

EDTA treated cells were suspended in 50mM Na-phosphate buffer at pH values indicated. Uptake studies were carried out as described in Section 2.2.13.3. Intracellular pH (Δ) and ΔpH (▲) represented in terms of mV (59 x ΔpH) were determined from the uptake of [14C] benzoic acid and [14C] methylamine. Δψ values (○) were calculated from the uptake of [3H] TPP⁺. Δp (●) was calculated from ΔpH and Δψ as described in Section 2.2.13.4.
over a range of external pH. This was largely the result of an increase in $\Delta \psi$ and a decrease in $\Delta \phi$ when the pH was increased from 6.0 to 8.5, thus a decrease in $\Delta \phi$ was compensated by an increase in $\Delta \psi$. In *Nitrobacter agilis* (Fig.47b), however, the contribution of $\Delta \phi$ decreased rapidly when the pH was increased (-73mV at pH 6.0 to +40mV at pH 8.5), while $\Delta \psi$ increased by 30mV only from pH 6 to 8.5, thus decreasing the total proton-motive force from 177mV at pH 6.0 to 95mV at pH 8.5.

### 3.5.2.5 Proton-motive force in cells of *Nitrosomonas europaea* harvested at various stages of growth

*Nitrosomonas europaea* grows slowly (mean generation time 10-12h). About 24h after inoculation the exponential stage of growth started and it lasted for another 4 days (Fig.48a,b). Because the cell yields were low, it was not possible to conduct uptake studies with the probes to determine $\Delta \phi$ and $\Delta \psi$ in growing cultures as described by Kashket for a number of bacteria (Kashket *et al.*, 1980; Kashket, 1981a,b). To assess whether there were any changes in $\Delta \phi$ and $\Delta \psi$ at various stages of growth, cultures (1 l) were harvested at various times as shown in Fig.48a and b. Thus $\Delta \phi$ and $\Delta \psi$ were determined at two pH values (6 and 8) after suspending the cells in fresh culture medium. The intracellular water volume was reasonably constant during growth (1.6 ± 0.2μl (mg dry wt.)^{-1}). Cells harvested at different stages of growth maintained a fairly constant pH (Fig.48a,b). Thus at pH 6.0, $\Delta \psi$ was approximately 122mV and $\Delta \phi$ 0.3 units (inside alkaline) and at pH 8.0, $\Delta \psi$ was approximately 165mV and $\Delta \phi$ 0.5 units (inside acid). A similar experiment with *Nitrobacter agilis* was not possible because of exceptionally low cell yields (40mg wet wt. l^{-1} culture after 5 days of growth).

### 3.5.2.6 Effects of some inhibitors on the components of $\Delta \phi$

To determine the relevance of respiratory potential to $\Delta \phi$ maintenance, the effects of respiratory inhibitors on $\Delta \phi$ and $\Delta \psi$ in *Nitrosomonas europaea* and *Nitrobacter agilis* were investigated. Sodium diethyldithiocarbamate (DIECA), a potent inhibitor of NH_{4}^{+} oxidation by *Nitrosomonas europaea* (Bhandari and Nicholas, 1979a,b) completely inhibited respiration at 20μM but did not have any effect on $\Delta \phi$ (Table 39). In *Nitrobacter agilis* (Table 40), sodium azide at 50μM completely
**FIG. 48:** INTRACELLULAR pH AND Δψ IN CELLS OF *NITROSONOMAS EUROPAEA* AT VARIOUS STAGES OF GROWTH DETERMINED AT pH VALUES OF (a) 6.0 AND (b) 8.0.

Cultures (18 ℓ) were grown in 20 ℓ Pyrex glass bottles at a constant temperature (28°C) and pH (8.0). Growth of the bacterium (●) was monitored throughout the incubation period by the rate of NH₄⁺ oxidation and by determining the protein contents of the cells. At the times indicated, 1-to 2 ℓ cultures withdrawn aseptically were harvested by centrifugation (10,000g for 30 min) in 250ml polycarbonate bottles. The cells were then washed and resuspended in growth medium at either pH 6.0 (a) or pH 8.0 (b). Uptake studies were carried out as described in Section 2.2.13.3. Δψ (○) was calculated from the uptake of [³H] TPP⁺. Intracellular pH (□) was determined by the uptake of [¹⁴C] benzoic acid and [¹⁴C] methylamine at pH 6.0 and 8.0 respectively.
FIG. 48

(a) [Graph showing mV vs. incubation (h)]

(b) [Graph showing pH vs. incubation (h)]

- mV
- pH
- Protein (mg/mL culture)⁻¹

incubation (h): 0, 40, 80, 120
TABLE 39: EFFECTS OF SOME INHIBITORS ON RESPIRATION AND PROTON-MOTIVE FORCE IN *NITROSOMONAS EUROPAEA*.

Washed cell suspensions in 50mM Tris-HCl at pH 6.9 were employed for $\Delta \psi$ and $\Delta \mathrm{pH}$ determinations. $\Delta \psi$ and $\Delta \mathrm{pH}$ were calculated from the uptake of $[^3\mathrm{H}] \text{TPP}^+$ and $[^{14}\mathrm{C}] \text{benzoic acid}$ respectively as described in Section 2.2.13. Respiration rates were determined at pH 7.8 by oxygen electrode technique (Section 2.2.11). Control rate of O$_2$ uptake was approximately 850 ng atom O min$^{-1}$ (mg protein)$^{-1}$.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (uM)</th>
<th>% Inhibition of ($\text{NH}_4^+$) respiration</th>
<th>$\Delta \psi^a$ (mV)</th>
<th>$\Delta \mathrm{pH}^b$</th>
<th>$\Delta \mathrm{p}^c$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>147</td>
<td>0.18</td>
<td>158</td>
</tr>
<tr>
<td>DIECA</td>
<td>20</td>
<td>100</td>
<td>148</td>
<td>0.00</td>
<td>148</td>
</tr>
<tr>
<td>CCCP</td>
<td>10</td>
<td>80</td>
<td>110</td>
<td>0.10</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>80</td>
<td>0.00</td>
<td>80</td>
</tr>
<tr>
<td>DCCD</td>
<td>200</td>
<td>50</td>
<td>117</td>
<td>0.16</td>
<td>128</td>
</tr>
<tr>
<td>DESB</td>
<td>50</td>
<td>25</td>
<td>187</td>
<td>0.50</td>
<td>216</td>
</tr>
</tbody>
</table>

$^a$ Inside negative  
$^b$ Inside alkaline  
$^c$ Inside negative
TABLE 40: EFFECTS OF SOME INHIBITORS ON RESPIRATION AND PROTON-MOTIVE FORCE IN NITROBACTER AGILIS.

Washed cell suspensions in 50mM Tris-HCl at pH 7.0 were employed for Δψ and ΔpH determinations. Δψ and ΔpH were calculated from the uptake of [3H]TPP⁺ and [14C] benzoic acid respectively as described in Section 2.2.13. Respiration rates were determined at pH 7.8 by oxygen electrode technique (Section 2.2.11). Control rate of O₂ uptake was approximately 650 ng atom O min⁻¹ (mg protein)⁻¹.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (µM)</th>
<th>% Inhibition of respiration</th>
<th>Δψa (mV)</th>
<th>ΔpHb (mV)</th>
<th>ΔpCc (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium azide</td>
<td>50</td>
<td>100</td>
<td>115</td>
<td>0.34</td>
<td>138</td>
</tr>
<tr>
<td>CCCP</td>
<td>10</td>
<td>70</td>
<td>114</td>
<td>0.05</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>100</td>
<td>82</td>
<td>0.20</td>
<td>94</td>
</tr>
<tr>
<td>DCCD</td>
<td>100</td>
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<td>75</td>
<td>0.12</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>100</td>
<td>116</td>
<td>0.38</td>
<td>138</td>
</tr>
<tr>
<td>DESB</td>
<td>20</td>
<td>45</td>
<td>124</td>
<td>0.31</td>
<td>142</td>
</tr>
<tr>
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<td>50</td>
<td>85</td>
<td>133</td>
<td>0.09</td>
<td>139</td>
</tr>
</tbody>
</table>

ₐInside negative  
₋Inside alkaline  
ₐInside negative
inhibited NO$_2^-$ oxidation (also see O'Kelly et al., 1970 and Section 3.1.6) and although it had no effect on $\Delta\psi$ it dissipated $\Delta p$H completely, thus lowering $\Delta p$ by about 20mV. Because respiration in nitrifying bacteria has been shown to be inhibited by uncouplers (Aleem, 1977; Bhandari and Nicholas, 1979a,b; Aleem and Sewell, 1981; Section 3.1.6) and this effect was related to the collapse of $\Delta\psi$ in *Nitrobacter* (Cobley, 1976a,b), the effects of the classical uncoupler CCCP on $\Delta p$ in both *Nitrosomonas europaeae* (Table 39) and *Nitrobacter agilis* (Table 40) were investigated. The respiration of both nitrifiers was completely inhibited by 50μM CCCP but $\Delta p$ was only partially collapsed (Tables 39,40). As shown in Section 3.1.6 several ATPase inhibitors restrict respiration in *Nitrobacter agilis* to an extent similar to ATPase itself. To investigate whether this effect was related to a collapse of $\Delta p$, two ATPase inhibitors, DCCD and DESB, were used in studies on $\Delta\psi$ and $\Delta p$H. DCCD at high concentrations (>200μM) affected $\Delta p$ in both nitrifiers by lowering $\Delta\psi$ in *Nitrosomonas europaeae* (Table 39) and $\Delta p$H in *Nitrobacter agilis* (Table 40). DESB had little or no effect on $\Delta p$ in *Nitrobacter agilis* (Table 40) but at 50μM it elevated $\Delta p$ by about 60mV (negative inside) in *Nitrosomonas europaeae*.

3.5.2.7 Effects of NH$_4^+$ and NH$_2$OH on $\Delta\psi$ and $\Delta p$H

Permeant amines and amine-like compounds have a tendency to redistribute across the membrane towards the acidic side in response to a pH gradient (Krogman et al., 1959; Good, 1960). It has been shown that high concentrations of substrate (NH$_4^+$, NH$_2$OH or N$_2$H$_5^+$) in *Nitrosomonas europaeae* tended to diminish proton pumping (Section 3.5.1.4). As shown in Fig.49, both NH$_4^+$ and NH$_2$OH at high concentrations diminished the small $\Delta p$H across the cell membranes of *Nitrosomonas europaeae* completely. Moreover, NH$_4^+$ also decreased $\Delta\psi$ (170mV to 140mV at 100mM NH$_4^+$) but relatively small concentrations of NH$_2$OH (≤20mM) rapidly dissipated $\Delta\psi$ by about 60mV. Increasing the NH$_2$OH beyond 20mM did not dissipate $\Delta\psi$ any further. *Nitrobacter agilis* does not oxidise NH$_4^+$ or NH$_2$OH, but it can assimilate small amount of NH$_4$Cl (Section 3.2.1). High concentrations of NH$_4^+$ (>10mM) dissipated $\Delta p$H completely and $\Delta\psi$ partially (20mV at 100mM NH$_4^+$).
FIG. 49: EFFECTS OF NH$_4^+$ AND NH$_2$OH ON $\Delta \psi$ AND $\Delta p$H IN WASHED CELLS OF NITROSOMONAS EUROPaea.

EDTA treated cells were suspended in 50mM Tris-HCl, pH 6.9. $\Delta \psi$ was determined from the uptake of $[^3H]$TPP$^+$ after treatment of the suspensions with either NH$_4$Cl (O) or NH$_2$OH (●) at indicated concentrations. $\Delta p$H was calculated from the distribution of $[^{14}C]$ benzoic acid in the presence of either NH$_4$Cl (▼) or NH$_2$OH (△). For details of uptake studies see Section 2.2.13.3.
FIG. 49

![Graph showing the relationship between mV and \( \Delta \text{pH} \) as a function of mM (NH\(_4^+\) or NH\(_2\text{OH}\)).](image)
3.5.3 ATP biosynthesis driven by artificially induced proton motive force

3.5.3.1 ATP biosynthesis in *Nitrosomonas europaea*

By use of $K^+$ ionophore valinomycin, ATP biosynthesis has been demonstrated in intact bacterial cells, under the conditions of artificially created proton-motive force (Maloney et al., 1974; Wilson et al., 1976). The addition of valinomycin to a cell suspension in $K^+$ free medium results in $K^+$ efflux creating a $\Delta \psi$ (inside negative) which in turn can drive ATP biosynthesis. A similar approach has been used in the present study to demonstrate ATP synthesis in *Nitrosomonas europaea*. Changes in intracellular ATP levels were monitored using the firefly luciferin-luciferase method of ATP determination (Stanley and Williams, 1969).

