NITROGEN ASSIMILATION AND ENERGY CONSERVATION

IN *NITROSOMONAS EUROPAEA* AND *NITROBACTER AGILIS*

by

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

Doctor of Philosophy

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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
FEMS Microbiol. Lett. 11: 202-206 (1981).

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
Proc. Aust. Biochem. Soc. 14: 48 (1981).

3). Assimilation of inorganic nitrogen compounds by *Nitrobacter agilis*.
S. Kumar and D.J.D. Nicholas
J. Gen. Microbiol. 128: 1795-1801 (1982).

4). Respiration-dependent proton transloction in *Nitrosomonas europaea* and its apparent absence in *Nitrobacter agilis* during inorganic oxidations.
T.C. Hollocher, S. Kumar and D.J.D. Nicholas
J. Bacteriol. 149: 1013-1020 (1982).

5). A proton-motive force dependent adenosine-5'-triphosphate synthesis in spheroplasts of *Nitrosomonas europaea*.
S. Kumar and D.J.D. Nicholas
FEMS Microbiol. Lett. 14: 21-25 (1982).

6). ATP biosynthesis in the nitrifying bacterium *Nitrosomonas europaea*.
S. Kumar and D.J.D. Nicholas
Abstracts of 12th International Congress of Biochemistry, Perth, p.299 (1982)

7). The proton-electrochemical gradients in washed cells of *Nitrosomonas europaea* and *Nitrobacter agilis*.
S. Kumar and D.J.D. Nicholas
J. Bacteriol. 154: 65-71 (1983).

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
FEBS Lett. 152: 71-74 (1983).

9). Adenylylation and deadenylylation of glutamine synthetase in the
nitrifying bacteria *Nitrosomonas europaea* and *Nitrobacter agilis.*
S. Kumar and D.J.D. Nicholas
Proc. Aust. Biochem. Soc. 15: 56 (1983).

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.

ACKNOWLEDGEMENTS

I record my deep and sincere thanks to my supervisor, Professor D.J.D. Nicholas, Chairman of the Department of Agricultural Biochemistry, Waite Agricultural Research Institute, The University of Adelaide, for his guidance, constant encouragement and constructive criticism throughout the progress of the present investigation and the preparation of this manuscript.

I would also like to thank Drs. M.E. Tate and T.C. Hollocher for helpful suggestions, technical advice and critical discussions during the course of present investigation and to Dr. E.H. Williams for the help with NMR spectroscopy.

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 nomenculature 1978).

Trivial Name	E.C. Name and Number
Adenosine triphosphatase (ATPase)	ATP phosphohydrolase E.C. 3.6.1.3
Glutamate dehydrogenase (GDH)	L-glutamate : NAD(P)$^+$ oxidoreductase (deaminating)
NAD^+-GDH	E.C. 1.4.1.2
$NAD(P)^+$-GDH	E.C. 1.4.1.3
$NADP^+$-GDH	E.C. 1.4.1.4
Glutamate synthase (GOGAT)	L-glutamate : NAD$^+$ oxidoreductase (transaminating) E.C. 1.4.1.14
Glutamine synthetase (GS)	L-glutamate : ammonia ligase (ADP forming) E.C. 6.3.1.2

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

Chemicals

ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CCCP	carbonyl cyanide *m*-chlorophenyl hydrazone
CDP	cytidine 5'-diphosphate
CMP	cytidine 5'-monophosphate
CTAB	cetyl trimethyl ammonium bromide
CTP	cytidine 5'-triphosphate
DBP	2.4 dibromophenol
DCCD	N,N'dicyclohexylcarbodiimide
DESB	diethylstilbestrol
DIECA	diethyl dithiocarbamate (sodium salt)
2,4 DNP	2.4-dinitrophenol
DBP	2.4-dibromophenol

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 ·	ionosine 5'-diphosphate
IMP	ionosine 5'-monophosphate
ITP	ionosine 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 phosphosphonium 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

A	absorbance
amu	atomic mass unit
°C	degree centrigrade (celcius)
Ci	curie
cm	centimeter
d	day(s)
Δp or $\tilde{\mu}_{H}{}^{+}$	proton-motive force or proton-electrochemical gradient
ΔpH	transmembrane pH gradient
$\Delta\psi$	transmembrane potential difference (membrane potential)
F	free energy or Faraday's constant
g	unit of gravitational field
g	gram
G	Gibb's energy constant
h	hour(s)
$\rightarrow H^{+}/O$	no. of protons translocated per $2e^{-}$ transferred to O
Hz	hertz
J	joule(s)
k	kilo
K	equilibrium constant
kcycle	kilo cycle
K_i	inhibitor constant
kJ	kilojoules
K_m	Michaelis constant
K_{sol}	solubility constant
kV	kilo volt
λ	wave length
l	litre
M	molar
m	meter
mCi	millicurie
mg	milligram
μCi	microcurie
min	minute(s)
ml	millilitre
mmol	millimole(s)
mM	millimolar
μg	microgram

μl	microlitre
μmol	micromole(s)
μM	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
$t\frac{1}{2}$	half time
T	thermodyamic temperature (Kelvin)
v	volume
V	velocity of the reaction
Vmax	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
$\simeq$	approximately equal

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SUMMARY

SUMMARY

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 NO_3^- production and O_2 uptake simultaneously and continuously during NO_2^- utilization by *Nitrobacter agilis*. The stoichiometry of NO_2^- oxidation by washed cells was $1NO_2^- : 0.5O_2 : 0.75 NO_3^-$ compared to $1NO_2^- : 0.5O_2 : 1NO_3^-$ for sphero-plasts and membrane vesicles of *Nitrobacter agilis*. The effects of several metabolic inhibitors on NO_2^- and O_2 utilization and 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 NO_2^- medium supplemented with $2mM-NH_4Cl$. Higher concentrations of NH_4Cl however competitively inhibited NO_2^- oxidation and the growth of the bacterium.

5. Washed cells of *Nitrobacter agilis* readily incorporated $^{15}NH_4^+$, $^{15}NH_2OH$, $^{15}NO_2^-$ and $^{15}NO_3^-$ 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 $^{15}NH_4^+$ or $^{15}NO_2^-$ into cell nitrogen, indicating that glutamate dehydrogenase pathway is the main route for the assimilation of 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 feed back inhibition involving amino acids and nucleotides, substrate

inhibition by NH_4^+ as well as by an adenylylation/deadenylylation 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 ie. 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 saltingin 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 $\rightarrow H^+/O$ ratio for NH_4^+ oxidation by *Nitrosomonas europaea* was 3.4 and that for both NH_2OH and $N_2H_5^+$ 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 $\rightarrow H^+/O$ 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}H] tetraphenyl phosphonium cation (TPP$^+$). Intracellular pH was determined by the distribution of [^{14}C] benzoic and [^{14}C] acetyl salicylic acids and [^{14}C] methylamine. At pHe of 7 for *Nitrosomonas europaea* and 7.5 for *Nitrobacter agilis* there was no detectable ΔpH so that only $\Delta\psi$ contributed to Δp. Intracellular pH (pHi) in cells of *Nitrosomonas europaea* varied from 6.3 at pHe 6 to 7.8 at pHe 8.5. In *Nitrobacter agilis* however pHi was relatively constant (7.3 to 7.8) over the pH range of 6 to 8.5. The components of Δp ($\Delta\psi$ and ΔpH) remained constant at various stages of growth of *Nitrosomonas europaea* so that the metabolic state of the cells did not affect Δp. 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 ΔpH. NH$_4^+$ was transported essentially as a neutral species (NH$_3$) by a simple diffusion mechanism. Experiments with ^{22}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}N (^{15}NO$_2^-$ and ^{15}NO$_3^-$) and ^{18}O (H$_2^{18}$O, ^{18}O$_2$ and P^{18}O$_4^{2-}$) it was shown by GC/MS and ^{15}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$_4^{2-}$.

I. 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 CO_2. 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 CO_2 by nitrifiers is well established in *Nitrosomonas* (Rao and Nicholas, 1966), *Nitrobacter* (Aleem, 1965) and *Nitrocystis* (Campbell et al., 1966). It seems that CO_2 fixation is common to all chemoautotrophs (Kelly, 1967, 1971). Delwiche et al. (1963) have shown that *Nitrosomonas* actively fixed ^{14}C-labelled CO_2 into compounds that were precipitated by trichloroacetic acid. Carboxydismutase, enzymes of the reductive pentose pathway and phosphoenol pyruvate carboxylase 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 carboyxlates 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 CO_2 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 O_2. 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 Kluyer and Donker (1926) that the oxidation of ammonia to nitrite occurs in two steps:

$$\text{(i)} \quad NH_3 \xrightarrow{\;2e^-\;} NH_2OH$$

$$\text{(ii)} \quad NH_2OH \xrightarrow{\;4e^-\;} NO_2^-$$
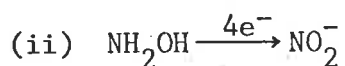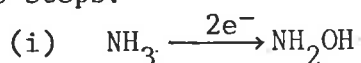
Engel and Alexander (1958a,b) reported that washed cells of *Nitrosomonas europaea* progressively lost their ammonia oxidising ability upon ageing. Oxidation of ammonia to hydroxylamine is inhibited by a variety of inhibitors (Lees, 1952a,b, 1955; Campbell and Aleem, 1965; Hooper and Terry, 1973; Ashworth *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_2OH 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 *Nitrosomonas europaea* which oxidised ammonia only in the presence of Mg^{2+} or NH_2OH. Rees and Nason (1966) found a small (7%) incorporation of ^{18}O from $^{18}O_2$ into NO_2^- (uncorrected for possible loss by exchange reactions) during ammonia oxidation by cells of *Nitrosomonas*. 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 NH_2OH to NO_2^-. Dua *et al.* (1979) reported an incorporation of ^{18}O from $^{18}O_2$ (0.3 atom % excess) into NH_2OH produced by the oxidation of ammonia by cells in the presence of hydrazine, but not from $H_2^{18}O$. This experiment was repeated with the yields of >92 atom % enrichment (Hollocher *et al.*, 1981). Using ^{18}O isotope shift in ^{15}N-NMR, Andersson *et al.* (1982) reported that NO_2^- produced by cells from NH_3 or NH_2OH has the isotopic oxygen composition of water. Cells were found to catalyse rapid exchange of both the oxygen atoms of NO_2^- with water. Thus the origin of second oxygen atom in NO_2^- was uncertain. All this evidence indicates that NH_3 oxidation to NH_2OH involves an oxygen insertion reaction via a monooxygenase. The origin of second oxygen in NO_2^- produced in the second step of NH_3 oxidation ($NH_2OH \rightarrow NO_2^-$) is still unclear.