The addition of $NH_4Cl$ to a cell suspension at pH 7.5 in 0.1M sodium phosphate buffer resulted in a rapid increase in intracellular ATP levels (Fig.50). Within 5 min of adding the substrate there was a 2.5 fold increase in ATP levels and this value decreased slowly, levelling out after 10 min (Fig.50). At pH 6.0 however, the addition of $NH_4Cl$ did not produce ATP since at this pH substrate oxidation is completely inhibited. The addition of valinomycin (100$\mu$g) did not induce any ATP biosynthesis in washed cells indicating that this compound is probably impermeable to the cells (also see Section 3.5.1.4). Thus experiments were conducted with spheroplasts prepared from washed cells according to the method of Bhandari and Nicholas (1979b). The addition of $NH_4Cl$ to a suspension of spheroplasts produced a synthesis of ATP at about half the rate of washed cells (Fig.51). The addition of valinomycin to spheroplasts in a potassium free medium resulted in a rapid increase in intracellular ATP levels followed by a slow decline (Fig.51). ATP synthesis reached a maximum within 1 min of adding valinomycin. Similar results were obtained when cells were pretreated with either DTECA (50$\mu$M), 2-chloro-6-trichloromethyl pyridine (N-serve) (100$\mu$M) or CO (20 min bubbling) to inhibit $NH_4^+$ oxidation, indicating that valinomycin induced ATP biosynthesis was not associated with endogenous $NH_4^+$ respiration or a possible $NH_4^+$ contamination. ATP synthesis induced by valinomycin was inhibited completely by DCCD (Fig.52) an inhibitor of $Ca^{2+}$, $Mg^{2+}$ ATPases indicating that a functional ATPase is required for
FIG. 50: ATP SYNTHESIS IN WASHED CELLS OF NITROSOMONAS EUROPAEA.

Washed cells (500mg wet weight) were suspended in 200ml of NH₄Cl free growth medium (Section 2.2.1) and aerated for 2h. Cells were harvested by centrifugation at 15,000g for 10 min, washed once with 0.1M sodium phosphate (pH 7.5) and then suspended in 100ml of the same buffer at appropriate pH. For NH₄⁺ induced ATP synthesis the buffer was at pH 7.5 and in all other experiments it was at pH 6.0. Five ml of each suspensions (5mg wet weight ml⁻¹) was used for each experiment. At zero time, 0.4ml aliquot was removed to determine the basal ATP level, then either 2mM NH₄Cl (●) or 100µg valinomycin (Ο) was added. At the times indicated 0.4ml aliquots were removed for ATP assay by firefly method as described in Section 2.2.175. All experiments were carried out at 30°C in a waterbath shaker.

FIG. 51: VALINOMYCIN INDUCED ATP SYNTHESIS IN SPHEROPLASTS OF NITROSOMONAS EUROPAEA.

Spheroplasts (400mg wet weight) prepared from starved cells (Fig.50) by the method of Bhandari and Nicholas (1979b) were suspended in 100ml of 0.1M sodium phosphate containing 0.2M sucrose and 1mM MgCl₂. For NH₄⁺ induced ATP biosynthesis experiments the pH of the suspending medium was 7.5 while for valinomycin experiments it was 6.0. Five ml of spheroplast suspension was used for each experiment. At zero time, either 2mM NH₄Cl (●) or 100µg valinomycin (Ο) was added. Further details are given in Fig.50.
FIG. 52: EFFECT OF DCCD ON VALINOMYCIN INDUCED ATP SYNTHESIS IN SPHEROPLASTS OF *NITROSOMONAS EUROPAEA*.

Spheroplasts were suspended (4 mg wet wt. ml⁻¹) in a reaction mixture described in Fig. 51, supplemented with DCCD (50-100 μM). Valinomycin (100 μg) was added at zero time. Experimental details as in Fig. 50 and 51.

without DCCD (●); with DCCD (○).

FIG. 53: EFFECT OF K⁺ ON VALINOMYCIN INDUCED ATP SYNTHESIS IN SPHEROPLASTS OF *NITROSOMONAS EUROPAEA*.

The reaction mixture described in Fig. 51 was supplemented with 2, 5, 7 or 10 mM KCl. Reaction was started by the addition of 100 μg valinomycin at zero time. Experimental details as in Fig. 50 and 51.

no KCl (●); 2 mM KCl (○); 5 mM KCl (▲); 7 mM KCl (▲); 10 mM KCl (▼).
ATP biosynthesis in *Nitrosomonas europaea*.

The synthesis of ATP is dependent on the magnitude of the membrane potential ($\Delta \psi$). In the absence of $K^+$, the addition of a $K^+$ ionophore (valinomycin) results in rapid extrusion of $K^+$ creating a proton-motive force towards the inside of cell. An inhibition of ATP synthesis mediated by valinomycin would be expected should the $\Delta \psi$ collapse on adding external $K^+$. The results in Fig.53 clearly show that this is so, since $K^+$ reduced valinomycin induced ATP biosynthesis and completely inhibited it at 10mM concentration.

As these experiments were conducted at pH 6.0 the cells would also have a $\Delta p$H (inside alkaline) to add to total $\Delta p$.

Nigericin, another $K^+$ ionophore which results in $K^+$/H$^+$ exchange across the membranes, would create a $\Delta p$H (inside acid) if added to cell suspension in $K^+$ free medium. Thus theoretically the addition of nigericin to cells should result in a decrease in intracellular ATP because inside acid $\Delta p$H would lower the $\Delta \psi$ (inside negative) so that ATPase would work in the direction of ATP hydrolysis instead of ATP synthesis. The results were, however, contrary to this anticipated result since nigericin appeared to act like valinomycin. In spheroplasts, nigericin induced ATP synthesis in $K^+$ free medium which reached a maximum at 30s after its addition, followed by a rapid decrease. Whether this unexpected result was due to membrane compartmentation and some localized pH gradient (inside alkaline) or due to some non-specific ion uptake is not clear. However in a fluorescence quenching experiment (Fig.54) nigericin appeared to carry out classical $K^+$/H$^+$ exchange. The addition of $NH_4^+$ to a spheroplast suspension resulted in fluorescence increase (Fig.54a) typical of results of Section 3.5.1, and as described by Bhandari and Nicholas (1979b). Addition of nigericin produced a slow decrease in fluorescence indicating an inwardly directed proton movement (Fig.54b). When nigericin was added first, followed by $NH_4$Cl the proton extrusion was inhibited but this effect was reversed by adding KCl (Fig.54b). When KCl was added first, subsequent addition of nigericin resulted in proton extrusion (Fig.54c). The addition of KCl to nigericin-treated spheroplasts produced an extrusion of protons (Fig.54d). The possible ion exchange reactions observed in the experi-
FIG. 54: NIGERICIN MEDIATED K⁺/H⁺ EXCHANGE IN SPHEROPLASTS OF NITROSOMONAS EUROPAEA.

Spheroplasts were prepared according to the method of Bhandari and Nicholas (1979b). To 2.5ml 0.1M Tris-HCl buffer (pH 7.5) supplemented with 0.2M sucrose and 1mM MgCl₂ in a 1cm cuvette were added 0.1µmol quinacrine hydrochloride and 0.1ml spheroplasts (50mg wet weight). The following additions (+) were made:

NH₄Cl (70µmol), nigericin (NIG) (50µg) or KCl (200µmol).

Fluorescence emission was determined (arbitrary units) in a Fluorispec model SF-1 fluorimeter (Baird Atomic, Massachusetts, U.S.A.) as described in Section 2.2.12.1.

FIG. 55: SCHEMATIC REPRESENTATION OF NIGERICIN MEDIATED K⁺/H⁺ EXCHANGE ACROSS THE SPHEROPLAST MEMBRANE.
FIG. 54

(a)  

(b)  

(c)  

(d)  

FIG. 55

(a)  

(b)  

(c)
ment are shown in Fig. 55. In $K^+$ free medium, the addition of nigericin would result in loss of intracellular $K^+$ and its replacement by $H^+$ (Fig. 55b) resulting in decrease in quinacrine fluorescence. However in $K^+$ containing medium, nigericin addition would result in $K^+$ uptake associated with $H^+$ extrusion (Fig. 55c) and hence increase in quinacrine fluorescence.

3.5.3.2 ATP biosynthesis in *Nitrobacter agilis*

Attempts to show valinomycin induced ATP synthesis were largely unsuccessful. Cells usually maintained high levels of intracellular ATP. Aerating cells for up to 10h in a $NO_2^-$ free medium had little effect on the intracellular ATP pool. The addition of $NO_2^-$ to a starved cell suspension resulted in an increase in intracellular ATP similar to that shown for *Nitrosomonas europaea* (Fig. 50) but about half the magnitude. The endogenous ATP varied between 0.5 to 2.0mM in ten separate batches of cells used in the study. Addition of valinomycin (10-50μg ml$^{-1}$) to a cell or spheroplast suspension in a $K^+$ free medium resulted in a slow and variable response. This response plus the $NO_2^-$ induced intracellular ATP changes were completely abolished by preincubating cells with 50-100μM sodium azide, an inhibitor of $NO_2^-$ oxidation (Section 3.1.6) indicating that valinomycin response was probably associated with some phenomenon other than the generation of an artificial $\Delta\psi$. Preincubation of cells with either DCCD (100-200μM) or DESB (20-100μM) drastically reduced the intracellular ATP levels (0.05 - 0.08mM) and these treated cells did not synthesize ATP when either $NO_2^-$ or valinomycin were added to a $K^+$ free medium.

3.5.4 $Na^+$ and $K^+$ transport

The apparent absence of proton translocation in *Nitrobacter agilis* posed a problem: how is energy conserved in this bacterium? One of the possible alternative mechanisms involves a respiration dependent primary $Na^+$ pump as has been reported in *Vibrio alginolyticus* (Tokuda and Unemoto, 1981). *Halobacterium halobium* also extrudes $Na^+$ by either a $Na^+/H^+$ antiporter (Lanyi and MacDonald, 1976) or a $Na^+$ pump, halorhodopsin (Lindley and MacDonald, 1979). In this Section the existence of such systems in *Nitrosomonas europaea* and *Nitrobacter agilis* is explored. Since $Na^+$ and $K^+$ transport systems play an
important role in overall bioenergetics of the cell, their effects on $\Delta p$ have been investigated.

3.5.4.1 Preparation of $K^+$ depleted cells

Bacterial cells usually contain high concentrations of $K^+$ and comparatively low amounts of $Na^+$. To characterize the $K^+$ and $Na^+$ transport systems it is necessary to deplete cellular $K^+$ and to manipulate the internal cation concentrations of cells without damaging the transport systems. A simple and novel method of $K^+$ depletion of bacterial cells has been described by Nakamura et al. (1982). The method involves treating cells with DEA at alkaline pH (see Section 2.2.14.1). The intracellular concentrations of $K^+$ and $Na^+$ in both *Nitrosomonas europaea* and *Nitrobacter agilis* determined by atomic absorption spectroscopy varied greatly from one batch of cells to another (Table 41). The variation was due in part to the number of cell washings and duration of storage prior to determining the $K^+$ and $Na^+$ contents of cells. When the cells of either *Nitrosomonas europaea* or *Nitrobacter agilis* were suspended in 50 mM Tris-Cl (pH 7.5 - 9.0) containing 150 mM NaCl, a slow extrusion of intracellular $K^+$ occurred (Fig.56). In the presence of 50 mM DEA-Cl (pH 9.2), both *Nitrosomonas europaea* and *Nitrobacter agilis* rapidly lost intracellular $K^+$. Thus after 10 min of DEA treatment there was a loss of about 80 and 95% intracellular $K^+$ from *Nitrosomonas europaea* and *Nitrobacter agilis* respectively (Fig.56). There was no net entry of $Na^+$ even when the reaction mixture contained 150 mM NaCl in addition to 50 mM DEA. The intracellular concentration of $Na^+$ in both bacteria remained constant during DEA treatment. The DEA treated cells contained <5 mM $K^+$.

3.5.4.2 Respiration in $K^+$ depleted cells

The respiration rates of untreated and DEA treated cells of both *Nitrosomonas europaea* and *Nitrobacter agilis* with and without KCl are shown in Table 42. The $K^+$ depleted (DEA treated) cells of *Nitrosomonas europaea* retained about 83% of the $NH_4^+$ oxidising capacity and 74% of the $NH_2OH$ oxidising activity of the untreated cells. The $NO_2^-$ oxidising activity of *Nitrobacter agilis* was little affected by $K^+$ depletion. The addition of $K^+$ had no effect on $NH_2OH$ oxidation by *Nitrosomonas*.
Freshly harvested cells were washed once in 50mM Tris-HCl buffer (pH 7.5) and then suspended in the same buffer (25 mg wet wt. ml⁻¹). Aliquots (100-200μl) filtered through Millipore filters (0.22μ) were washed once with 2ml, 0.2M choline chloride and Na⁺ and K⁺ were determined in TCA extracts of cells by atomic absorption spectroscopy, as described in Section 2.2.14.2.