The first attempts to prepare cell free-extracts of *Nitrosomonas* were made by Imshenetskii and Ruban (1952, 1954a,b, 1956, 1957). Very slow rate of ammonia oxidation was observed when the autolysates of *Nitrosomonas* 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 NH_3 to NO_2^-. The cell-free preparations by Nicholas and Jones (1960), however oxidised NH_2OH to 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 *Nitrosomonas 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 catalysed the oxidation of NH_2OH. 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_2OH produced from NH_4^+ by ammonia mono-oxygenase (Suzuki et $al.$, 1974; Hollocher et $al.$, 1981) is further oxidised to NO_2^- by hydroxylamine oxidoreductase (HAO). Hofman and Lees (1952, 1953) found that the oxidation of NH_2OH by whole cells was inhibited by hydrazine and under these conditions NH_2OH was accumulated. Three two-electron steps for the oxidation process were then postulated (Lees, 1952b; Hofman and Lees, 1953):

$$NH_4^+ \xrightarrow{2e^-} NH_2OH \xrightarrow{2e^-} (NOH) \xrightarrow{2e^-} NO_2^-$$
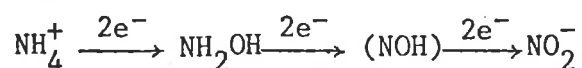
Chromatographic methods were used to identify NH_2OH as an intermediate during the oxidation of NH_4^+ to NO_2^- (Yoshida and Alexander, 1964).

Hydroxylamine oxidation in cell-free extracts was first achieved by Nicholas and Jones (1960). Cell-free extracts oxidised NH_2OH 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_2OH as well as by hydrazine but N_2 gas instead of NO_2^- was produced in the presence of hydrazine. They demonstrated that hydrazine competitively inhibited NH_2OH oxidation to NO_2^-. Based on these and other investigations, Nicholas and Jones (1960) and Falcone et al. (1963) further suggested that the oxidation of NH_2OH 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 hydroxy-lamine 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., 1979b; 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 Nitrosomonas (Hooper et al., 1978). Rees and Nason (1965) reported an unusual absorption maximum at 465nm from the dithionite reduction of extracts of Nitrosomonas. 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_2OH or NH_2NH_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_2O_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 a_1 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 a_1.

Aleem *et al.* (1962) suggested that nitrohydroxylamine is the intermediate formed by initial oxidative condensation between NH_2OH and NO_2^- as follows:

$$NH_2OH \longrightarrow (NOH) + 2(H)$$
$$2(H) + 2 \text{ Cyt. } c \text{ } Fe^{3+} \longrightarrow 2 \text{ cyt. } c \text{ } Fe^{2+} + 2H^+$$
$$(NOH) + HNO_2 \longrightarrow NO_2 \cdot NHOH$$
$$NO_2 \cdot NHOH + \tfrac{1}{2} O_2 \longrightarrow 2HNO_2$$
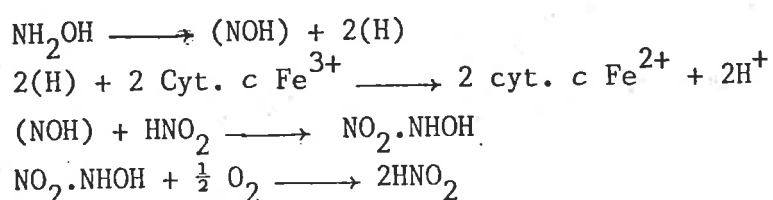
It was then proposed that dehydrogenation of the nitroxyl to NO and N_2O occurs (Falcone *et al.*, 1962, 1963; Anderson, 1964a, 1965a). It was observed that under anaerobic conditions when NH_2OH was added to extracts of *Nitrosomonas* equivalent amounts of both N_2O and NO were formed (Anderson, 1964a, 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 N_2O production (Bremner and Blackmer, 1978; Blackmer *et al.*, 1980) or N_2O 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:

$$\text{Cyt 551. } Fe^{3+} + NO_2^- \longrightarrow \text{Cyt 551. } Fe^{2+} + \text{"}NO_2\text{"}$$
$$\text{Cyt 551. } Fe^{2+} + \text{"}NO_2\text{"} + \tfrac{1}{2} O_2 \longrightarrow \text{Cyt 551.} Fe^{3+} + NO_3^-$$
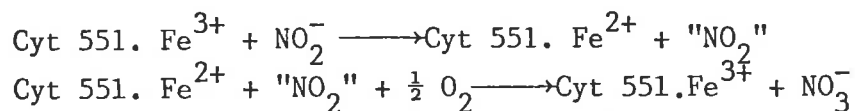
According to the scheme proposed by Lees and Simpson (1957) and Butt and Lees (1958), NO_2^- oxidation is mediated by cytochrome c and NO_3^- is formed as a result of an O insertion from molecular O_2. Aleem and Nason (1959) further characterized NO_2^- oxidase that catalysed the following reaction:

$$NO_2^- \rightarrow \text{cytochrome } c \text{ reductase} \rightarrow \text{cytochrome } c \rightarrow \text{cytochrome } a_1 \rightarrow$$
$$\text{cytochrome oxidase} \rightarrow O_2.$$
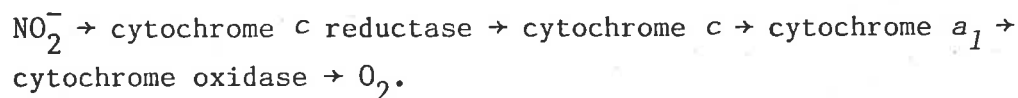
As this scheme involves the reduction of O_2 to H_2O by the electrons supplied by NO_2^-, it was proposed that molecular oxygen could not possibly supply an O atom to NO_2^- for the formation of NO_3^-. Subsequently Aleem *et al.* (1965) provided evidence from ^{18}O isotope studies that H_2O but not O_2 contributed O during NO_2^- oxidation to NO_3^-. They proposed the following mechanism to account for their results:

(i) $\qquad NO_2^- + H_2^{18}O \longrightarrow NO_2^-.H_2^{18}O$

(ii) $\qquad NO_2^-.H_2^{18}O + 2 \text{ cyt } a_1.Fe^{3+} \longrightarrow N^{18}O_3^- + 2 \text{ cyt } a_1.Fe^{2+} + 2H^+$

(iii) $\qquad 2 \text{ cyt } a_1.Fe^{2+} + 2 \text{ cyt } aa_3.Fe^{3+} \longrightarrow 2 \text{ cyt } a_1.Fe^{3+} + 2 \text{ cyt } aa_3.Fe^{2+}$

(iv) $\qquad 2 \text{ cyt } aa_3.Fe^{2+} + 2H^+ + \frac{1}{2}O_2 \longrightarrow 2 \text{ cyt } aa_3.Fe^{3+} + H_2O$

$\qquad$ sum: $\quad NO_2^- + \frac{1}{2}O_2 \longrightarrow NO_3^-$
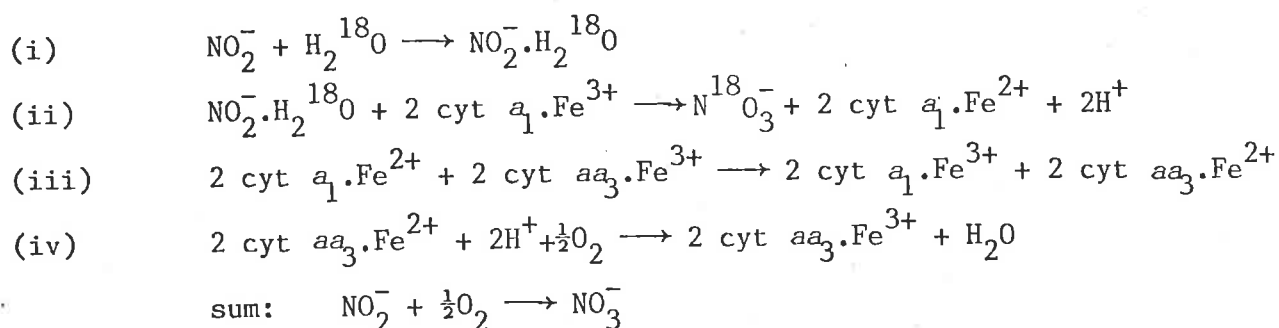
The four steps in the scheme are as follows (Aleem, 1977): (i) hydration of the NO_2^- molecule prior to removal of electrons from NO_2^- and protons from H_2O; (ii) electrons are transferred from hydrated NO_2^- molecule to cytochrome a_1 and NO_3^- is formed in turn as a result of the incorporation of 'O' from H_2O ; (iii) a proton current is generated; (iv) finally electrons and protons are transferred to molecular O via a cyt aa_3 type oxidase.