<table>
<thead>
<tr>
<th>Batch</th>
<th><strong>Nitrosomonas europaea</strong></th>
<th><strong>Nitrobacter agilis</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na⁺</td>
<td>K⁺</td>
</tr>
<tr>
<td>1</td>
<td>80</td>
<td>85</td>
</tr>
<tr>
<td>2</td>
<td>59</td>
<td>146</td>
</tr>
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<td>3</td>
<td>73</td>
<td>170</td>
</tr>
<tr>
<td>4</td>
<td>62</td>
<td>99</td>
</tr>
<tr>
<td>5</td>
<td>65</td>
<td>135</td>
</tr>
</tbody>
</table>

Nd - Not determined.
FIG. 56: TIME COURSE FOR $K^+$ EXTRUSION FROM THE CELLS OF (a) NITROSOMONAS EUROPaea AND (b) NITROBACTER AGILIS DURING DIETHANOLAMINE (DEA) TREATMENT.

Freshly harvested cells (approx. 500mg wet wt.) washed twice with 100mM $K^+$ phosphate buffer (pH 7.5) were suspended in 4ml of 10mM HEPES-NaOH (pH 8.0). To start the reaction, this cell suspension was added to 2ml of 50mM DEA-HCl, 150mM NaCl (pH 9.2) containing 4 $\mu$Ci ml$^{-1} \text{^{22}NaCl}$. Aliquots withdrawn at times indicated were dispensed into 1ml of 0.4M choline chloride and filtered immediately through Millipore filters (0.45μ). The cells on the filter were washed twice with 2ml of 0.4M choline chloride. $K^+$ was determined in TCA extracts as described in Section 2.2.14.2. In control experiments (O) the cell suspension in HEPES buffer was diluted with 50mM Tris-HCl buffer (pH 7.5-9.0) instead of DEA-HCl (●). The uptake of $\text{^{22}Na}^+$ (□) was determined after drying the filters for 1h at 100°C and then immersing them in 10ml of toluene based scintillation counting fluid (0.3% w/v POPOP and 0.03% w/v PPO in toluene). Radioactivity ($\text{^{22}Na}^+$) was measured in a Packard Tricarb 460 CD scintillation spectrometer.
TABLE 42: EFFECTS OF $K^+$ DEPLETION AND $K^+$ ADDITION ON RESPIRATION IN NITROSOMONAS EUROPAEA AND NITROBACTER AGILIS.

$K^+$ depleted cells were prepared by DEA treatment as described in Section 2.2.14.1. Respiration rates of bacteria determined by Clarke-type oxygen electrode (Section 2.2.11) are expressed as ng atom 'O' min$^{-1}$ (mg protein)$^{-1}$.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>DEA Treatment</th>
<th>Substrate</th>
<th>$K^+$ addition</th>
<th>Respiration rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrosomonas europaea</td>
<td>-</td>
<td>NH$_4$Cl</td>
<td>-</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>NH$_4$Cl</td>
<td>+</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>NH$_2$OH</td>
<td>-</td>
<td>760</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>NH$_2$OH</td>
<td>+</td>
<td>740</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>NH$_4$Cl</td>
<td>-</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>NH$_4$Cl</td>
<td>+</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>NH$_2$OH</td>
<td>-</td>
<td>560</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>NH$_2$OH</td>
<td>+</td>
<td>540</td>
</tr>
<tr>
<td>Nitrobacter agilis</td>
<td>-</td>
<td>NaNO$_2$</td>
<td>-</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>NaNO$_2$</td>
<td>+</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>NaNO$_2$</td>
<td>-</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>NaNO$_2$</td>
<td>+</td>
<td>110</td>
</tr>
</tbody>
</table>

$^a$The concentration of each substrate was 2.5mM

$^b$K$^+$ was added as KCl (25mM).
150

europaea and NO$_2^-$ oxidation by *Nitrobacter agilis* in either untreated or DEA treated cells, but it inhibited NH$_4^+$ oxidation in both treated and untreated cells of *Nitrosomonas europaea*. Thus 25mM KCl inhibited NH$_4^+$ oxidation to a similar extent in untreated and DEA treated cells of *Nitrosomonas europaea*.

3.5.4.3 Proton-motive force in K$^+$ depleted cells and the effect of added K$^+$

The changes in the components of $\Delta p$, viz. $\Delta pH$ and $\Delta \psi$, in K$^+$ depleted cells of *Nitrosomonas europaea* and *Nitrobacter agilis* as a function of external pH (pHe) are illustrated in Fig.57. The overall pattern of variation in $\Delta pH$ and $\Delta \psi$ in response to pHe in untreated cells of both bacteria was similar to that shown in Section 3.5.2. In K$^+$ depleted cells of *Nitrosomonas europaea* $\Delta \psi$ varied from 126mV at pHe 6.2 to 155mV at pH 8.2 while in *Nitrobacter agilis* it varied from 100mV to 135mV over the pHe range 6.2 to 8.2. The addition of 20mM KCl resulted in depolarization of $\Delta \psi$ by about 5mV in *Nitrosomonas europaea* and 10mV in *Nitrobacter agilis*. This depolarization of $\Delta \psi$ was independent of pHe in both bacteria (Fig.57). During the pHe changes from 6.2 to 8.2, $\Delta pH$ in K$^+$ depleted cells of *Nitrosomonas europaea* and *Nitrobacter agilis* varied from -34 to -5mV and from -44 to +12mV respectively. The addition of K$^+$ (20mM KCl) to K$^+$ depleted cells resulted in a concomitant increase in $\Delta pH$ (alkaline inside) by about 5mV in *Nitrosomonas europaea* and 10mV in *Nitrobacter agilis*. Again these changes were independent of pHe. Thus the partial depolarization of $\Delta \psi$ by K$^+$ in K$^+$ depleted cells of both bacteria was compensated by an equivalent increase in $\Delta pH$ so that the total proton-motive force remained almost constant in K$^+$ depleted cells and in those supplemented with K$^+$ (Fig.57). These results indicate that the inward movement of K$^+$ in both bacteria is electrogenic which leads to depolarization of the membrane (decrease in $\Delta \psi$, inside negative), which in turn allows the cells to pump more protons into the medium (increase in $\Delta pH$, inside alkaline).

3.5.4.4 $^{22}$Na$^+$ loading of K$^+$ depleted cells

The results in Fig.56 indicate that there was no intake of Na$^+$ associated with K$^+$ exit by the cells of either *Nitrosomonas europaea* or
**FIG. 57:** EFFECTS OF EXTERNAL pH (pHe) ON ΔpH, Δψ AND Δp IN K⁺ DEPLETED CELLS OF (a) *NITROSOMONAS EUROPAEA* AND (b) *NITROBACTER AGILIS* AND THE EFFECTS OF ADDED K⁺.

K⁺ depleted cells were suspended in 50mM sodium phosphate at the pH values indicated. Uptake studies were carried out as described in Section 2.2.13.3. ΔpH, represented in terms of mV i.e. 59 x ΔpH(●•) was determined with [14C] benzoic acid; Δψ values(▲●) were calculated from the uptake of [3H]TPP⁺; Δp(□■) was determined from the ΔpH and Δψ values (Δp = Δψ-59ΔpH). Open symbols, without KCl; closed symbols, +20mM KCl.
Nitrobacter agilis during DEA treatment. If DEA is removed from the reaction mixture by washing with a Na⁺ containing buffer (eg. Tris, Hepes or phosphate) the cells readily take up Na⁺. The time course of uptake of $^{22}\text{Na}^+$ by DEA treated cells of *Nitrosomonas europaea* and *Nitrobacter agilis* respectively are shown in Fig.58. Thus when the amine loaded cells of either bacterium were exposed to $^{22}\text{Na}^+$ there was an immediate uptake of $^{22}\text{Na}^+$. The extent of accumulation of $^{22}\text{Na}^+$ was dependent on the concentration of DEA in the external medium. Thus with 50mM DEA, no net entry of $^{22}\text{Na}$ was observed in either bacterium. In the absence of DEA however, both *Nitrosomonas europaea* and *Nitrobacter agilis* accumulated Na⁺. Thus after about 20 min incubation with $^{22}\text{Na}^+$ (+Na⁺) cells reached an equilibrium state and contained 150-200mM intracellular Na⁺.

3.5.4.5 $^{22}\text{Na}^+$ extrusion from $^{22}\text{Na}^+$ loaded cells

$^{22}\text{Na}^+$ loaded cells were prepared as described above (Fig.58) and an active extrusion of $^{22}\text{Na}^+$ from the cells was determined by a filtration method at 25°C (Fig.59). In *Nitrosomonas europaea* only about 20% $^{22}\text{Na}^+$ was extruded from cells when K⁺ was added to $^{22}\text{Na}^+$ loaded cells (Fig.59a). In the presence of CCCP, the addition of K⁺ to $^{22}\text{Na}^+$ loaded cells of *Nitrosomonas europaea* did not result in an extrusion of $^{22}\text{Na}^+$. In *Nitrobacter agilis* $^{22}\text{Na}^+$ loaded cells, the extrusion of $^{22}\text{Na}^+$ (Fig.59b) required K⁺ as a counter ion permitting overall neutrality. Thus the addition of K⁺ resulted in about 80% loss of Na⁺ from the cells in 15 min. The addition of CCCP completely inhibited $^{22}\text{Na}^+$ extrusion from the cells of *Nitrobacter agilis* indicating that the driving force for Na⁺ extrusion is Δp. To check whether there was any respiration driven Na⁺ pump in these bacteria, the effects of NH₄⁺ on *Nitrosomonas europaea* and NO₂⁻ in *Nitrobacter agilis* on the $^{22}\text{Na}^+$ system were studied. The addition of NH₄Cl to $^{22}\text{Na}^+$ loaded cells of either *Nitrosomonas europaea* or *Nitrobacter agilis*, resulted in a rapid extrusion of $^{22}\text{Na}^+$ (Fig.59a,b). However this loss was not respiration dependent because the addition of diethylidithiocarbamate, an inhibitor of NH₄⁺ oxidation by *Nitrosomonas europaea* (Table 39), prior to NH₄Cl did not prevent the extrusion of $^{22}\text{Na}^+$ in *Nitrosomonas europaea*. This was also confirmed by replacing NH₄⁺ with CH₃NH₂ (which is not oxidised by the cells), which
FIG. 58: UPTAKE OF $^{22}\text{Na}^+$ BY DIETHANOLAMINE (DEA) LOADED CELLS OF (a) NITROSOMONAS EUROPaea AND (b) NITROBACTER AGILIS.

$K^+$ depleted cells, prepared by DEA treatment as described in Section 2.2.14.1, were washed once in 50mM Tris-HCl (pH 7.5) and then suspended in the same buffer (450mg wet wt. ml$^{-1}$). For $^{22}\text{Na}^+$ uptake studies 100μl of this cell suspension was diluted to 1ml with 100mM, Na-HEPES buffer (pH 7.5) containing $^{22}\text{NaCl}$ (4 μCi ml$^{-1}$). Aliquots (100μl) were then withdrawn at various times and diluted with 1ml of cold 0.2M choline-chloride, 10mM Tris -HCl (pH 7.25) and filtered immediately through Millipore filters (0.45μ) and washed with 2ml of choline chloride buffer. The filters, dried at 100°C for 1h were immersed in 10ml scintillation fluid and radioassayed as described in Fig.56. Radioactivity was corrected for filter bound $^{22}\text{Na}^+$.
FIG. 58

% $^{22}$Na$^+$ uptake

incubation time (min)
FIG. 59: $^{22}$Na$^+$ EXTRUSION FROM $^{22}$Na$^+$ LOADED CELLS OF (a) NITROSONOMAS EUROPAEA AND (b) NITROBACTER AGILIS.

$^{22}$Na$^+$ loaded cells were prepared as described in Fig. 58. The amounts of $^{22}$Na$^+$ retained by the cells were determined by the filtration method described in Fig. 56 and Fig. 58. The following compounds were added at zero time:

- 20mM KCl (■);
- 20mM KCl + 20μM CCCP (□);
- 10mM NH$_4$Cl (○);
- 5mM NaNO$_2$ (●);
- 10mM NH$_4$Cl + 20μM CCCP (▼);
- 10mM NH$_4$Cl + 200μM DIECA (▲);
- 10mM methylamine-HCl (◇).

In the absence of any additions there was no net loss of $^{22}$Na$^+$ during the period of incubation.
FIG. 59

(a) % ^22Na retained by the cells

(b) incubation time (min)
also resulted in $^{22}\text{Na}^+$ extrusion from the cells. This, together with NH$_4$Cl dependent $^{22}\text{Na}^+$ loss from *Nitrobacter agilis* cells indicates that NH$_4^+$ acts as a permeant amine like diethanolamine and methylamine and is transported into the cells of both *Nitrosomonas europaea* and *Nitrobacter agilis* in its unprotonated form (NH$_3$) by passive diffusion. There was no evidence of a respiratory driven Na$^+$ pump in *Nitrobacter agilis* because the addition of NO$_2^-$ to $^{22}\text{Na}^+$ loaded cells did not result in the extrusion of $^{22}\text{Na}^+$ (Fig.59b). It should be noted here that Na$^+$ loaded cells of both bacteria actively respire in the presence of an appropriate oxidisable nitrogen substrate, so the apparent lack of a respiration dependent Na$^+$ pump could not have been caused by respiration loss.

### 3.5.5 Stable isotope experiments with $^{15}\text{N}$ and $^{18}\text{O}$ labelled compounds to study NO$_2^-$ oxidation by cells of *Nitrobacter agilis*

The inability of *Nitrobacter agilis* to translocate protons during respiration led to postulation of alternate energy conserving mechanisms in this bacterium. Dr. T.C. Hollocher of Brandeis University (U.S.A.) suggested (private communication) that the bacterium might synthesize ATP by a substrate type phosphorylation involving a mixed anhydride between either NO$_3^-$ and PO$_4^{2-}$ or NO$_3^-$ and ADP. Should this concept be correct, the 'O' in NO$_3^-$ produced by NO$_2^-$ oxidation in *Nitrobacter agilis* would come from PO$_4^{2-}$ as follows:

**Pi as nucleophile**

\[
\text{Pi} \rightarrow \text{ADP} \rightarrow \text{ATP} + \text{NO}_2^-
\]

**ADP oxygen as nucleophile**

\[
\text{ADP-}^{-} + \text{NO}_2^+ \rightarrow \text{ADP-}^{-} \rightarrow \text{ADP-PO}_3^- + \text{NO}_3^-
\]

**Cellular ATP hydrolysis**

\[
\text{ADP-}^{-} + \text{PO}_4^{2-} \rightarrow \text{ADP-}^{-} + \text{Pi}
\]

**ADP-PO$_4^-$**

\[
\text{ADP-}^{-} + \text{NO}_2^+ \rightarrow \text{ADP-}^{-} \rightarrow \text{ADP-PO}_3^- + \text{NO}_2^-
\]
Thus this mechanism was suggested to involve \( \text{NO}_2^- \) as an intermediate during \( \text{NO}_2^- \) oxidation. It has been shown that the source of oxygen during \( \text{NO}_2^- \) oxidation by \textit{Nitrobacter} is \( \text{H}_2\text{O} \) (Aleem et al., 1965), based on the incorporation of 0.044 to 0.078 atom % \(^{18}O\) into \( \text{NO}_3^- \) from 82 atom % \( \text{H}_2^{18}O \). The following experiments were designed to check the source of '0' in \( \text{NO}_3^- \) produced as a result of \( \text{NO}_2^- \) oxidation by \textit{Nitrobacter agilis}. Cells were incubated with nitrite (\( \text{NO}_2^- \) or \( ^{15}\text{NO}_2^- \)) and one of the following \(^{18}O \) compounds: \( \text{H}_2^{18}O \) (90-97 atom %), \( ^{18}\text{O}_2^- \) (99 atom %) and \( ^{18}\text{O}_4^- \) (approximately 90 atom %). Two distinct methods (GC/MS and \(^{15}\text{N}-\text{NMR} \) were used for isotope analysis of the product of \( \text{NO}_2^- \) oxidation (nitrate). For GC/MS studies, nitrate was converted to \( \text{N}_2\text{O} \) and phosphate to trimethyl phosphate which were then analysed as described in detail in Section 2.2.15.1. For \(^{15}\text{N}-\text{NMR} \) analysis, the reaction mixtures were analysed as such, after separation of cells by centrifugation (Section 2.2.15.2).

3.5.5.1 GC/MS studies of \( \text{NO}_2^- \) oxidation

3.5.5.1.1 Changes in \(^{18}O \) contents of phosphate during \( \text{NO}_2^- \) oxidation

The mass spectra of a commercially available sample of trimethyl phosphate and one prepared by the methylation of \( \text{H}_3\text{P}^{18}\text{O}_4^- \) are shown in Fig.60A and B respectively. Fig.60C illustrates the spectrum of trimethyl phosphate prepared from \( \text{P}^{18}\text{O}_4^- \) which had previously been incubated with \( \text{NO}_2^- \) and the cells of \textit{Nitrobacter agilis} (Section 2.2.15.1). By estimating the abundance of trimethyl phosphate molecule (\( m^+ \)) peak at 140 amu in Fig.60A and five other fragmentation ions at 110,109,95,80 and 79 amu and comparing them with corresponding amu values in Fig.60B and 60C, it was established that there was no apparent loss of \(^{18}O \) content of phosphate during \( \text{NO}_2^- \) oxidation by \textit{Nitrobacter}.

3.5.5.1.2 \(^{15}\text{N}^{18}O \) contents of nitrate produced by the oxidation of nitrite

A summary of the results of isotope experiments in which the product, nitrate, was analysed for \(^{15}\text{N} \) and \(^{18}O \) following its conversion to \( \text{N}_2\text{O} \) gas (Section 2.2.15.1) is given in Table 43. When unlabelled \( \text{NO}_2^- \) was oxidised by cells of \textit{Nitrobacter agilis} the only source of \(^{18}O \) which was significantly incorporated with \( \text{NO}_3^- \) was \( \text{H}_2^{18}O \) (Fig. 61). In this experiment, the normalized integral at 46 amu(N.\(^{18}O \)) of 7.48 was in reasonable agreement with the value of 7.88 expected if the \( \text{H}_2^{18}O \) which was used in
FIG. 60: MASS SPECTRA OF TRIMETHYL PHOSPHATE.

Commercial sample (A) was purchased from Sigma Chemical Co., St. Louis, U.S.A. $^{18}O$, trimethyl phosphate (B) was prepared by the methylation of $\text{H}_3\text{P}^{18}\text{O}_4$ with diazomethane as described in Section 2.2.15.1. Spectrum (C) was obtained for trimethyl phosphate prepared from the $^{18}O$ phosphate recovered from a NO$_2^-$ oxidising system (Section 2.2.15.1) at the end of incubation period. For further details see Section 2.2.15.1.
TABLE 43: STABLE ISOTOPE EXPERIMENTS WITH WASHED CELLS OF NITROBACTER AGILIS.

The incubation mixtures were prepared as described in Section 2.2.15.1. Each mixture contained 100mg (wet wt.) of cells. Following the complete oxidation of NO\textsubscript{3}, the NO\textsubscript{3} was recovered and converted to N\textsubscript{2}O as described in Section 2.2.15.1. The details of GC/MSE technique are described in Section 2.2.15.1. Data were collected, background corrected and processed under the Selected Ion Monitor or Peakfinder programme which assigns an integral of 100 to the largest (base) peak of the set. For comparison values were calculated (in parenthesis) assuming that only one of the three O atoms arose directly from water during oxidation of NO\textsubscript{3} and that of stocks of H\textsubscript{2}NO\textsubscript{2} and H\textsubscript{2}O\textsubscript{18}0 used were as specified in Section 2.1.2. Null values at the five amu values indicated were typically 0 to 0.2 after background correction.

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Normalized integrals for N\textsubscript{2}O species at the indicated amu values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N\textsubscript{2}O</td>
</tr>
<tr>
<td>NO\textsubscript{2} + H\textsubscript{2}O + PO\textsubscript{4}\textsuperscript{2-} + air</td>
<td>100</td>
</tr>
<tr>
<td>NO\textsubscript{2} + H\textsubscript{2}18O + PO\textsubscript{4}\textsuperscript{2-} + air</td>
<td>100</td>
</tr>
<tr>
<td>NO\textsubscript{2} + H\textsubscript{2}O + PO\textsubscript{4}\textsuperscript{2-} + air</td>
<td>100</td>
</tr>
<tr>
<td>NO\textsubscript{2} + H\textsubscript{2}O + PO\textsubscript{4}\textsuperscript{2-} + air</td>
<td>100</td>
</tr>
<tr>
<td>15\textsubscript{NO}\textsubscript{2} + H\textsubscript{2}O + PO\textsubscript{4}\textsuperscript{2-} + air</td>
<td>0.82 (1.00)</td>
</tr>
<tr>
<td>15\textsubscript{NO}\textsubscript{2} + H\textsubscript{18}O + PO\textsubscript{4}\textsuperscript{2-} + air</td>
<td>0.79 (1.02)</td>
</tr>
<tr>
<td>15\textsubscript{NO}\textsubscript{2} + H\textsubscript{2}O + PO\textsubscript{4}\textsuperscript{2-} + air</td>
<td>0.82 (1.00)</td>
</tr>
<tr>
<td>15\textsubscript{NO}\textsubscript{2} + H\textsubscript{2}O + PO\textsubscript{4}\textsuperscript{2-} + air</td>
<td>0.83 (1.00)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Due largely to spillover from the base peak at 45 amu as the result of drifts in tuning. This was confirmed by use of the sweep mode of data collection (Peak Finder Programme).
FIG. 61: MASS SPECTRA OF N₂O GAS

(A) a commercial sample (B) and (C) prepared by the pyrolysis of ammonium nitrate obtained from a NO₂⁻ oxidising system with H₂O and H₂¹⁸O respectively (Section 2.2.15.1). Data were obtained under the Peak Finder programme. Spectra do not include ions below 35 amu (lower cut off level was 35 amu) and thus fragments of N₂O i.e. NO⁺ (ion 30) and N₂⁺ (ion 28) are absent in these spectra. Details of the experiments and GC/MS technique are described in Section 2.2.15.1.
the experiment had contained 95 atom % $^{18}O$, assuming that only one oxygen atom of $\text{NO}_3^-$ had been derived from water and that the $\text{NO}_2^-$ stock contained 10% (w/w) $\text{NO}_3^-$ initially. Similarly in experiments, when 99 atom % $^{15}\text{NO}_2^-$ was used instead of unlabelled $\text{NO}_2^-$, only $^{18}O$ from $\text{H}_2^{18}O$ was significantly incorporated in $^{15}\text{NO}_3^-$. The observed value (7.80) and calculated value (7.95) are in close agreement (Table 43).

3.5.5.2 $^{15}N$-NMR studies of $\text{NO}_2^-$ oxidation

One of the advantages of using $^{15}N$-NMR is that with the aid of stable isotopes ($^{15}N$ and $^{18}O$) the reactants and the products of a biochemical reaction can be analysed directly. This overcomes any dilution or exchange reactions associated with the processing of the samples. The results in Fig. 62 are for an experiment similar to the one recorded in Table 43, except that the samples were analysed directly by $^{15}N$-NMR (Section 2.2.15.2). The signals of various $^{15}N$ and $^{18}O$ nitrate standards (Fig. 62A) are essentially as reported by Andersson et al. (1982) but only three peaks were observed, corresponding to $^{15}N^{16}O_3^-$, $^{15}N^{16}O_2^{18}O^-$ and $^{15}N^{16}O^{18}O_2^-$ as confirmed by spiking $^{15}N^{16}O_3^-$ resonance. The peaks were well resolved and separated by 1.71 Hz (0.0563 ppm). A visible signal was observed after a few scans when the concentration of $^{15}\text{NO}_3^-$ was more than 40mM. Smaller concentrations required longer accumulation time.

When the cells were incubated with $^{15}\text{NO}_2^-$ with $\text{H}_2^{16}O$ in 100mM $^{16}O_2^-$ buffer, only one resonance was observed which corresponded to $^{15}N^{16}O_3^-$. In Fig. 62B, the NMR spectrum of the product of $^{15}\text{NO}_2^-$ oxidation in the presence of 100% $^{18}O_2$ is shown. Again only one peak was observed with an isotopic configuration of $^{15}N^{16}O_3^-$ indicating that none of the '0' in nitrate produced by $^{15}\text{NO}_2^-$ oxidation was derived from $^{18}O_2$. When the cells were incubated with $^{15}\text{NO}_2^-$ and $\text{H}_2^{18}O$ two major peaks and a minor one were observed, separated by 1.71 Hz and representing $^{15}N^{16}O_3^-$, $^{15}N^{16}O_2^{18}O^-$ and $^{15}N^{16}O^{18}O_2^-$ respectively (Fig. 62C). The ratio of areas of three peaks was 31.6 : 6 : 1. Thus $^{15}N^{16}O_2^{18}O^-$ and $^{15}N^{16}O^{18}O_2^-$ isotope combinations were about 19% and 3.2% respectively, of $^{15}N^{16}O_3^-$. The spectrum of the product of $^{15}\text{NO}_2^-$ oxidation in the presence of $^{18}O$ phosphate (all 4 '0' atoms labelled with $^{18}O$) is shown in Fig. 62D. Only one peak was observed which corresponded to $^{15}N^{16}O_3^-$ indicating that none of the '0' in $^{15}\text{NO}_3^-$ are derived from $^{18}O_4$ during nitrite oxidation by *Nitrobacter agilis.* In another experiment in which cells were incubated for 18h with $^{18}O_4$
FIG. 62: NMR SPECTRA OF $^{18}O/^{16}O$ DERIVATIVES OF NITRATE.