Aleem (1968) and Sewell and Aleem (1969) established that there is a second NO_2^- oxidising system in *Nitrobacter* which is coupled to the generation of reduced pyridine nucleotides and is highly endergonic:

$$NO_2^- + H_2O + NAD(P)^+ \longrightarrow NO_3^- + NAD(P)H + H^+$$
$$\Delta G^{o\prime} = +35 \text{ kcal.mol}^{-1}$$
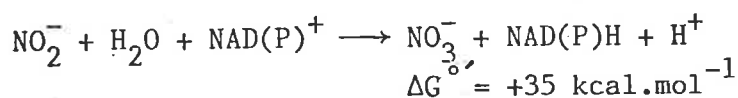
Sewell *et al.* (1972) reported that there are five different types of cytochromes present in *Nitrobacter* : cytochrome c (Em_7 = 274mV), cytochrome a (Em_7 = 240mV), cytochrome a_3 (Em_7 = 400mV) and two a_1 type cytochromes (Em_7 = 352mV and Em_7 = 100mV). Similar results were subsequently reported by Cobley (1973). The oxidation-reduction potentials of cytochromes c, a and a_3 showed no pH dependence but a high potential component of cytochrome 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 NO_2^- / NO_3^- couple in extracts of *Nitrobacter*. The Em became more positive from pH 9.0 (Em = 328mV) to pH 6.8 (Em = 414mV). Because the Em for NO_2^- and of the high potential component of cytochrome a_1 at pH 8.0 (optimum for NO_2^- oxidation) are at 360mV and 327mV respectively, the reduction of high potential a_1 component by NO_2^- poses no thermodynamic barrier. Moreover since the Em of cytochrome c is not affected by pH changes, its reduction by NO_2^- and cytochrome a_1 would be endergonic.

It is well established now that the site of entry of NO_2^- in the electron

transport chain is cytochrome a_1 and the reduction of cytochrome c by 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 aa_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 600nm in the oxidised form and at 443 and 606nm when reduced. Yamanaka et al. (1981) reported that this terminal oxidase contained 1 mole of haem a, 1.6 g-atom of Cu per 41,000g. 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 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 NH_2OH to NO_2^- is an energy yielding

step. The free energy changes during NH_4^+ oxidation are as follows:

		$\Delta G_1^{\circ\prime}$	$\Delta G_2^{\circ\prime}$
(i)	$NH_4^+ + \frac{1}{2}O_2 \longrightarrow NH_2OH + H^+$	−0.7	+ 3.85
(ii)	$NH_2OH + O_2 \longrightarrow NO_2^- + H_2O + H^+$	−83.3	−68.98
	Sum: $NH_4^+ + 1.5\ O_2 \longrightarrow NO_2^- + H_2O + 2H^+$	−84.0	−65.04
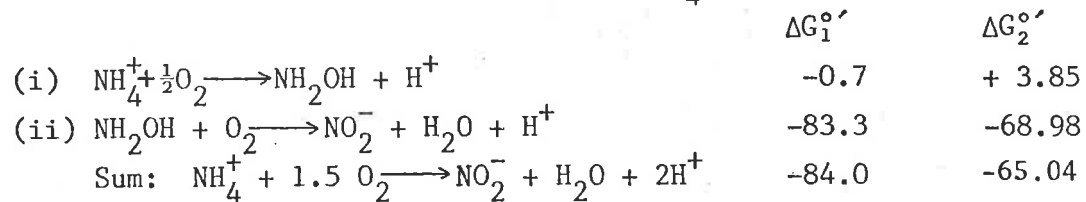
Where $\Delta G_1^{\circ\prime}$ and $\Delta G_2^{\circ\prime}$ denote the free energy values according to Gibbs and Schiff (1961) and Aleem (1970) respectively.

Burge *et al.* (1963) were unable to detect ATP synthesis during NH_2OH oxidation, based on ^{32}P experiments. Ramaiah and Nicholas (1964) however demonstrated phosphorylation in particulate fractions with P/O ratio of around 0.20. Drozd (1976), reported $\rightarrow H^+/O$ ratio of about 2 for either NH_4^+ or NH_2OH 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 re-actions associated with CO_2 fixation in chemolithotrophic bacteria are well established (Wallace and Nicholas, 1969b). The generation of reduced nicotinamide adenine nucleotides coupled to oxidation of NH_4^+ or NH_2OH would be thermodynamically unfavourable due to positive values of $\Delta G^{\circ\prime}$ 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 *Nitrosomonas* and *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:

$$NH_2OH \longrightarrow Cyt\ b \longrightarrow cyt\ c \longrightarrow cyt\ oxidase \longrightarrow O_2$$

ATP —— ——ATP

Flavins

Pyridine nucleotides
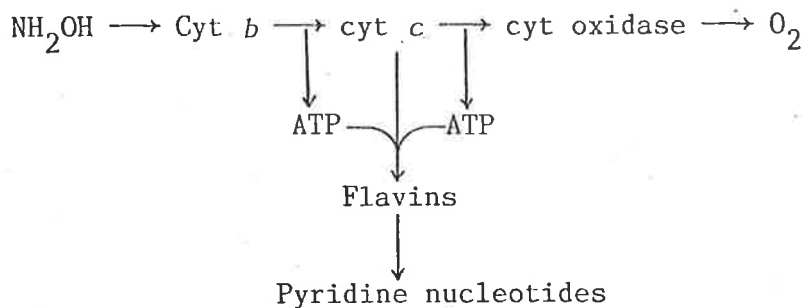
1.1.5.2 *Nitrobacter* species

The oxidation of NO_2^- 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_2^-, 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 bio-synthesis 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 genera-tion 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 (≈ 0.1) were reported for NO_2^- oxidation by membrane particles. The following mechanism of proton translocation was put forward by Cobley (1976b):
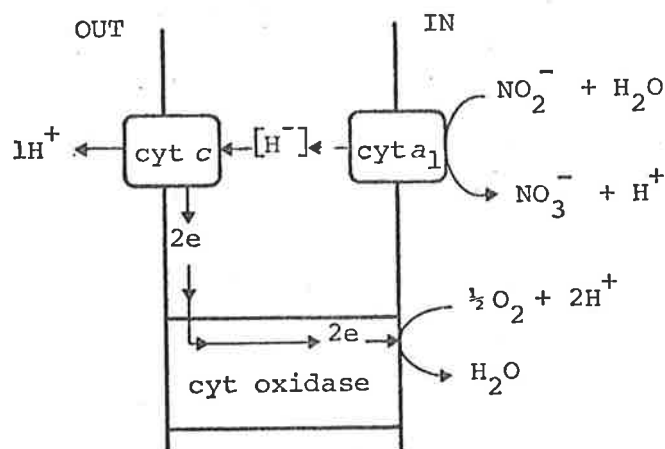
An alternative mechanism proposed by Aleem (1977) predicts the movement of two protons and two net positive charges out of the cell for the flow of two electrons
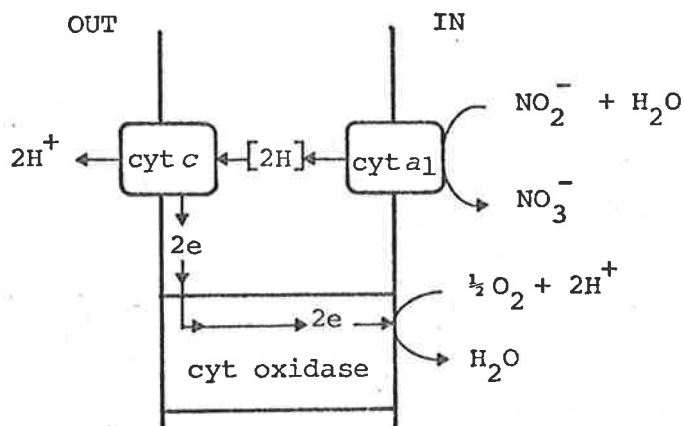
Aleem's scheme (Aleem, 1977) did not account for the stimulation of NO_2^- oxidation by $\Delta\psi$ whereas Cobley's scheme (Cobley, 1976b) predicted an unusual H^- (hydride) transfer. Ferguson (1982) proposed that cytochrome oxidase (aa_3) can alternatively act as a proton pump in *Nitrobacter* to generate proton-motive force:

OUT   IN

cyt c ←—2e←— cyt a_1

$NO_2^- + H_2O$

$NO_3^- + 2H^+$

2e

2e →

cyt oxidase

$\frac{1}{2}O_2 + 2H^+$

H_2O

$2H^+$ $2H^+$
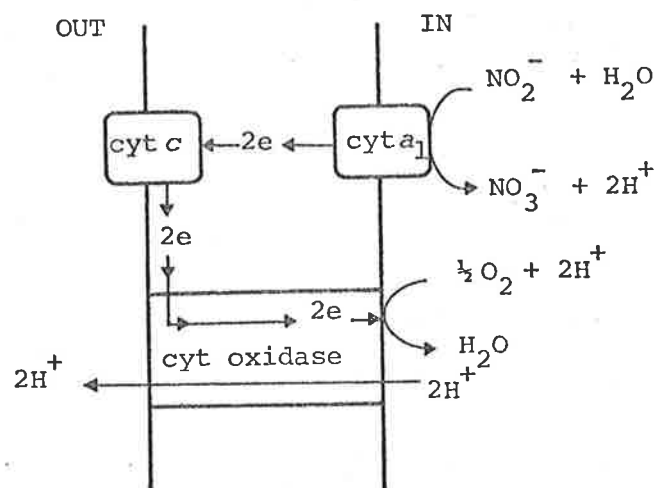
Interestingly, a recent report (Sone *et al.*, 1983) indicates that unlike the cytochrome *c* oxidase of other bacteria (Solioz *et al.*, 1982) and mitochondria (Wikstrom and Krab, 1980) the cytochrome *c* oxidase from *Nitrobacter agilis* lacked the proton-pump activity.

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 NO_2^- and NH_2OH reduction by either bacterium was found to be ammonia (Wallace and Nicholas, 1968). Both *Nitrobacter* and *Nitrosomonas* incorporated $^{15}NH_4^+$, $^{15}NH_2OH$ and $^{15}NO_2^-$ into cell nitrogen and in addition *Nitrobacter* but not *Nitrosomonas* also incorporated $^{15}NO_3^-$ (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_2OH 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 ($^{15}NO_2^-$, $^{15}NO_3^-$, $^{15}NH_2OH$ 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 $\rightarrow H^+/O$ ratio for specific substrate.

(v)   Determination of proton-motive force (Δp) in *Nitrosomonas europaea* and *Nitrobacter agilis* and the role of Δp in mediating ATP biosynthesis.