(A) 100mM standard $^{15}N-^{18}O$ nitrate derivatives produced by chemical exchange (Section 2.1.2).
(B) 40mM, $^{15}N-^{16}O_3^-$ produced by cells in presence of $^{18}O_2$.
(C) 20mM $^{15}N-^{16}O_3^- + ^{15}N-^{16}O_2^{18}O^- + ^{15}N-^{16}O_2^{18}O_2^-$ produced by cells in presence of $H_2^{18}O$.
(D) 50mM, $^{15}N-^{16}O_3^-$ produced by cells in presence of $P^{18}O_4^-$. 

Experimental details are described in Section 2.2.15.2.
and $^{15}\text{NO}_2^-$, the NMR spectrum was similar to that observed in Fig.62D. Thus there appeared to be no measurable biological or chemical exchange of $^{18}O$ between either $P^{18}O_4^-$ and $H_2O$, $P^{18}O_4^-$ and $^{15}\text{NO}_3^-$ or $P^{18}O_4^-$ and $^{15}\text{NO}_2^-$. The observations of GC/MS and NMR studies taken together indicate that one and only one oxygen in $\text{NO}_3^-$ produced as a result of $\text{NO}_2^-$ oxidation by *Nitrobacter agilis* originates from $H_2O$ and not from $O_2$ or $\text{PO}_4^-$, ruling out the possibility of substrate level oxidative phosphorylation involving a P-O-N type intermediate.
4. DISCUSSION
4. DISCUSSION

4.1 NITRITE OXIDATION BY WASHED CELLS, SPHEROPLASTS AND MEMBRANE VESICLES OF NITROBACTER AGILIS

The oxidation of NO$_2^-$ by Nitrobacter has been studied in intact cells (Lees and Simpson, 1957), cell free extracts (Aleem and Nason, 1959) and membrane particles (O'Kelly et al., 1970; Cobley, 1976a,b; Aleem and Sewell, 1981). In most of these studies, the utilization of NO$_2^-$ has been followed by a colorimetric method for NO$_2^-$ or by O$_2$ uptake. In the present work electrode methods have been developed to monitor O$_2$ uptake and NO$_3^-$ production simultaneously and continuously during NO$_2^-$ oxidation by cells, spheroplasts and membrane vesicles respectively. Utilization of NO$_2^-$ has been followed by a colorimetric method (Nicholas and Nason, 1957). This approach to study NO$_2^-$ oxidation is particularly advantageous because all the three parameters of NO$_2^-$ oxidation, i.e. NO$_2^-$ and O$_2$ utilization and NO$_3^-$ production can be measured conveniently in the same reaction mixture.

The following stoichiometry of NO$_2^-$ oxidation by washed cells, 1NO$_2^-$ : 0.5O$_2$ : 0.75 NO$_3^-$ indicates that some of the NO$_3^-$ (or NO$_2^-$) is retained by the cells and probably reduced to NH$_4^+$ via assimilatory nitrate and nitrite reductases (Wallace and Nicholas, 1968). The different stoichiometry for NO$_3^-$ in spheroplasts and vesicles (1NO$_2^-$ : 0.5O$_2$ : 1NO$_3^-$) as compared with that for washed cells (1NO$_3^-$ : 0.5O$_2$ : 0.75 NO$_3^-$) may be associated with impairment of the nitrate and nitrite reducing systems in spheroplasts and vesicles.

O'Kelly et al. (1970) found that the oxidation of NO$_2^-$ by membrane particles of Nitrobacter agilis was sensitive to a variety of metal inhibitors, however Aleem (1977) reported that the metal binding agents, 8-hydroxy quinoline (8HQ), 0-phenanthroline, a-a'dipyridyl and salicylaldoxime had little or no effect on NO$_2^-$ oxidation. The results recorded in this thesis indicate that although NO$_2^-$ oxidation was sensitive to inhibitors such as 2-trichloromethyl-pyridine (2TMP) and 8HQ, their effects could not be reversed by the addition of any metal salts and thus may be non-specific. The inhibitors of cytochrome oxidase viz. azide and CO also inhibited NO$_2^-$ oxidation in washed cells, spheroplasts and membrane vesicles, and these results are consistent with earlier findings (O'Kelly et al., 1970; Aleem, 1977; Aleem and Sewell, 1981). In washed cells and in spheroplasts NO$_2^-$ oxidation was restricted by inhibitors of electron transport but these effects were not observed in membrane vesicles.
indicating that in cells and spheroplast these inhibitors can affect a variety of metabolic functions. The fact that electron transport inhibitors do not affect NO₂⁻ oxidation in membrane vesicles substantiates the idea that NO₂⁻ does not enter the electron transport chain at the level of either NADH, ubiquinone or cytochrome b (Aleem, 1977; Aleem and Sewell, 1981). The observations in the present work that NO₂⁻ oxidation by washed cells, spheroplasts and membrane vesicles was inhibited by uncouplers are similar to those of Cobley (1976a), Aleem (1977) and Aleem and Sewell (1981).

A Mg²⁺ dependent ATPase has been detected in membrane vesicles of Nitrobacter agilis (Section 3.1.7). ATPase activity requiring either Mg²⁺ or Ca²⁺ has been found in a large number of bacteria (Machtiger and Fox, 1973; Abrams and Smith, 1974). The enzyme, associated with membranes, plays an important role in ion transport and ATP synthesis. Unlike the ATPase of Nitrosomonas europaea (Bhandari and Nicholas, 1980), that of Nitrobacter agilis was unaffected by uncouplers (e.g. CCCP, DNP) but was strongly inhibited by classical ATPase inhibitors (e.g. DCCD). One important observation in the present study is that the ATPase inhibitors (DCCD, vanadate, diethylstilbestrol and NBD chloride) also restrict NO₂⁻ oxidation to approximately the same extent as for ATPase itself. The ATPase however was not affected by the inhibitors of NO₂⁻ oxidation (e.g. uncouplers). This indicates a possible functional relationship between NO₂⁻ oxidase and ATPase in the bacterium. The delipidation of membranes by phospholipase A₂ treatment results in a loss of ATPase activity suggesting a possible role of membrane conformation and phospholipids in maintaining the activity of this enzyme.

4.2 ASSIMILATION OF INORGANIC NITROGEN COMPOUNDS IN NITROBACTER AGILIS AND NITROSOMONAS EUROPAEA

4.2.1 Pathways of nitrogen assimilation in Nitrobacter agilis

Although numerous studies have been done on the mechanism of NO₂⁻ oxidation by Nitrobacter agilis, the further assimilation of NO₂⁻ (or NO₃⁻) into cell nitrogen is not well understood. An attempt has been made to delineate the pathways of assimilation of inorganic nitrogen compounds in this bacterium.

The experiments reported in Section 3.2 clearly show that washed cells of Nitrobacter agilis incorporated ¹⁵NO₂⁻, ¹⁵NO₃⁻, ¹⁵NH₂OH and ¹⁵NH₄⁺ respectively into cell nitrogen; of these ¹⁵NH₄⁺ was most readily utilized. These results are in agreement with those of Wallace and Nicholas (1968). This also
confirms the finding that \( \text{NH}_4^+ \) enhances the growth of this bacterium in pure cultures (Section 3.2.1). Thus in its natural habitat, \( \text{NH}_4^+ \) would be an important source of nitrogen for *Nitrobacter agilis*.

Coble (1976a) reported that \( \text{NO}_2^- \) oxidation by electron transport particles of *Nitrobacter winogradskyi* was stimulated by \( \text{NH}_4\text{Cl} \) (maximum stimulation 35% at 2mM). In the present study with washed cells by *Nitrobacter agilis*, no such stimulation was observed. Although 2mM \( \text{NH}_4\text{Cl} \) stimulated the growth of the bacterium, higher concentrations inhibited \( \text{NO}_2^- \) oxidation. This effect was reversed by increasing the concentration of \( \text{NO}_2^- \), indicating that it was competitive (Section 3.2.2). This phenomenon could have ecological significance in regulating the overall nitrification of ammonia to nitrite. High concentrations of \( \text{NH}_4^+ \) would inhibit \( \text{NO}_2^- \) oxidation until such time as enough \( \text{NO}_2^- \) was produced (e.g. from the oxidation of \( \text{NH}_4^+ \) by Nitrosonomas) to overcome the inhibition. Since \( \text{NH}_4^+ \) even at relatively high concentrations does not inhibit \( \text{NO}_2^- \) oxidation completely (Fig. 12), its accumulation would only retard and not completely inhibit the rate of \( \text{NO}_2^- \) conversion to \( \text{NO}_3^- \).

In most bacteria assimilating ammonia directly as the source of nitrogen, glutamate dehydrogenase (GDH) is usually a key enzyme, producing glutamate. Under these conditions glutamine synthetase (GS) has a relatively low activity (Woolfolk et al., 1966; Bender et al., 1977; Ely et al., 1978). In *Nitrosomonas europaea*, GDH functions primarily in the direction of glutamate production (Hooper et al., 1967). This was confirmed by Bhandari and Nicholas (1981) who also found that GS activity in *Nitrosonomas europaea* was relatively low compared with that of GDH whereas glutamate synthase (GOGAT) was not detected. In the present study with *Nitrobacter agilis* although GS had appreciable activity (transferase), relatively low activity was recorded for GOGAT. Glutamate is the major amino acid in the cytoplasm of *Nitrobacter agilis*, viz. about 25% (w/w) of the total amino acids (Wallace et al., 1970). It is likely that GDH is the main pathway for the synthesis of glutamate in this bacterium. The evidence in support of this conclusion comes from experiments reported in Section 3.2.5. The inhibition of both GS and GOGAT by L-methionine DL-sulfoximine (MSX) and azaserine respectively did not have any effect on the incorporation of \( ^{15}\text{NO}_2^- \) or \( ^{15}\text{NH}_4^+ \) into cell nitrogen indicating that either this pathway is not mandatory or following its inhibition, GDH takes over the adequate production of glutamate for cell growth.

*Nitrobacter* has active nitrate, nitrite and hydroxylamine reductases (Wallace and Nicholas, 1968) and these enzymes are probably required for the
assimilation of $\text{NO}_2^-$ and $\text{NO}_3^-$ via $\text{NH}_4^+$ when there is no exogenous $\text{NH}_4^+$ available to the bacterium. Based on the available information, the following scheme is proposed for the assimilation of inorganic nitrogen compounds by *Nitrobacter agilis*.

1) nitrite oxidase
2) nitrate reductase
3) nitrite reductase
4) hydroxylamine reductase
5) glutamate dehydrogenase
6) glutamine synthetase
7) glutamate synthase

4.2.2 Purification, properties and regulation of glutamine synthetase and glutamate dehydrogenase from *Nitrobacter agilis* and *Nitrosomonas europaea*

Glutamine synthetase (L-glutamate : ammonia ligase ADP forming, EC6. 3.1.2) is a key regulatory enzyme of inorganic nitrogen metabolism in many organisms (Tyler, 1978). The enzyme has been characterized and its properties determined in a variety of bacteria (Nagatani et al., 1971; Brown et al., 1974; Kleiner and Kleinschmidt, 1976; Bender et al., 1977; Stacey et al., 1977; Johansson and Gest, 1977; Darrow and Knotts, 1977; Siedal and Shelton, 1979; Alef et al., 1981; Alef and Zumft, 1981; Bhandari and Nicholas, 1981; Khanna and Nicholas, 1983a; Murrell and Dalton, 1983). There is no information available about this enzyme and its possible role in the nitrogen metabolism of the nitrifying bacterium *Nitrobacter agilis*.

The properties of GS purified from *Nitrobacter agilis* described in this thesis are similar to those for a variety of bacteria in terms of requirement for divalent cations, molecular weight, number of subunits and inhibition by $\text{NH}_4^+$, amino acid and nucleotides (Hubbard and Stadtman, 1967; Deuel and Stadtman, 1970; Hachimori et al., 1974; Bhandari and Nicholas, 1981). The $K_m$ values for the substrates of the transferase and biosynthetic reactions of the purified *Nitrobacter* enzyme are comparable to those reported for other bacteria (eg. Shapiro and Stadtman, 1970a). The molecular weight of GS from *Nitrobacter agilis* was found to be 700,000 which is similar to that reported for this enzyme from *Rhodopseudomonas palustris* (=700,000) (Alef et al., 1981) but higher than that of the *Escherichia coli* enzyme (592,000) (Shapiro and Stadtman, 1970a). The subunit molecular weight of 58,000 for the enzyme from
Nitrobacter indicates that the native enzyme is composed of 12 homologous sub-units as in other bacteria eg. *E. coli* (Shapiro and Stadtman, 1970a).

The inhibition by NH$_4$Cl of the transferase activity of GS from *Nitrobacter agilis* (Section 3.3.2.6) supports a postulated model that glutamine reacts with the enzyme in such a way that its -NH$_2$ group occupies the NH$_4^+$ binding site, while the "oxygen-binding" site to which glutamate is normally bound is required for the attachment of the corresponding oxygen group of glutamine (Gass and Meister, 1970). The results also indicate that the inactivation of the NH$_4^+$ binding site by glutamine would preclude the binding of NH$_2$OH at this locus.