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

(vii)   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 $P^{18}O_4^{2-}$) 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 tetra-phenyl boron ($NaB(C_6H_5)_4$), triphenyl methyl phosphonium bromide ($TPMP^+Br^-$) dimethyl sulfate, trimethyl phosphate, N-methyl-N-nitroso-p-toluene sulfonamide (for the preparation of diazomethane), α-ketoglutarate, hydroxy-lamine hydrochloride, azaserine, L-methionine-DL-sulphoximine (MSX), 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-TMP) 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 Chemial Co. (Midland, U.S.A.) and Drughouse Ltd., (Adelaide, Australia).

2.1.2 *Stable isotopes*

$^{15}NH_4Cl$ (30 atom % excess), $K^{15}NO_3$ and $Na^{15}NO_2$ (both 32.5 atom % excess) were purchased from L'office National Industriel de `l´ Azote (ONIA), Marseille, France. $^{15}NH_2OH$ ($\simeq$97 atom % excess) and $H_2^{18}O$ (97 atom % excess) were from Merck, Sharpe and Dohme, Montreal, Canada. $H_2^{18}O$ (98.3 atom % excess) was from Prochem, London, England and $^{18}O_2$ (99 atom % excess) from Yeda Research and Development, Israel. $H^{15}NO_3$ (99 atom % excess) was obtained from Isomet

Corp. N.J., U.S.A. $K^{15}NO_3$ (99 atom % excess) was prepared by neutralizing $H^{15}NO_3$ with KOH. $K^{15}NO_2$ (99 atom % excess) was prepared through reduction of $K^{15}NO_3$ (99 atom % excess) by lead (Pb) at $420°C$ in silica crucible (Jolly, 1964). The $K^{15}NO_2$ so prepared was dissolved in distilled water, filtered and dried. The stock solutions of $K^{15}NO_2$ contained 10 and 30% $K^{15}NO_3$ and rest $K^{15}NO_2$ in two separate preparations used for GC/MS and NMR studies respectively. $H_3P^{18}O_4$ was prepared by the reaction of excess $H_2^{18}O$ (97 atom excess) (0.5ml) with PCl_5 (1.3g) : $PCl_5 + 4H_2^{18}O \longrightarrow H_3P^{18}O_4 + 5HCl$.   Hydrochloric acid and water were evaporated from the reaction mixture in a $100°C$ 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 [^3H] tetraphenyl phosphonium bromide (TPP^+Br^-) (23.7 $Ci.mmol^{-1}$), acetyl [carboxyl-^{14}C] salicylic acid (20m$Ci.mmol^{-1}$), and [^{14}C] inulin (5.6 $Ci.mmol^{-1}$) were from Amersham, Bucks, England. ^3H_2O (1$Ci.mol^{-1}$), [7-^{14}C], benzoic acid (22.6 m$Ci.mmol^{-1}$), [^{14}C] methylamine hydrochloride (51.8 m$Ci.mmol^{-1}$), [^{14}C] sucrose (1 m$Ci.mmol^{-1}$) and $^{22}NaCl$ (293.4 m$Ci.mg^{-1}$) were purchased from New England Nuclear (NEN), Boston, U.S.A.

2.1.4 *Solutions, buffers and solvents*

Unless stated otherwise, the aquous 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. Luois, U.S.A.

2.1.6 *Enzymes and marker proteins*

Phospholipase A_2, 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.1^{-1}): $(NH_4)_2SO_4$,4; KH_2PO_4,0.5; $CaCl_2.2H_2O$,0.004; $MgSO_4.7H_2O$,0.05; chelated iron (methyldiamine bis-orthohydroxy-phenylacetic acid), 0.0001 and $CuSO_4$, 0.00002. The pH of the cultures were continuously adjusted to 7.8 with sterile 20% (w/v) K_2CO_3 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.1^{-1}): $NaNO_2$,1; KH_2PO_4,1; NaCl,0.3; $MgSO_4.7H_2O$,0.5; $MnSO_4$,0.002 and $Fe_2(SO_4)_3$,0.005. After sterilization the pH of the medium was adjusted to 7.5 with sterile 20% (w/v) K_2CO_3.

Unless stated otherwise, the media were inoculated with 10% (v/v) of an exponantially 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 $12\ell.h^{-1}$.   Cells were washed several times with 50mM Tris-HCl buffer (pH 7.5 to 7.8) and either used immediately or stored at $4^{o}C$.

2.2.2 *Preparation of spheroplasts and membrane vesicles*

The spheroplasts and membrane vesicles of *Nitrobacter agilis* were pre-pared as follows: 1g wet weight of cells suspended in 50ml of 50mM Tris-HCl buffer (pH 8.0) containing 0.2M sucrose, 1mM Na_2EDTA and 80mg lysozyme in a 100ml Erlenmeyer flask were incubated at $30^{o}C$ in a waterbath shaker (60 rev. min^{-1}) for 2h.   Then 2 µg DNAse and 2ml of 2mM Mg-acetate were added and in-cubation continued for 1h.   A pellet of spheroplasts was then obtained by centrifuging the suspension at 8000g for 15 min at $4^{o}C$.   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 spheroplats (1g wet weight) for 15 min in 25ml 50mM Tris-HCl buffer containing 10mM Na_2EDTA, 10mM $MgSO_4$ and 100µg 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 $4^{o}C$ in an icebath.   After centrifuging at 5000g for 20 min to re-move intact spheroplasts the supernatant fraction was futher 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 Na_2EDTA and finally suspended in the same buffer.   Both spheroplasts and membrane vesicles were stored at $2^{o}C$.

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 $4^{o}C$.   Unless stated otherwise, the sonicated suspensions were centrifuged at 30,000g for 20 min at $4^{o}C$ and the supernatant (S_{30}) used for study.

2.2.4 *Incorporation of ^{15}N labelled compounds into cell nitrogen*

The incubation mixture contained: washed cell suspension (25mg protein), ^{15}N-labelled substrate (either $Na^{15}NO_2$, $K^{15}NO_3$, $^{15}NH_2OH.HCl$ or $^{15}NH_4Cl$, 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 $30^{\circ}C$. The reaction was terminated by adding 30ml chilled water and the contents were immediately centrifuged at 30,000g for 10 min at $4^{\circ}C$. 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_2SO_4 and 2g of the digestion mixture (7gHgO + 93g Na_2SO_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_2SO_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_5) 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 $50^{\circ}C$ 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 50ml h^{-1}) until the absorbance (A_{280}) was close to zero.   Glutamine synthetase was eluted from the column with 2mM 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 70cm) pre-equilibrated with the buffer.   The enzyme was eluted with the same buffer (flow rate 12ml.h^{-1}) and the active fractions pooled and concentrated as before.

In *Nitrosomonas europaea*, the S_{30} was heat-treated at $65^{o}C$ 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 1M acetic acid, was allowed to stand on ice for 30 min and then centrifuged at 30,000g for 30 min.   The pellet was discarded and the pH of the supernatant fraction was adjusted to 4.2 with 1M acetic acid.   After standing on ice for 20 min, it was centrifuged at 30,000g for 30 min and the pellet homogenised in 10ml of 10mM Tris-HCl, 1mM $MnCl_2$ buffer (pH 7.2).   To this suspension was added half volume of 20% (w/v) polyethylene glycol (PEG) in 50mM Tris-HCl buffer (pH 7.5), drop by drop with constant stirring and after standing on ice for 15 min it was centrifuged at 30,000g for 15 min.   The pellet resuspended in 4M NaCl in 50mM Tris-HCl buffer (pH 7.5) was added drop by drop with constant stirring to an equal volume of 20% (w/v) PEG in 50mM Tris-HCl buffer (pH 7.5) and after standing on ice for 20 min it was centrifuged at 30,000g for 20 min. Most of the glutamine synthetase was recovered in the supernatant fraction which was dialysed overnight against 10mM Tris-HCl, 1mM $MnCl_2$ 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 50mM Tris-HCl, 1mM β mercaptoethanol, pH 7.5) S_{30}, was centrifuged at

at 110,000 x g for 1h 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 con-
taining enzyme activity were pooled and dialysed overnight against the
same buffer.   All purified enzymes were stored at -15°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°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 γ glutamyl transferase and biosynthetic activities of
glutamine synthetase were determined by the method of Shapiro and
Stadtman (1970à). 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; $MnCl_2.4H_2O$,
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_4Cl,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°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; NH_4Cl,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 ($\approx$2,000,000) was used to determine the void volume. The distribution coefficient (Kd) was calculated by the formula,

$$Kd = \frac{Ve}{Vo}$$

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):

$$A + \frac{B}{100} \quad (100-A)$$

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, nondenaturing 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 $FeCl_3$ reagent (0.4g $FeCl_3$, 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 $37^{\circ}C$ in a reaction mixture containing: 40ml 0.1M Tris-HCl buffer (pH 8.25), 3ml 1M sodium glutamate, 1ml 0.0065M phenzine methosulfate, 2ml 0.0057M nitroblue 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 NH_4^+, NH_2OH and $N_2H_5^+$ by *Nitrosomonas europaea* and NO_2^- 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 O_2 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) $NH_4^+ + 3O \longrightarrow NO_2^- + H_2O + 2H^+$

(ii) $NH_2OH + 2O \longrightarrow NO_2^- + H_2O + H^+$

(iii) $N_2H_5^+ + 2O \longrightarrow N_2 + 2H_2O + H^+$
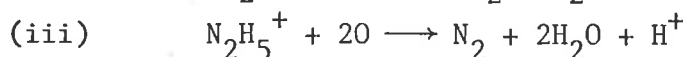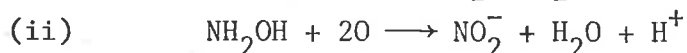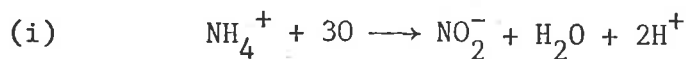
and for *Nitrobacter agilis* is (Nicholas, 1963; Aleem, 1965):

(iv) $NO_2^- + O \longrightarrow NO_3^-$
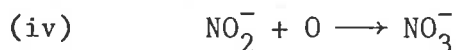
Unless stated otherwise, for electrode measurements, the buffer was 50mM Tris-HCl buffer (pH 7.5).