The GS from *E. coli* is regulated by a complex set of mechanisms (Magasanik *et al*., 1974) involving feed back inhibition, repression/derepression and adenlyylation/deadenylylation (Woolfolk *et al*., 1966; Ginsburg and Stadtman, 1973; Wohlhueter *et al*., 1973). Subsequent reports indicate that a similar type of regulation exists in a variety of other bacteria (Bender *et al*., 1977; Johansson and Gest, 1977; Darrow and Knotts, 1977; Alef and Zumft, 1981; Khanna and Nicholas, 1983a,b; Michalski *et al*., 1983). Bhandari and Nicholas (1981) reported that the enzyme from *Nitrosomonas europaea* is inhibited by several feed back inhibitors. The inhibition of GS from *Nitrobacter agilis* by amino acids and nucleotides reported in this thesis indicates that this enzyme is also regulated by similar feed back mechanisms. Mixtures of various amino acids showed cumulative inhibition of enzyme activity suggesting that each modifier is completely independent in its action and thus it is possible that separate binding sites on the enzyme are present for each of the feed back inhibitors as proposed for the *E. coli* enzyme (Stadtman *et al*., 1968).

In general, GS from Gram-negative bacteria are regulated by adenlyylation/deadenylylation (Ginsburg and Stadtman, 1973). The adenlylated and de-adenlylated forms of GS differ in their regulatory properties (Bender *et al*., 1977). It is well known that the adenlylation state of the enzyme depends on the nitrogen source in the growth medium. Thus bacterial cells growing with NH$_4^+$ contain GS largely repressed and in an adenlylated form (Wohlhueter *et al*., 1973). As expected *Nitrosomonas europaea* grown with NH$_4^+$ contains GDH as the main enzyme for the assimilation of NH$_4^+$ (Hooper *et al*., 1967) and has relatively little GS activity (Bhandari and Nicholas, 1981 and the present study). On the other hand *Nitrobacter agilis* has appreciable activities of GS and GDH but relatively low GOGAT (Section 3.2).
It is of interest that the *Nitrobacter* enzyme was highly adenyllylated even when the cells were grown with NO$_3^-$, without any added NH$_4^+$ in the culture medium. Cetyl trimethyl ammonium bromide (CTAB) treatment of the cultures prior to harvest had no substantial effect on the state of adenyllylation of GS. Relatively low concentrations of CTAB (2.5μg ml$^{-1}$) were used compared to those employed for other bacteria (Bender et al., 1977; Davies and Ormerod, 1982; Michalski et al., 1983) because higher concentrations resulted in cell lysis. This lysis may be associated with the low cell density of exponentially grown cultures of nitrifiers. The native adenyllylated form of GS from *Nitrobacter agilis* could be deadenyllylated by treatment with snake venom phosphodiesterase. This confirms that the *Nitrobacter* enzyme is indeed regulated by an adenyllylation/deadenyllylation mechanism. Another line of evidence to support this conclusion is that differentially adenyllylated forms of *Nitrobacter* GS, prepared by controlled phosphodiesterase treatments of GS differ in their pH optima. The isoactivity pH of 7.4 for GS from *Nitrobacter agilis* lies between 7.15 for *E. coli* (Stadtman et al., 1970) and 7.55 for *Klebsiella aerogenes* (Bender et al., 1977). This isoactivity pH of *Nitrobacter* GS was independent of the purification stage of the enzyme as was also found in *K. aerogenes* (Bender et al., 1977).

*Nitrosomonas europaea* growing with high concentrations (~120mM) of NH$_4^+$ would be expected to have a highly adenyllylated form of GS. The results presented in this thesis indicate that the enzyme from *Nitrosomonas europaea* has some unusual features, viz. stimulation of transferase activity by Mg$^{2+}$ in crude extracts, and the inability of phosphodiesterase to reverse the Mg$^{2+}$ effect. However there are three lines of evidence indicating that the enzyme is adenyllylated: (a) the biosynthetic activity was Mn$^{2+}$ dependent, (b) the Mg$^{2+}$ effect on the transferase activity was dependent on the amount of NH$_4^+$ present in the medium (Fig. 32), and (c) the enzyme was progressively inhibited by increasing concentrations of urea (Bhandari, 1981). The biosynthetic activity of a fully adenyllylated GS from *E. coli* has been shown to be Mn$^{2+}$ dependent (Shapiro and Stadtman, 1970b). Moreover in *E. coli* (Kingdon et al. 1967) and in *Rhodopseudomonas capsulata* (Johansson and Gest, 1977) adenyllylation of the enzyme resulted in a change in metal specificity from Mg$^{2+}$ to Mn$^{2+}$ for the biosynthetic activity. The Mg$^{2+}$ effect on transferase activity in *Nitrosomonas europaea* may be related to the adenyllylation state of the enzyme since it was directly affected by the amount of NH$_4^+$ present in the growth medium. It should be noted that Mg$^{2+}$ stimulation of transferase activity was independent of the concentration of Mn$^{2+}$ in the assay mixture because an increase in Mn$^{2+}$
(up to 20mM) did not modify the Mg$^{2+}$ (20–100mM) effect on GS transferase activity. There was no effect of Mg$^{2+}$ on transferase activity in purified enzyme preparations. It is conceivable however that during the purification procedure the adenylylation state of the enzyme may have changed.

The observation that snake venom phosphodiesterase did not affect the Mg$^{2+}$ effect on transferase activity of GS from *Nitrosomonas europaea*, is comparable to recent reports for *Chlorobium limicola* (Davies and Ormerod, 1982) and *C. vibrioforme* (Khanna and Nicholas, 1983a,b). Davies and Ormerod (1982) reported that although the transferase activity of GS from *C. limicola* was inhibited by Mg$^{2+}$, phosphodiesterase treatment had no effect and they concluded that the enzyme was not regulated by an adenylylation/deadenylylation type mechanism. Khanna and Nicholas (1983b) however have shown that when toluene permeabilized cells of *C. vibrioforme* were incubated with $[^{14}\text{C}]$ ATP followed by an ammonia shock, the $[^{14}\text{C}]$ label was associated with GS, but phosphodiesterase treatment of this enzyme did not remove the $[^{14}\text{C}]$ adenine moiety. In a similar experiment with toluene treated cells of *Nitrosomonas europaea* $[^{14}\text{C}]$ ATP did not label GS, presumably because of very low amounts of enzyme in this bacterium. However from the results for *Chlorobium* (Khanna and Nicholas, 1983a,b) and for *Nitrosomonas europaea* (present study) it would appear that the inability of phosphodiesterase to reverse the Mg$^{2+}$ inhibition of transferase activity does not necessarily imply that the enzyme is not adenylylated. It is of interest that both *Chlorobium* and *Nitrosomonas* enzymes did not interact with Cibacron Blue F3G-A dye (in Blue Sepharose CL-6B column) which normally binds to the nucleotide binding site of GS. It is possible therefore that the nucleotide binding site of the enzymes from *Chlorobium* and *Nitrosomonas* differ from those of GS from other bacteria (eg. *Nitrobacter*).

As discussed in Section 4.2.1 the cell-free extracts of *Nitrobacter agilis* contained NAD$^{+}$ and NADP$^{+}$ dependent glutamate dehydrogenase (GDH) activities. It is clear from the results reported in this thesis that these two activities were associated with two distinct protein fractions. This report is similar to those for *Thiobacillus novellus* (Le'John et al., 1968), *Hydrogenomonas* H16 (Krämer, 1970) and *Micrococcus aerogenes* (Kew and Woolfolk, 1970; Johnson and Westlake, 1972) which also have two GDH isozymes dependent on NAD$^{+}$ and NADP$^{+}$ respectively. The NAD$^{+}$-GDH of *Nitrobacter agilis* appears to operate in either direction ie. amination of α-ketoglutarate to glutamate and deamination of glutamate to α-ketoglutarate, whereas NADP$^{+}$-GDH functions mainly in the direction of glutamate (ammonia assimilation). It is of interest that the
amination reaction of NADP⁺-GDH from *Nitrobacter agilis* was stimulated by NH₄⁺ and NADPH (substrate stimulation) so that two distinct Kₘ values were obtained for either substrate. Cells of *Nitrobacter agilis* contained a high concentration of NH₄⁺ (approximately 30mM) and this explains why (a) GS is highly adenylylated (Section 3.3.3) and (b) NADP⁺-GDH (Kₘ NH₄⁺, 6.3 to 33mM) operates in the direction of glutamate production even when no exogenous NH₄⁺ is available to the bacterium. *Nitrosomonas europaea* has only biosynthetic GDH activity (NADP⁺-GDH) which can function in either direction i.e. amination and deamination (Hooper *et al.*, 1967). It is likely however that under physiological growth conditions, the enzyme is essentially unidirectional since the deamination reaction is almost completely inhibited (80 to 90%) by 10mM NH₄Cl (Hooper *et al.*, 1967). No such regulation of NADP⁺-GDH from *Nitrobacter* seems necessary because the deamination activity of the enzyme is only about 4% of that of the amination activity and the enzyme would thus appear largely biosynthetic. Unlike the NADP⁺-GDH of *Nitrosomonas europaea* (Hooper *et al.*, 1967) the amination reaction of NADP⁺-GDH from *Nitrobacter agilis* was unaffected by high concentrations (100mM) of α-ketoglutarate and by nucleotides. In fact NADPH stimulated the amination activity as shown in Fig. 39.

Attempts to purify NAD⁺-GDH from *Nitrobacter agilis* by affinity chromatography on Blue-Sepharose CL-6B were unsuccessful. Low activity of the NAD⁺ enzyme as well as minimal cell yields of *Nitrobacter agilis* make it difficult to purify this enzyme in sufficient amounts to study its properties and regulation. The bacterial NAD⁺-GDH are usually catabolic in function i.e. they utilize glutamate to produce α-ketoglutarate required as a substrate for transamination reactions and for the tricarboxylic acid cycle (Smith *et al.*, 1975). Although NAD⁺-GDH from *Nitrobacter agilis* can carry out both deamination and amination functions, its main role may be the production of α-ketoglutarate.

The high intracellular NH₄⁺ concentration observed under laboratory growth conditions support the view that GDH is the main pathway for NH₄⁺ assimilation in *Nitrobacter agilis*. Accumulation of NH₄⁺ via the assimilatory NO₂⁻ reductase (Wallace and Nicholas, 1968) appears to be under minimal regulatory control. Under natural growth conditions in the soil, the availability of N source (NO₂⁻ and/or NH₄⁺) for *Nitrobacter agilis* can vary greatly and this may account for the fact that the bacterium has two NH₄⁺ assimilation pathways i.e. GDH and GS/GOGAT. The following schemes are suggested assuming that NO₂⁻ reductase is responsible for the production of NH₄⁺ in *Nitrobacter agilis* when exogenous NH₄⁺
is unavailable to bacteria. When NO$_2^-$ is limiting, the nitrite oxidase would predominate, generating ATP and reducing equivalents, resulting in minimal reduction of NO$_2^-$ to NH$_4^+$. Under these conditions GS would be unadenylylated (active form) and would readily assimilate the small amounts of NH$_4^+$ available ($K_m$ NH$_4$ = 0.2mM).

When an ample supply of NO$_2^-$ is available (e.g., culture growth conditions), NO$_2^-$ reductase would produce sufficient amounts of NH$_4^+$ that would result in adenylylation of GS. The NH$_4^+$ thus produced would then be predominately assimilated by the GDH pathway.

4.3 ENERGY CONSERVATION IN NITROSOMONAS EUROPaea AND NITROBACTER AGILIS

4.3.1 Respiration driven proton translocation

It is well known that the operation of the respiratory chain results in the translocation of protons across the cell membrane thus developing an electrical potential ($\Delta\psi$, inside negative) as well as a pH gradient ($\Delta$pH, inside alkaline). This results in a proton-motive force ($\Delta$p) which drives active transport and ATP synthesis (Mitchell, 1966).
The subject of respiratory driven proton translocation in nitrifying bacteria is relatively unexplored. Drozd (1976) reported \( \rightarrow H^+/0 \) ratios of about 2 for the oxidation of \( NH_4^+ \) and \( NH_2OH \) by *Nitrosomonas europaea*. The results for \( NH_4^+ \) appear to be in error, because 0.15M KSCN, which was used as the permeant ion, completely inhibits the oxidation of \( NH_4^+ \) (Bhandari and Nicholas, 1979a, and present study). Although the value reported for the oxidation of \( NH_2OH \) is correct for the particular \( NH_2OH \) concentration used in the \( O_2 \) pulse experiments, no account was taken of phenomenon that might diminish the \( \rightarrow H^+/0 \) ratio, namely the tendency of permeant amines to follow protons across the membranes.