2.2.12 *Proton translocation*

2.2.12.1 Fluorescence quenching method

The fluoresence emission was followed as described by Tuovinen

et al. (1977). Measurements were made in a 1cm 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 O_2 (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 equili-
brate under N_2 for 10 min at $25^{\circ}C$, 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 O_2 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 O_2 by bacteria involved a rapid acidifica-
tion of the medium, followed immediately thereafter by a slow ($t\frac{1}{2}$ =
approximately 1 min) passive diffusion of protons back across the cell
membrane. The latter process tended to diminish the amplitude of the
initial rapid pH transient by 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 extra-
polated back to a time, approximately 1 to 2s after O_2 injection, at
which the initial transient had reached half its final amplitude, as
suggested by Scholes and Mitchell (1970b).

The solubility of O_2 in 0.15M KCl was taken as 2.32mg atom $O.1^{-1}$
at latm. 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 con-
ditions 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 NH_4^+, NH_2OH or $N_2H_5^+$ in the case of *Nitrosomonas
europaea* were injected into a system under pure O_2. Because the initial
concentration of reductant was typically well below its K_m value (eg.
30 nmol NH_4^+ in 1.5ml = 20μM; K_m for NH_4^+ at pH 7.4 is approximately
1.0mM) respiration rates were relatively slow. The response, especially
in the absence of a permeant ion, was typically a progressive acidifi-
cation 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 $\rightarrow H^+/O$ ratio in oxygen pulse studies for the protons trans-
located by the proton pump it was necessary to determine the yield of
stoichiometric protons and substract 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 3-5μM 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 5μM was in-
sufficient 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 observes (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 ClO_4^-
was used, it was added as $NaClO_4$ at a final concentration of 0.3M to a
solution already 0.15M in K^+. Inasmuch as $KClO_4$ is relatively in-
soluble ($K_{sol} \simeq 10^{-2}$ at 25°C) much of the K^+ and some of the ClO_4^- were
precipitated to give actual concentrations of K^+, Na^+ and 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 (ΔpH) 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 *Nitro-
bacter agilis*) for 10 min at 37°C. The cells collected by centrifuga-
tion 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 ^3H_2O (for total pellet water), [^{14}C]
sucrose (for total pellet water - intracellular water, which does not
include periplasmic space) and [^{14}C] inulin (for extra cellular 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 ± 0.2 and 1.2 ± 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 $25^{o}C$ 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_2O_2 (1μl ml^{-1}).
For *Nitrosomonas*, 5mM NH_4Cl and for *Nitrobacter* 5mM $NaNO_2$ was the sub-
strate. 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 ΔpH were measured. For $\Delta\psi$
determination $[^3H]$ TPP^{+} Br^{-}(20-50 $nCi.ml^{-1}$) was added to a cell suspen-
sion (1-1.5mg dry weight. ml^{-1}). For ΔpH determination $[^{14}C]$ benzoic
acid (2 $\mu Ci.ml^{-1}$), $[^{14}C]$ acetyl salicylic acid (2 $\mu Ci.ml^{-1}$) or $[^{14}C]$
methylamine-hydrochloride (1 $\mu Ci.ml^{-1}$) was added. ^3H_2O was used to
determine total pellet water.

2.2.13.4 Calculations of proton motive force (Δp or $\tilde{\mu}_H{+}$)

The calculations of $\Delta\psi$ and ΔpH 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:

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

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

At $\quad 27°C$ (T = 300°K) 2.303 RT/F $\simeq$ 60mV

$\therefore \quad \Delta\psi = 60 \times \log \frac{[TPP^+]in}{[TPP^+]out}$

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

Intracellular pH (pHi) was calculated from the distribution of ^{14}C weak acids (benzoic and acetyl salicylic);

pHi = pK + log [Ain/Aout (10$^{(pHe-pK)}$+ 1)-1]

or by the distribution of weak base ^{14}C-methylamine;

pHi = pK - log [Bin/Bout (10$^{(pK-pHe)}$+ 1)-1]

ΔpH was obtained from the difference of pHi and pHe (ΔpH = pHi - pHe). Proton-motive force (Δp) was calculated as:

$$\Delta p = \Delta\psi - 2.303 \frac{RT}{F} \Delta pH \text{ or at } 27°C \text{ } \Delta p = \Delta\psi - 60 \text{ } \Delta pH$$

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 ($\simeq$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 0.22μ or 0.45μ filters (Type GS or HWAP), 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 ^{15}N and ^{18}O labelled compounds to study NO_2^- oxidation by cells of Nitrobacter agilis*

The aim of the experiments was to determine the ^{18}O contents of NO_3^- produced during the aerobic oxidation of NO_2^- at pH 7.8 by *Nitrobacter agilis* in the presence of $^{18}O_2$, $H_2^{18}O$ or [^{18}O] phosphate. Two techniques were used for isotopic analysis namely gas chromatography combined with mass spectrometry (GC/MS) and ^{15}N-nuclear magnetic resonance (NMR) spectroscopy. The two sets of studies were carried out independently.

2.2.15.1 GC/MS studies

For experiments involving $^{18}O_2$ or $H_2^{18}O$ washed cells were suspended in either 0.1M Tris-HCl (pH 7.8) or 0.1M K-phosphate (pH 7.8). For experiments involving [^{18}O] phosphate, cells were incubated in 0.1M [^{18}O] 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 1atm 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 $NaNO_2$ or $K^{15}NO_2$. 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 NO_2^- (or $^{15}NO_2^-$). All incubations were at 28^oC in a waterbath shaker (120 rev.min^{-1}). After utilization of the final addition of nitrite, each reaction mixture was chilled to 0^oC 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 NH_4OH and $MgCl_2$ in order to precipitate $MgNH_4PO_4$, which was removed by centrifugation. This step was omitted if Tris-HCl was the buffer. The phosphate depleted super-natant 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 NH_4OH 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 CH_2N_2 (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 H_3PO_4 and $H_3P^{18}O_4$ to yield trimethylphosphate ($(CH_3)_3PO_4$ or $(CH_3)_3P^{18}O_4$). Methylation of HNO_3 was in the same manner.

but it was found necessary to add an approximately equal volume of concentrated H_2SO_4 to the HNO_3. This procedure generated a mixture of dimethyl sulfate and methyl nitrate which could be separated by GC/MS. Ether and excess CH_2N_2 were removed from the methylation mixtures by evaporation at 45^oC. The methylated products were dissolved in CH_2Cl_2 for GC/MS analysis.

The yields of methyl nitrate produced by CH_2N_2 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 N_2O had the following retention times respectively: 3.6 min at 160^oC, 1.8 min at 160^oC, 4.8 min at 50^oC and 0.3 min at 25^oC. The degree of ionization-induced fragmentation of N_2O^+ into NO^+ and N_2^+ was similar to those reported previously, (Cady and Bartholomew, 1960; St. John and Hollocher, 1977). Trimethyl phosphate and dimethyl sulfate were dissolved in CH_2Cl_2 (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 CH_2Cl_2 on the column. The M^+ peak for CH_3NO_3 (77 amu) was generally not observed; identification was based on the base (integral = 100) peak (NO_2^+), NO^+ and a weak $(M-1)^+$ peak at 76 amu.

2.2.15.2 ^{15}N-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: (i) 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) 1 ml 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_2O_2 (5μl) (ecept for experiment ii) followed by incubation at $28^\circ C$ 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 $4^\circ C$ 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 ^1H 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
60cm) near the cathode. The paper was moistened with 100mM 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 100mM Na-tetraoxalate (pH 4.2). The
perclean 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, (100mA
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 aquous 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 $2^{o}C$ 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 1ml with double distilled water. The red azodye was developed by adding 1ml of 1% (w/v) sulphanilamide in 1N-HCl, followed by 1ml of 0.01% (w/v) N-(1-naphthyl) ethylene diamine hydrochloride. After 15 min, the absorbance was read at 540nm in 1cm 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 NH_4^+ was diluted to 0.5ml with distilled water. Color was developed by adding 1ml of reagent A (10% w/v, sodium potassium tartarate) and 2ml of reagent B (2.272g mercuric iodide, 1.826g KI and 4g NaOH in 100ml distilled water). After 20 min the absorbance was read at 435nm in 1cm glass cuvettes employing a Shimadzu (QV-50) spectrophotometer. The concentration of NH_4^+ 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 1cm 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 2ml with distilled water and 0.5ml of acid molybdate (2.5% w/v ammonium molybdate in 2.5M H_2SO_4) was then added followed by 0.1ml of colour reagent. The colour reagent was prepared by mixing 1g 1-amino 2-naphthol 4-sulphonic acid, 3g anhydrous sodium sulphite and 6g sodium metabisulphite and stored at $4^{\circ}C$ in the dark. The colour reagent was prepared fresh before use by dissolving 0.25g of the mixture in 10ml 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 (0.4ml) were dispensed from the reaction mixture into 0.1ml 3M perchloric acid in a test tube (1 x 5cm) kept in ice. After 15 min, 0.3ml 1M KOH was added and then 20µl of this neutralized extract was assayed for ATP. The reaction mixture in a scintillation vial (15 x 45mm) contained 1ml 10mM sodium phosphate (pH 7.5), 0.9ml distilled water, 0.1ml 5mM $MgCl_2$ and 50µl 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 (20µl) 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 NO_3^- production and O_2 uptake*

A technique has been developed to measure O_2 uptake and NO_3^- production simultaneously and continuously during 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 O_2 electrode (Department of Biochemistry, University of Bristol, U.K.), a NO_3^- sensitive electrode (Orion model 93-07-01) and a reference electrode (Orion model 90-02). The NO_3^- and reference electrods 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 recorder connected to the pH meter through a Unicam SP45 concentration readout unit to convert the log response of the 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 NO_3^- electrode was calibrated before each experiment using a standard 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 NO_2^-, after NO_2^- additions.