The results reported in this thesis indicate that *Nitrosomonas europaea* responded in a classical fashion in oxygen pulse experiments and showed efficiencies of proton translocation with amine-like substrates (\( \rightarrow H^+/0 \propto 4 \)) comparable to those for enteric bacteria (Garland et al., 1975) and for the reduction of nitrogen oxides by denitrifying bacteria (Kristjansson et al., 1978). The \( \rightarrow H^+/0 \) ratios for the oxidation of endogenous substrate, which is presumed to represent a set of organic compounds, were in the range of 4 to 6. High concentrations of \( NH_4^+ \), \( NH_2OH \) and \( N_2H_5^+ \) appeared to diminish the proton pumping activity of the cells. The most likely cause of this is the well known tendency of the permeant amines to redistribute across the membrane towards the acidic side in response to a pH gradient (Krogman et al., 1959; Good, 1960; Cobley, 1976a,b).

The \( \rightarrow H^+/0 \) ratios recorded for *Nitrosomonas europaea* are quantitatively in accord with the finding that the first step in \( NH_4^+ \) oxidation (\( NH_4^+ +0 + NH_2OH + H^+ \)) is mediated by a mono-oxygenase (Dua et al., 1979; Hollocher et al., 1981; Andersson et al., 1982) and could not thermodynamically support proton translocation (\( \Delta G^o' = -0.7 \) kcal). The observed \( \rightarrow H^+/0 \) ratio for \( NH_4^+ \) is 3.4. Subtracting 0.66 stoichiometric protons per 0 atom gives a ratio of 2.74. Inasmuch as the first of the three 0 atoms is utilized in an 0 insertion reaction, the effective \( \rightarrow H^+/0 \) ratio becomes 3/2 x 2.74 = 4.1 for the two 0 atoms reduced via electron transport system. The corresponding ratio for \( NH_2OH \) and \( N_2H_5^+ \) oxidation by *Nitrosomonas europaea* would be 4.4-0.5 = 3.9. Thus the \( \rightarrow H^+/0 \) ratio ascribable to pumped protons is about the same for these three substrates. These data also indicate that \( NH_4^+ \), \( NH_2OH \) and \( N_2H_5^+ \) enter
the cells of *Nitrosomonas europaea* as neutral species and NO$_2^-$ exits as an
electronneutral species with one proton. This pattern is analogous to that
inferred from oxidant pulse studies of NO$_2^-$ reduction by denitrifying bacteria
(Kristjansson *et al.*, 1978) and would prevent the internal acidification of
*Nitrosomonas europaea*.

If the reaction $\text{NH}_2\text{OH} + 20 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + \text{H}^+$ ($\Delta G^\circ = -83$ kcal or
$-347.4$ kJ mol$^{-1}$) is the relevant energy yielding reaction in NH$_4^+$ oxidation by
*Nitrosomonas europaea*, the average free energy available to each of the 8.2
protons translocated is about 10 kcal (approximately 42 kJ) per proton. The
transformation of only half of this available free energy into an electrical and
pH gradient could support a proton-motive force of about 220 mV and this ought
to be sufficient for ATP synthesis (Wilson *et al.*, 1976; Some *et al.*, 1977;
Hinkle and Yu, 1979).

In *Nitrobacter* the free energy available in the aerobic oxidation of a
high potential reductant NO$_2^-$ (NO$_2^- + O \rightarrow NO_3^-; \quad \Delta G^\circ = -18$ kcal or $-75.4$ kJ mol$^{-1}$)
is used to drive the reduction of a low potential oxidant NAD$^+$ and to produce

$$\text{NO}_2^- + \text{NAD}^+ + \text{H}_2\text{O} \rightarrow \text{NO}_3^- + \text{NADH} + \text{H}^+ \quad \Delta G^\circ = 38 \text{ kcal}$$

It has been shown with membrane vesicles that the aerobic oxidation of both
NO$_2^-$ and NADH is linked to ATP synthesis (Aleem and Alexander, 1958; Aleem and
Nason, 1960; Butt and Lees, 1960; Cобley, 1976b). The coupling of NO$_2^-$
respiration to phosphorylation and NAD$^+$ reduction is generally assumed to be
by way of the proton-motive force (Cобley, 1976a,b; Aleem, 1977; Aleem and
Sewell, 1981; Ferguson, 1982). Thus the apparent inability of *Nitrobacter agilis*
to translocate protons in O$_2$ pulse studies is remarkable and interesting.

The only report of respiration dependent proton translocation in *Nitro-
bacter* is that of Cобley (1976b) who demonstrated with electron transport
particles of *Nitrobacter winogradskyi* that the aerobic oxidation of NO$_2^-$ resulted
in a small but reversible alkalization of the medium. Although the effect
was abolished by agents known to collapse $\Delta \mu_p$, the amplitude of the effect
showed saturation with respect to O$_2$, was not appreciably enhanced by valinomycin/K$^+$, and showed an extremely low H$^+/O$ ratio of $\simeq 0.1$. Whether this effect
resulted directly from the operation of a respiratory proton pump in inverted
membrane particles is uncertain. The data reported in this thesis also show
small respiration dependent changes in external pH. These changes and the
subsequent slower ones in external proton concentration were not much enhanced by permeant ions and in general exhibited properties and kinetics distinctly different from the classical picture. No convincing evidence is thus available for a respiratory proton pump in *Nitrobacter agilis* to support Cobley's prediction (Cobley, 1976b) that the $\Delta^+ / \Delta^0$ ratio should be 1. The data of Cobley (1976a,b) show that NO$_2^-$ oxidation in *Nitrobacter winogradskyi* membrane vesicles is inhibited by agents that collapse the membrane potential, but whether the effect is mediated entirely through the membrane potential is not clear. The extent of inhibition imposed by the uncoupler CCCP at high concentrations (180µM) and the K$^+$ carrier valinomycin was not complete (Cobley, 1976b). The uncoupling concentration of CCCP is usually between 5 to 20µM. In any case, the rates of NO$_2^-$ oxidation in the *Nitrobacter agilis* preparations used in this thesis were adequate for oxygen pulse studies even in the presence of permeant ion concentrations known to be effective in collapsing the membrane potential in *Nitrosomomas europaea*. There was no evidence in these studies that the membranes of *Nitrobacter agilis* were abnormally permeant to protons (uncoupled). The relaxation of protons across the membranes following acid pulses was slow (Fig. 44) and typical of that observed with *Nitrosomomas europaea* (Fig. 41) and other bacteria (Kristjansson et al., 1978). While it is conceivable that none of the nominally permeant ion was in fact permeant, this requires *Nitrobacter agilis* to possess a unique membrane system, which is unlikely. Moreover the lipophilic cation TPMP$^+$, which is a well known permeant ion and has been used to determine membrane potential in bacteria (Rottenberg, 1979) and to collapse the membrane potential in oxidant pulse experiments (Boogerd et al., 1981; Castignetti and Hollocher, 1983), was effective with *Nitrosomomas europaea* but not with *Nitrobacter agilis*. Valinomycin should have penetrated the cytoplasmic membrane in spheroplasts and membrane vesicles of *Nitrobacter agilis* even if it could not do so in intact cells. Based on the results for *Nitrosomomas europaea* and a variety of other bacteria there is no evidence that permeant ions inhibit the respiratory proton pump per se.

4.3.2 Proton-motive force and ATP biosynthesis

According to Mitchell's chemiosmotic hypothesis (Mitchell, 1966), the electrochemical gradient of protons ($U_{H^+}$) gives rise to a proton-motive force ($\Delta p$). This proton gradient consists of an electrical potential ($\Delta \Psi$) and a pH gradient ($\Delta p\text{H}$). The two components of $\Delta p$ have the following relationship: $\Delta p = \Delta \Psi - 2.3RT/F \Delta p\text{H}$. Whereas $\Delta p$ has been determined in a variety of bacteria
(Padan et al., 1976; Guffanti et al., 1978; Deutsch and Kula, 1978; Kashket et al., 1980; Kashket, 1981a,b; Jarrell and Sprott, 1981; Matin et al., 1982) no information is available for the nitrifying bacteria. The measurement of Δp in *Nitrosomonas europaea* and *Nitrobacter agilis* is reported in this thesis.

The results in Section 3.5.2 indicate that at an external pH (pHe) of 7.0 for *Nitrosomonas europaea* and 7.5 for *Nitrobacter agilis* there was no ΔpH (inside alkaline) because at these pH values neither weak acids nor weak base were accumulated by these bacteria. The pH optima for NH$_4^+$ and NO$_2^-$ oxidation by *Nitrosomonas europaea* and *Nitrobacter agilis* respectively were between 7.5 and 8.0, indicating that optimally respiring cells of these bacteria do not have a ΔpH. *Nitrosomonas europaea* appeared to have a limited capacity to maintain a constant intracellular pH (pHi). This result contrasts with those reported for *Micrococcus lysodeikticus* (Friedberg and Kaback, 1980) and *E. coli* under aerobic conditions (Padan et al., 1976), but is comparable to anaerobic bacteria, namely, *Methanospirillum hungatei* (Jarrell and Sprott, 1981), *Clostridium pasteurianum* (Riebeling et al., 1975) and even *E. coli* grown under anaerobic conditions (Kashket and Wong, 1969). In *Nitrobacter agilis* pH did not remain relatively constant (7.3 to 7.8) over a range of pHe values (6 to 8.5) which is in agreement with the results for *E. coli* (Padan et al., 1976), *Micrococcus lysodeikticus* (Friedberg and Kaback, 1980), *Thiobacillus acidophilus* (Matin et al., 1982) and *Bacillus subtilis* (Khan and Macnab, 1980) (for a review see Padan et al., 1981).

The weak base methylamine, used as a probe for the determination of ΔpH, is not oxidised by either nitrifier. Since ammonia and its analogues are probably taken up by *Nitrosomonas europaea* as neutral species (Drozd, 1976) it is unlikely that the cells would accumulate methylamine in response to a Δψ (inside negative) as reported for *Azotobacter vinelandii* (Laane et al., 1980).

At a pHe of 6.0, neither *Nitrosomonas europaea* nor *Nitrobacter agilis* oxidised its respective substrate, but they still maintained a reasonable ΔpH and Δψ, and thus Δp. In fact, in *Nitrobacter agilis*, Δp was maximal at pHe 6.0 (or less than 6.0), and it decreased linearly with an increase in pHe. However at pH 7.0 when both *Nitrosomonas europaea* and *Nitrobacter agilis* retained about half of their respiratory activities, the small ΔpH was dissipated by uncouplers and compounds which inhibit respiration. It is known that *Nitrosomonas europaea* and *Nitrobacter agilis* have appreciable rates of endogenous respiration (Section 3.5.1; Drozd, 1976; Sewell and Aleem, 1979;
Hyman and Wood, 1983) involving complex organic substrates. In Nitrosomonas europaea endogenous respiration has been shown to be coupled to proton translocation (Section 3.5.1 and Drozd, 1976). It is likely that this endogenous respiration enables the cells to maintain a reasonable Δp in the absence of exogenous substrates, or when the exogenous respiration is inhibited. This phenomenon could have ecological significance for nitrifiers, because these soil bacteria in their natural habitat may encounter conditions that preclude respiration for extended periods of time.

In Nitrosomonas europaea the uncoupler CCCP severely inhibited respiration (80% at 10μM CCCP) but lowered Δp by about 40mV. At higher concentrations (100 μM CCCP) the respiration was completely inhibited but Δp was reduced by only 78mV. In Nitrobacter agilis; CCCP (50μM) completely restricted respiration, but non-respiring cells still maintained a Δp of 82mV (inside negative). Uncouplers are known to restrict respiration in nitrifying bacteria (Cobley, 1976b; Aleem, 1977; Bhandari and Nicholas, 1979a,b, 1980; Aleem and Sewell, 1981) but it is only recently they have been shown to inhibit respiration in other bacteria e.g. Thiobacillus acidophilus (Matin et al., 1982) and denitrification in Pseudomonas denitrificans and Pseudomonas aeruginosa (Walter et al., 1978). The effects of uncouplers on denitrification were not linked to the collapse of Δp but rather to their detergent-like effects on the cell membranes (Walter et al., 1978). It is possible that the mechanism of inhibition of respiration by CCCP and other uncouplers in Nitrosomonas europaea and Nitrobacter agilis is similar to that in the denitrifying bacteria.

Because the inhibitors known to collapse Δψ also inhibited NO$_2^-$ oxidation by membrane particles of Nitrobacter winogradskyi, Cobley (1976a,b) predicted that NO$_2^-$ oxidation requires Δψ. The way in which NO$_2^-$ oxidation is mediated by Δψ is however not understood (discussed in Section 4.3.1). Cobley also reported that NH$_4^+$ stimulated NO$_2^-$ oxidation by collapsing ΔpH. The results of the present study also indicate that ΔpH is collapsed by NH$_4^+$, but NH$_4^+$ also lowered Δψ. Thus Cobley's prediction (Cobley, 1976b) that NH$_4^+$ stimulation of NO$_2^-$ oxidation resulted from a collapse of ΔpH only is not substantiated by the data presented in this thesis.