3.1.2 *Stoichiometry of NO_2^- oxidation by washed cells*

The addition of 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 O_2 and extrusion of NO_3^- (Fig.2). Nitrite was determined in aliquots of the reaction mixture by the method of Nicholas and Nason (1957). The stoichiometry of NO_2^- oxidation by washed cells was $1NO_2^- : 0.5O_2 : 0.75NO_3^-$ (Fig.3). The maximum rates of NO_2^- and O_2 uptake and NO_3^- production were approximately 0.3, 0.15 and 0.26 μmol min^{-1}. (mg protein)$^{-1}$ respectively and varied from one batch of cells to another. The uptake of NO_2^- and production of NO_3^- were dependent on O_2 and when all the O_2 from medium was consumed,

FIG.1: ELECTRODE ASSEMBLY FOR MEASURING NO_3^- PRODUCTION AND O_2 UPTAKE

A. 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.1
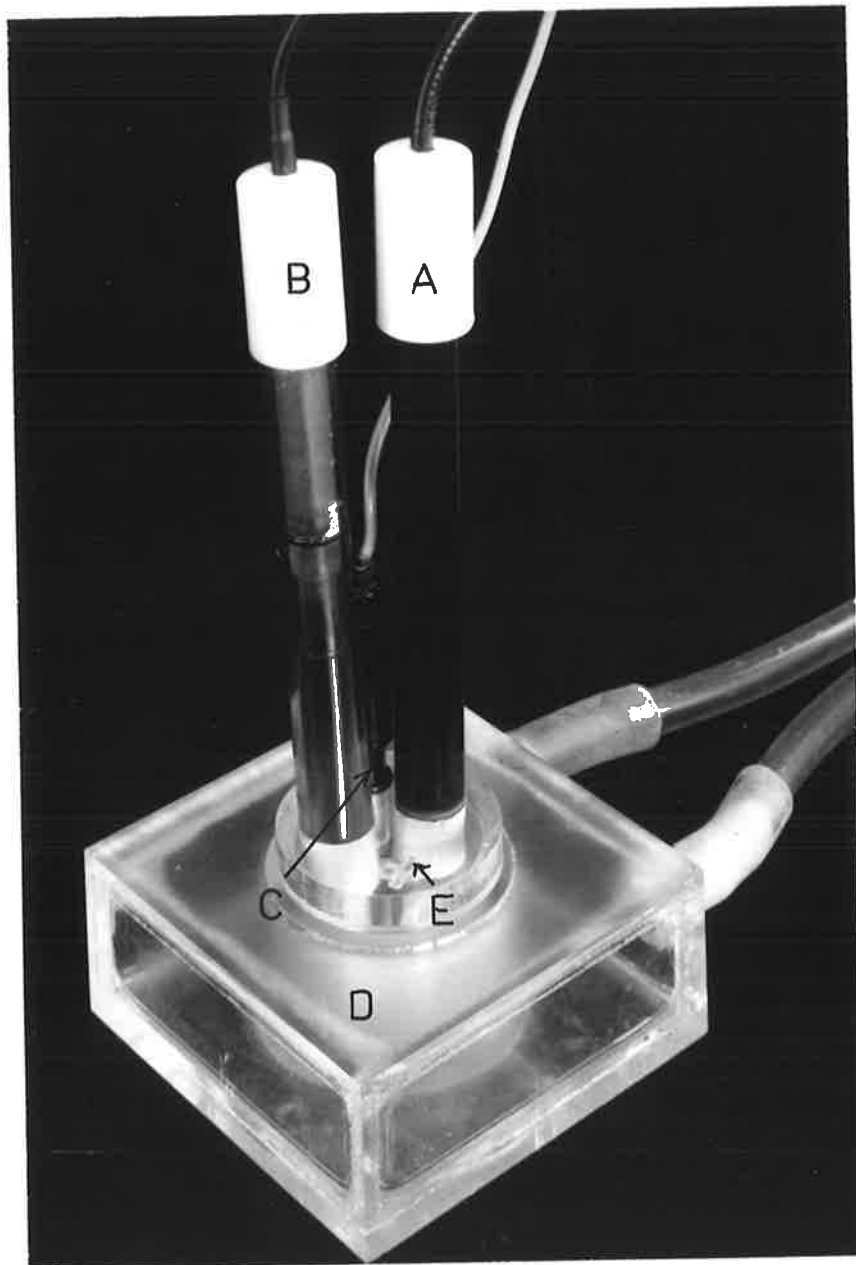
FIG.2: UPTAKE OF O_2 AND PRODUCTION OF NO_3^- BY WASHED CELLS

100μl of washed cell suspension (40 mg wet weight) was added to a perspex vessel containing 5μl catalase (2mg. ml^{-1}) and 0.25 m mol Tris-HCl buffer (pH 8.0) in a final volume of 5ml. The reaction mixture was maintained at 25°C. Reaction was started by adding 10μmol of NO_2^- *via* a Hamilton microsyringe through a port in the lid of the vessel. O_2 was regenerated by injecting $\simeq$10μl, 2% (v/v) H_2O_2 into the reaction mixture. The reaction mixture was continuously stirred with a magnetic flea$_1$. The maximum rates alongside the traces are in μmol. 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 uprake (□).

FIG. 2

FIG. 3
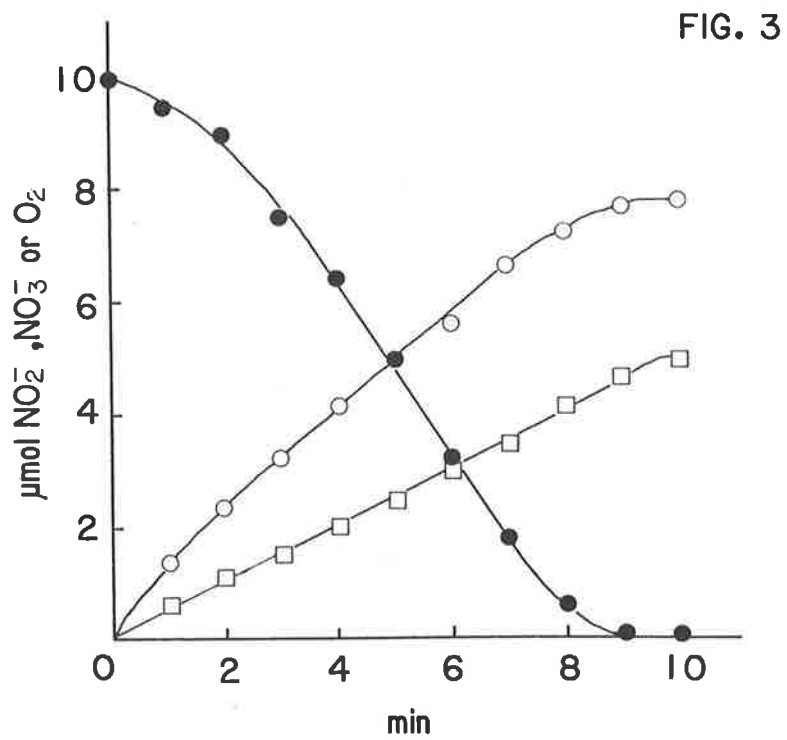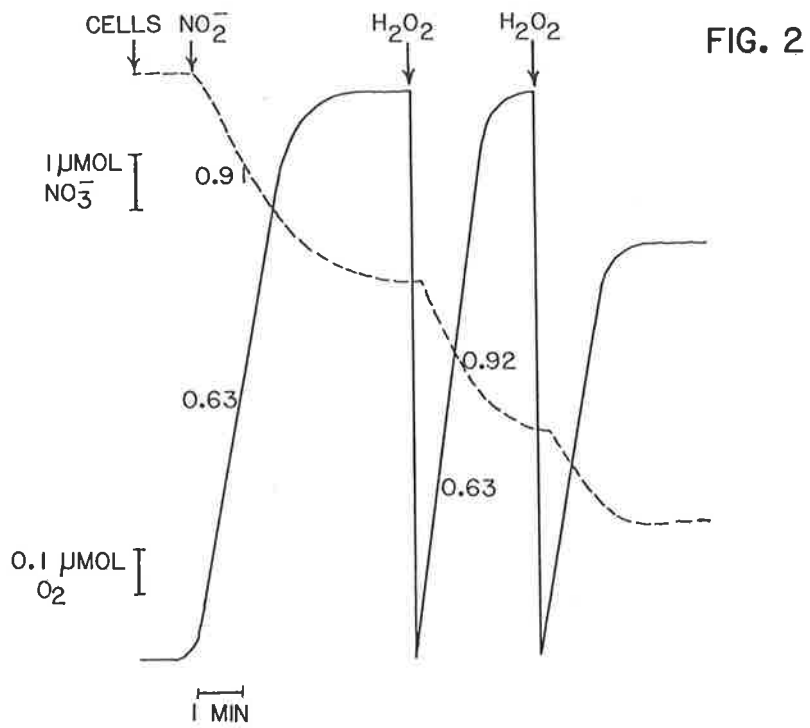
both NO_2^- uptake and NO_3^- production ceased. Thus oxygen was regenerated in these experiments by means of catalase and H_2O_2.

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 sphero-plasts 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 vasicularization 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_2^- oxidation by spheroplasts and membrane vesicles*

Spheroplasts and membrane vesicles prepared as described in Section 2.2.2 were tested for NO_2^- oxidising activity by the electrode method. Both preparations oxidised NO_2^- at about 1/8th of the rate of washed cells. Stoichiometry of $1NO_2^- : 0.5O_2 : 1NO_3^-$ was recorded for both spheroplasts and membrane vesicles (Fig.6a and 6b respectively).

3.1.5 *Optimum conditions for NO_2^- oxidation by membrane vesicles*

The optimum pH for NO_2^- oxidation by membrane vesicles was 7.5 and the oxidation rate decreased rapidly above this pH. The optimum temperature was $23^{\circ}C$. The K_m values of 0.8mM and 20μM were obtained for NO_2^- and O_2 respectively. Additions of any of the metal salts listed in Table 1, except for Ni^{2+} and Cu^{2+}, did not affect NO_2^- oxidation while Ni^{2+} and Cu^{2+} inhibited NO_2^- uptake by about 25 and 20% respectively at 1mM final concentration.

3.1.6 *Effects of metabolic inhibitors on NO_2^- oxidation*

It is known that NO_2^- 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. 4
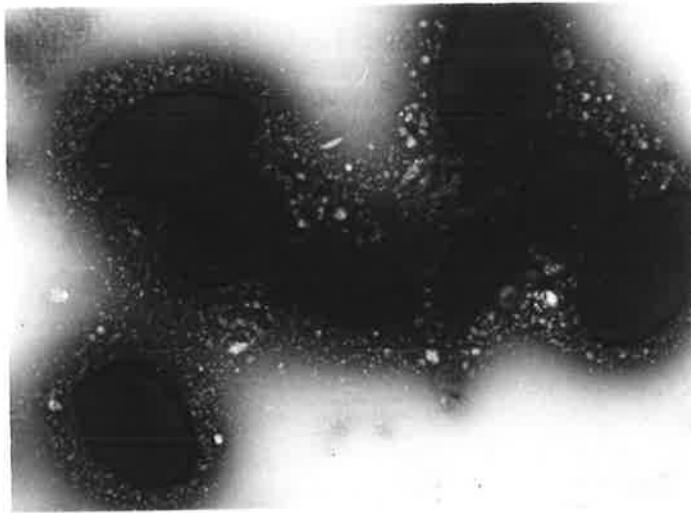
A
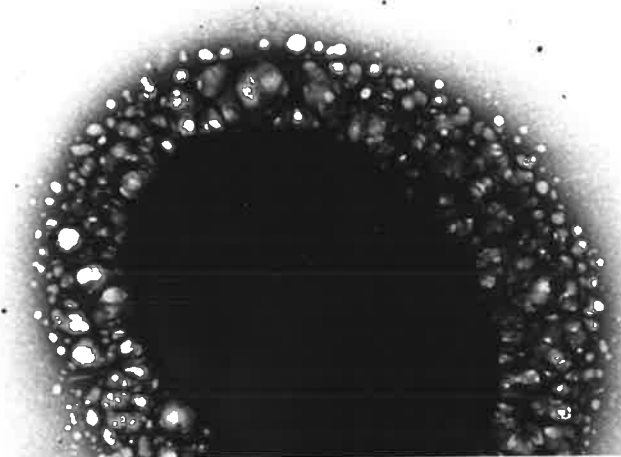
B
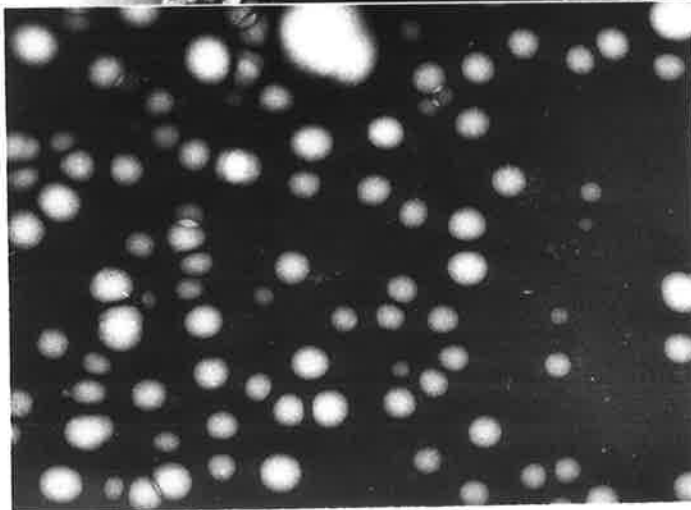
C

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
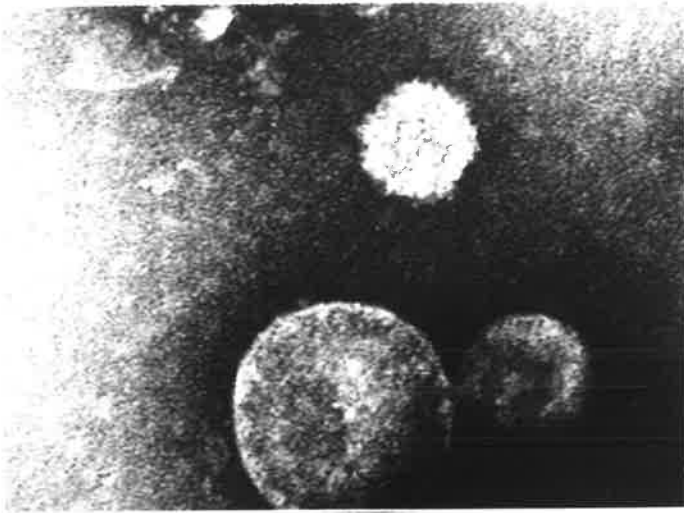
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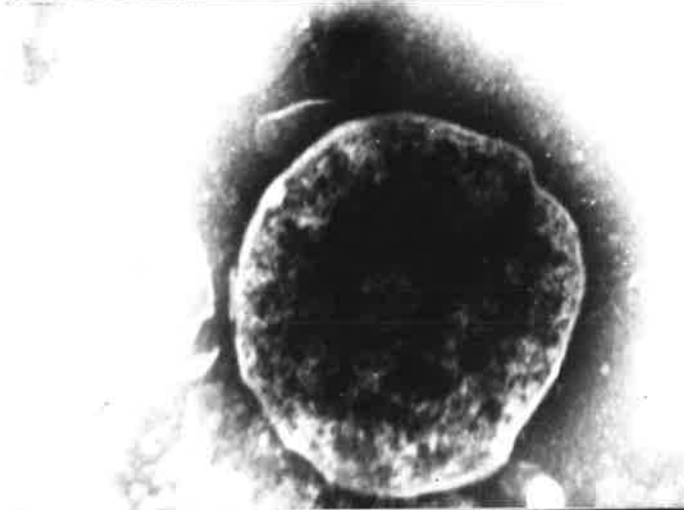
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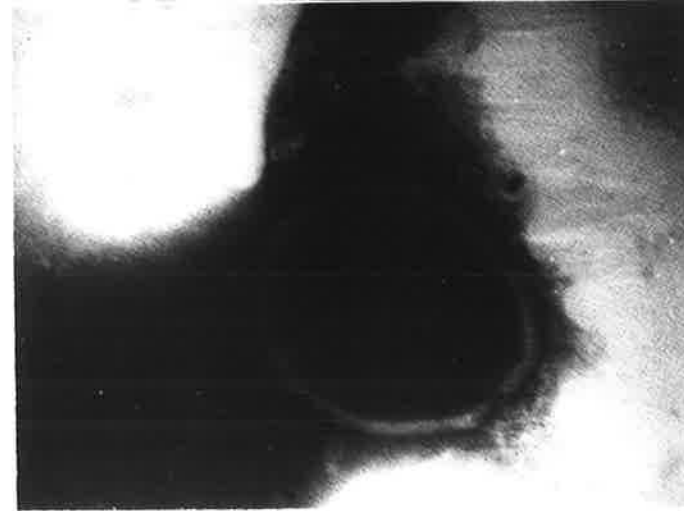
C

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 (□).

FIG. 6a
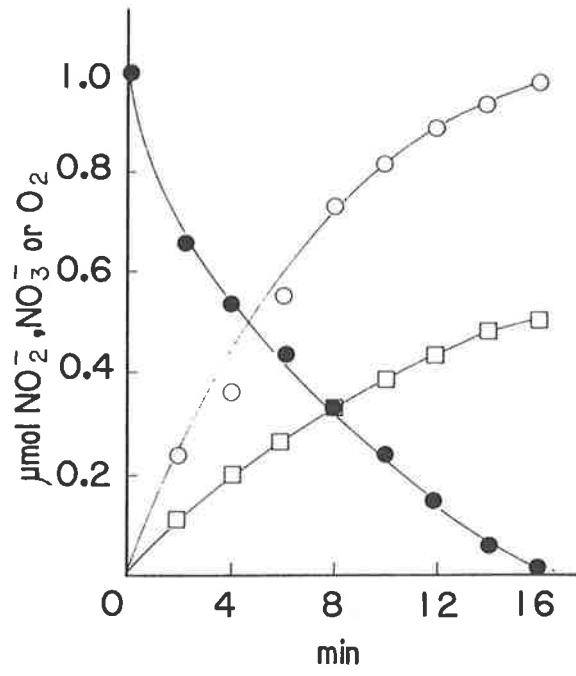
FIG. 6b
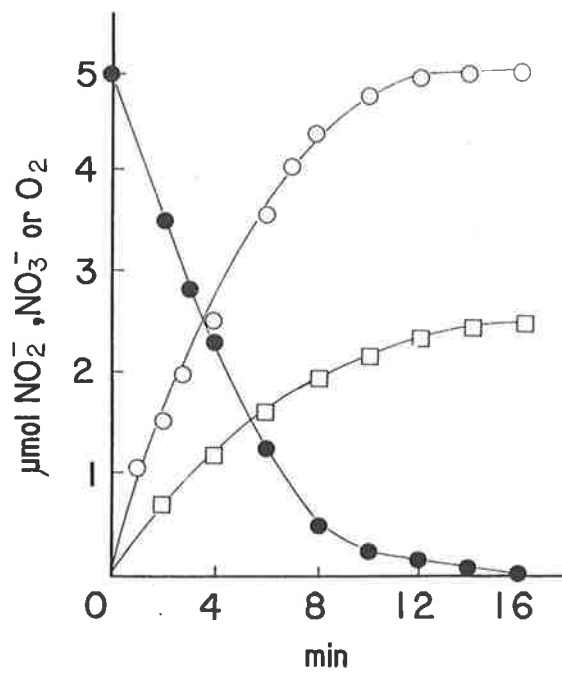
TABLE 1: EFFECTS OF VARIOUS METALLIC IONS ON O_2 DEPENDENT NO_2^- UPTAKE AND NO_3^- PRODUCTION BY MEMBRANE VESICLES.