As discussed in Section 4.1, ATPase inhibitors strongly inhibited NO$_2^-$ oxidation by Nitrobacter agilis. Similarly NH$_4^+$ oxidation by Nitrosomonas europaea was also restricted by ATPase inhibitors but to a lesser extent (Section 3.5.2). This inhibition did not appear to be associated with a collapse of Δp, because DESB elevated Δp in Nitrosomonas europaea rather than lowering it.
The results of the present study indicate that the total Δp at pH 7.5 would be approximately 150mV and 125mV (inside negative) in *Nitrosomonas europaea* and *Nitrobacter agilis* respectively. In *E. coli* Δp is composed of both ΔΨ and ΔpH (Padan *et al.*, 1976); however ΔpH appears to be absent in *Methanobacterium thermoautotrophicum* and *Methanospirillum hungatei* (Jarrell and Sprott, 1981). The overall behaviour of ApH and ΔΨ in nitrifying bacteria is quite similar to that reported for other bacteria e.g. *E. coli* (Padan *et al.*, 1976).

In this thesis, it is shown that spheroplasts of *Nitrosomonas europaea* synthesize ATP in response to an artificially created ΔΨ. It was shown earlier (Reid *et al.*, 1966) that rapid immersion of mitochondria in an acidic medium resulted in ATP synthesis. Subsequently, Maloney *et al.* (1974) and Wilson *et al.* (1976) demonstrated that both *Streptococcus lactis* and *E. coli* synthesize ATP in response to a valinomycin induced ΔΨ. The results of ATP synthesis by *Nitrosomonas europaea* are comparable to those for *S. lactis* and *E. coli* (Maloney *et al.*, 1974; Wilson *et al.*, 1976). The ATP synthesis induced by valinomycin was inhibited by DCCD indicating the involvement of ATPase. The inhibition of valinomycin induced ATP synthesis in spheroplasts of *Nitrosomonas europaea* by increasing concentrations of KCl (Fig. 53) indicated that it was dependent on the magnitude of ΔΨ as reported for *E. coli* and *S. lactis* (Maloney *et al.*, 1974; Wilson *et al.*, 1976). Valinomycin induced ATP synthesis was always transient presumably because of (a) ATP hydrolysis following the increase in intracellular ATP levels and (b) collapse of ΔΨ by inward movement of cations. Thus the results reported in this thesis provide evidence that, as in other bacteria, *Nitrosomonas europaea* also has a respiratory driven proton-motive force coupled to an energy conserving system as expected from Mitchell's chemiosmotic hypothesis (Mitchell, 1966). For reasons given in Section 3.5.3.2 similar experiments of valinomycin induced ATP synthesis with washed cells and spheroplasts of *Nitrobacter agilis* were inconclusive.

### 4.3.3 Na⁺ and K⁺ transport

In recent years a substantial amount of information has accumulated on the role of Δp in living systems. In addition to pumps which extrude protons to generate a Δp, bacteria also possess several genetically distinct cation transport systems (Harold and Atendorf, 1974; Rhoads *et al.*, 1976; Epstein and Laimins, 1980). Transport systems which derive their energy from previously formed electrochemical gradients utilize three basic mechanisms for energy coupling, as described by Mitchell (1973), namely, symports, uniports and anti-
ports. Of the several ion transport systems, those for Na⁺ and K⁺ play an important role in regulating intracellular pH (Krulwich et al., 1979; Beck and Rosen, 1979; Brey et al., 1980; Plack and Rosen, 1980; Tokuda et al., 1981) and active transport of nutrients (Stock and Roseman, 1971; Thomson and McLeod, 1971; Lanyi et al., 1976; Tokuda and Kaback, 1977; Eddy, 1978). As there is no information available on these transport systems in nitrifying bacteria, an attempt has been made to characterize some of these cation transport systems in *Nitrosomonas europaea* and *Nitrobacter agilis*.

The results of K⁺ depletion experiments with the cells of *Nitrosomonas europaea* and *Nitrobacter agilis* respectively are similar to those reported for *Vibrio alginolyticus* and *E. coli* (Nakamura et al., 1982). The amine treatment method for K⁺ depletion was effective for both nitrifiers. There was no net entry of Na⁺ during the extrusion of K⁺ in either nitrifier indicating that the uptake of unprotonated amine into the cells by passive diffusion and its subsequent protonation inside allows for the K⁺ extrusion via K⁺/H⁺ antiporter even in the absence of Na⁺ entry.

![Diagram of K⁺/H⁺ antiporter](image)

Since the internal pH is decreased by the extrusion of K⁺ and the antiporter is relatively inactive at lower pH (Nakamura et al., 1982), a high concentration of amine is required for the bulk release of cellular K⁺. It appears that the cells of *Nitrosomonas europaea* grown with high NH₄⁺ have relatively low and variable intracellular K⁺ (Table 41) which could well be associated with K⁺/H⁺ antiporter system and NH₄⁺ transport into the cell.

The depolarization of ΔΨ by K⁺ has been shown in *Streptococcus faecalis* and *E. coli* (Bakker and Mangerich, 1981). These authors reported that the addition of K⁺ to K⁺ depleted cells of *S. faecalis* resulted in depolarization of ΔΨ by about 60mV but this depolarization of ΔΨ was compensated by an appropriately equivalent increase in ΔpH so that the total proton-motive force remained reasonably constant. Thus the electrogenic K⁺ influx results in an interconversion between the components of Δp. Similar results were obtained for *E. coli* but the extent of depolarization of ΔΨ by K⁺ was much lower than in *S. faecalis*.
Bakker and Mangerich, 1981). It appears that the extent of depolarization of $\Delta \psi$ by $K^+$ depends on the ability of cells to take up $K^+$. Thus the results reported in this thesis indicate that a similar mechanism exists in both Nitrosomonas europaea and Nitrobacter agilis. In the absence of other permeant ions the proton pumps of the cytoplasmic membranes tend to develop a large outwardly directed $\Delta \psi$ and a small $\Delta \varphi$ (Mitchell, 1966). Inward movement of $K^+$ will decrease $\Delta \psi$, which allows more protons to be pumped out with the result that in steady state $\Delta \psi$ is partially converted into $\Delta \varphi$. Such interconversions are well known in energy transducing membranes eg. lipid soluble cation $\text{TPMP}^+$ causes an extensive conversion of $\Delta \psi$ into $\Delta \varphi$ in illuminated cell suspensions of Halobacterium halobium (Bakker et al., 1976). Thus increasing concentration of a permeant ion continues to decrease $\Delta \psi$. Up to a certain point the depolarization of $\Delta \psi$ can be compensated by an increase of $\Delta \varphi$ until the membrane becomes leaky to protons due to secondary effects such as swelling (Padan and Rottenberg, 1973).

A respiration dependent primary Na$^+$ extrusion system functioning at alkaline pH has been demonstrated in the marine bacterium Vibrio alginolyticus (Tokuda and Unemoto, 1981). Halobacterium halobium also extrudes Na$^+$ either by a Na$^+/H^+$ antiporter system (Lanyi and MacDonald, 1976) or a Na$^+$ pump, halorhodopsin (Lindley and MacDonald, 1979). Using $^{22}\text{Na}^+$ loaded cells it is shown in the present work that both Nitrosomonas europaea and Nitrobacter agilis lack the respiration dependent Na$^+$ pump. Extrusion of $^{22}\text{Na}^+$ from $^{22}\text{Na}^+$ loaded cells required $K^+$ as a counter-ion in both Nitrosomonas europaea and Nitrobacter agilis but the extent of $^{22}\text{Na}^+$ extrusion by $K^+$ addition in Nitrosomonas europaea was much less than in Nitrobacter agilis (Fig. 59). It appears that the $K^+$ uptake system of Nitrosomonas europaea is not as efficient as in Nitrobacter agilis and this observation is supported by the fact that $K^+$ uptake results in about twice the depolarization of $\Delta \psi$ in Nitrobacter agilis than in Nitrosomonas europaea (Fig.57). Amines and NH$_4$Cl also resulted in the extrusion of $^{22}\text{Na}^+$, probably via a Na$^+/H^+$ antiporter system.

In summary the results reported in Section 3.5.4 indicate that Nitrosomonas europaea and Nitrobacter agilis have several distinct cation transport systems including antiporters for $K^+/H^+$, $K^+/\text{Na}^+$ and Na$^+/\text{H}^+$. At least one of these antiporters ($K^+/\text{Na}^+$) requires an electrochemical gradient of protons for its operation. $K^+$ can also be transported by an electrogenic mechanism. The possible cation transport systems in Nitrosomonas europaea and Nitrobacter agilis are summarized in the following scheme:
4.3.4 $^{15}N,^{18}O$ isotope studies of NO$_2^-$ oxidation by Nitrobacter agilis

The results reported in this thesis clearly indicate that *Nitrosomonas europaea* follows the classical chemiosmotic pattern for energy conservation. Although in *Nitrobacter agilis* the pattern of components of proton-motive force ($\Delta\Psi$ and $\Delta$pH) and cation transport systems appear quite similar to those in other bacteria (See Padan et al., 1981 and the references therein), the apparent absence of demonstrable proton translocation in fluorescence quenching and oxygen pulse experiments is unclear. During the course of this study, Dr. T.C. Hollocher (Biochemistry Department, Brandeis University, U.S.A.) suggested (personal communication) that in the absence of a respiratory proton
pump the bacterium might synthesize ATP by substrate level phosphorylation involving a mixed anhydride between either \( \text{NO}_3^- \) and \( \text{PO}_4^{2-} \) or \( \text{NO}_3^- \) and ADP, by analogy with the oxidation of sulfite by *Thiobacillus* and reverse of dissimilatory reduction of sulfate by *Desulfovibrio*, both of which proceed by way of adenosine 5'-phosphosulfate (Roy and Trudinger, 1970). The validity of this hypothesis was checked by means of \( ^{15} \text{N}-\text{NMR} \) and GC/MS studies using stable isotopes of \( ^{15} \text{N} \) and \( ^{18} \text{O} \) (Section 3.5.5). The results of both \( ^{15} \text{N}-\text{NMR} \) and GC/MS studies clearly indicate that the third '0' in \( \text{NO}_3^- \) produced from \( \text{NO}_2^- \) originated from \( \text{H}_2\text{O} \) and not from either \( \text{O}_2 \) or \( \text{PO}_4^{2-} \). These findings are in agreement with those of Aleem *et al.* (1965). The GC/MS studies of \( \text{NO}_2^- \) oxidation reported in Section 3.5.5.1 indicate that there was no apparent exchange of \( ^{18} \text{O} \) between \( \text{H}_2\text{O} \) and \( \text{P}^{18} \text{O}_4^- \) so that it is unlikely that \( ^{18} \text{O} \) would have been lost from \( \text{P}^{18} \text{O}_4^- \) during the experiments. The results in Section 3.5.5 also rule out the possibility of the formation of a P-O-N type intermediate as suggested by Hollocher.

The results of the \( ^{15} \text{N}-\text{NMR} \) study of \( \text{NO}_2^- \) oxidation by *Nitrobacter agilis* (Section 3.5.5.2) indicate that when the cells were incubated with \( ^{15} \text{NO}_3^- \) and \( \text{H}_2\text{O} \), the three resonances in the NMR spectrum represented \( ^{15} \text{N}^{16} \text{O}_3^-(100\%) \), \( ^{15} \text{N}^{16} \text{O}_2^{18} \text{O}^-(19\%) \) and \( ^{15} \text{N}^{16} \text{O}^{18} \text{O}^-(2.3\%) \). Since the \( ^{15} \text{NO}_2^- \) stock contained about 30\% \( ^{15} \text{NO}_3^- \), the ratio of \( ^{15} \text{N}^{16} \text{O}_3^- \): \( (^{15} \text{N}^{16} \text{O}_2^{18} \text{O}^- + ^{15} \text{N}^{16} \text{O}^{18} \text{O}^-) \) is close to the theoretical value (100 : 32). This result proves that all the '0' in nitrate was derived from water. The oxidation of \( \text{NO}_2^- \) to \( \text{NO}_3^- \) by *Nitrobacter agilis* requires only one oxygen atom which is supplied by water (Aleem *et al.*, 1965 and the present study). The appearance of a \( ^{15} \text{N}^{16} \text{O}^{18} \text{O}^- \) resonance in the \( ^{15} \text{N}-\text{NMR} \) spectrum (Fig.62) is thus unusual. A chemical exchange of \( ^{18} \text{O} \) between \( \text{H}_2^{18} \text{O} \) and \( ^{15} \text{N}^{16} \text{O}^{18} \text{O}^- \) at alkaline pH is unlikely. A possible explanation of the appearance of \( ^{15} \text{N}^{16} \text{O}^{18} \text{O}^- \) could be associated with the recycling of \( ^{15} \text{NO}_2^- \) by nitrite oxidase (I) and dissimilatory type nitrate reductase (II) (Sewell and Aleem, 1979) in thick cell suspensions which tend to become anaerobic.
In conclusion, it is possible that *Nitrobacter agilis* conforms to the classical chemiosmotic coupling mechanism for ATP biosynthesis but the non-detection of a respiratory proton pump in this bacterium is unusual and further work is awaited to resolve this interesting anomaly.
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