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

Metal ion	% Inhibition		
	NO_2^- uptake	NO_3^- production	O_2 uptake
Fe^{2+}	0	3	Nd
Fe^{3+}	2	6	Nd
Co^{2+}	0	0	0
Zn^{2+}	0	0	0
Mn^{2+}	3	5	6
Mg^{2+}	0	0	0
Ni^{2+}	27	25	22
Cu^{2+}	17	19	17
Ca^{2+}	0	0	2

Nd – Not determined because of interference with determination.

some of these inhibitors on the utilization of both NO_2^- and O_2 and the production of NO_3^- by washed cells, spheroplasts and membrane vesicles of *Nitrobacter agilis* were investigated. The effects of some metal inhibitors on NO_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_2^- and O_2 uptake and NO_3^- production by washed cells (Table 2). DIECA which inhibits NH_4^+ oxidation completely at very low concentrations ($13\mu M$) in *Nitrosomonas europaea* (Bhandari and Nicholas, 1979a) restricted NO_2^- and O_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^{2+}. Sodium azide at $40\mu M$ inhibited NO_3^- production and O_2 uptake completely in washed cells. The overall pattern of inhibition of NO_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 phosphory-lation (uncouplers) and ATPase on NO_2^- and O_2 uptake and NO_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_2^- oxidising systems but again the extent varied. The inhibitors of electron transport *viz*. rotenone, amytal and HOQNO, all inhibited NO_2^- and O_2 uptake and NO_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_2^- and O_2 utilization and NO_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_2^- and O_2 uptake and NO_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_2^- AND O_2 AND PRODUCTION OF NO_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 $NaNO_2$. The following procedure was used for each compound: 1-25μl of inhibitor was injected into the reaction vessel which was magnetically stirred and after 5 min, 2μmol $NaNO_2$ was added to start the reaction. In the absence of inhibitor the uptake values for NO_2^- and O_2 were 0.41 and 0.20μmol min^{-1} respectively and the extrusion rate of NO_3^- was 0.35μmol min^{-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.

% Inhibition

	KSCN (mM)		Thiourea (mM)		2TMP (mM)		8-hydroxy quinoline (mM)	DIECA (μM)		Azide (μM)		Toluene Dithiol (μM)
	0.1	0.5	0.1	0.5	0.1	0.5	0.1	20	200	40	200	40
NO_2^- uptake	0	27	42	50	21	50	47	12	33	Nd	Nd	40
NO_3^- production	0	27	50	59	23	49	Nd	17	Nd	100	100	47
O_2 uptake	0	10	40	46	17	38	52	10	30	84	100	34

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.

% Inhibition

	2TMP (mM)		8-Hydroxy quinoline (mM)		DIECA (µM)		Toluene dithiol (µM)		CO (15 lb for 15 min)
	0.1	1.0	0.1	1.0	20	200	50	100	
NO_2^- uptake	49	100	45	50	15	35	52	60	37
NO_3^- production	50	100	Nd	Nd	17	Nd	56	70	42
O_2 uptake	56	100	50	56	15	35	53	68	39

Nd — Not determined because of interference with electrode.

TABLE 4: EFFECTS OF METAL INHIBITORS ON THE UPTAKE OF NO_2^- AND O_2 AND EXTRUSION OF 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.

% Inhibition

	2TMP (mM)		8-hydroxy quinoline (mM)	Sodium azide (mM)	Toluene dithiol (mM)
	0.5	1.0	0.5	0.1	0.05
NO_2^- uptake	52	65	75	57	33
NO_3^- production	50	62	Nd	55	40
O_2 uptake	55	65	75	60	40

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.

% Inhibition

	a Rotenone (μM)		b Amytal (μM)		c HOQNO (μM)		d CCCP (μM)		e 2,4-DNP (μM)		f 2,4-DBP (μM)		g NBD Chloride (μg)		h DCCD (μM)	
	20	60	6	15	28	54	2	5	50	100	50	100	50	100	10	50
NO_2^- uptake	7	54	15	35	25	60	23	58	27	63	34	42	39	39	31	69
NO_3^- production	2	60	12	39	28	65	22	56	30	66	36	45	33	36	34	65
O_2 uptake	22	30	12	26	22	30	25	70	20	36	23	48	46	68	32	72

TABLE 6: EFFECTS OF INHIBITORS OF ELECTRON TRANSPORT (a-c) OXIDATIVE PHOSPHORYLATION (d-f) AND ATPase (g,h) ON THE UPTAKE OF NO_2^- AND O_2 AND THE PRODUCTION OF NO_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

	a Amytal (µM)		b HOQNO (µM)		c KCN (µM)			d PCP (µM)		e CCCP (µM)		f 2,4-DNP (µM)		g NBD Chloride (µg)		h DCCD (µM)	
	10	20	20	50	50	150	200	20	40	5	10	50	100	50	100	50	100
NO_2^- uptake	10	38	16	51	2	60	100	25	75	62	71	26	42	43	50	58	74
NO_3^- production	10	39	17	56	2	Nd	Nd	26	76	62	70	30	46	46	52	60	76
O_2 uptake	0	22	11	42	12	63	100	20	72	68	73	30	45	53	72	69	78

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_2^- AND O_2 AND THE PRODUCTION OF NO_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.

% Inhibition

	a Rotenone (mM)		b Amytal (µM)	c HOQNO (mM)	d CCCP (µM)		e 2,4-DBP (µM)		f 2,4-DNP (µM)		g PCP (µM)		h NBD Chloride (µg)	i DCCD (µM)	
	0.5	1.0	50	1.0	10	20	50	100	50	100	50	100	100	10	20
NO_2^- uptake	24	36	10	5	62	76	85	100	27	43	51	85	80	41	46
NO_3^- production	25	34	15	10	60	76	82	100	30	40	50	85	80	40	45
O_2 uptake	20	30	10	7	65	75	80	100	31	42	52	87	79	42	47

Sulfhydryl group inhibitors, NEM and pCMB which did not restrict NO_2^- and O_2 uptake and NO_3^- 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 -SH groups for O_2 dependent NO_2^- utilization.

3.1.7 *ATPase activity in membrane vesicles*

A Mg^{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^{-1}. $(mg protein)^{-1}$. Since the ATP hydrolysing F_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_2 on nitrite oxidase and ATPase activities in membrane vesicles*

Phosphalipase A_2 results in delipidation of membranes by breaking phospholipids into lysophosphatides and fatty acids:

$$\text{membranes (phospholipids)} \xrightarrow{PL-A_2 + Ca^{2+}} \underset{\text{membranes}}{\text{(lysophosphatides + fatty acids)}}$$
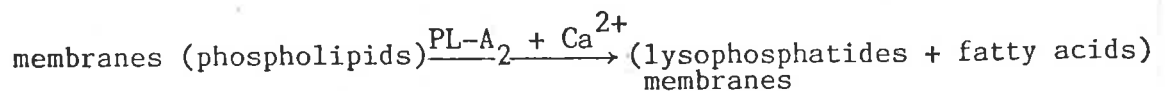
lysophosphatides and fatty acids can be removed by washing the membranes with serum albumin solution:

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

		NEM (mM)		pCMB (mM)	
		0.5	1.0	0.5	1.0
Spheroplasts	NO_2^- uptake	0	16	26	39
	NO_3^- production	11	13	24	31
	O_2 uptake	7	14	20	36
Membrane vesicles	NO_2^- uptake	65	81	32	72
	NO_3^- production	58	80	30	71
	O_2 uptake	69	72	24	70

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 MgSO$_4$. 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-A$_2$ TREATMENT ON ATPase AND NO$_2^-$ 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 CaCl$_2$, 0.1 mg bovine serum albumin and 20 units of
phospholipase-A$_2$. Reaction mixture without phospholipase-A$_2$
was preincubated for 5 min at room temperature (25°C) then phos-
pholipase-A$_2$ 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 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 NO$_2^-$ 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.

NO$_2^-$ oxidase (○); ATPase (●); residual lipid phosphate (□).

FIG. 7

FIG. 8
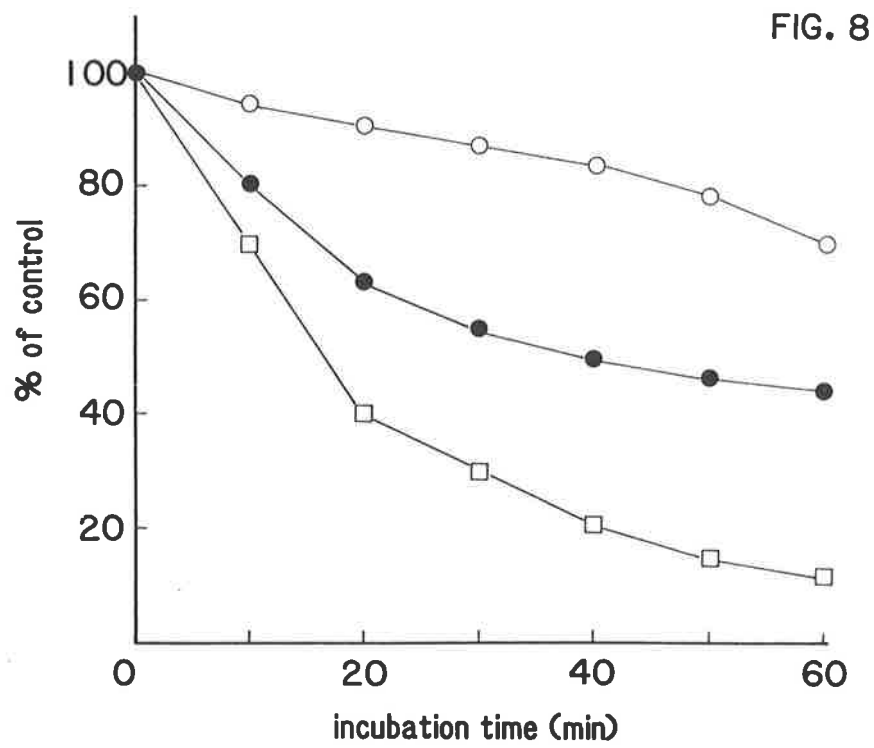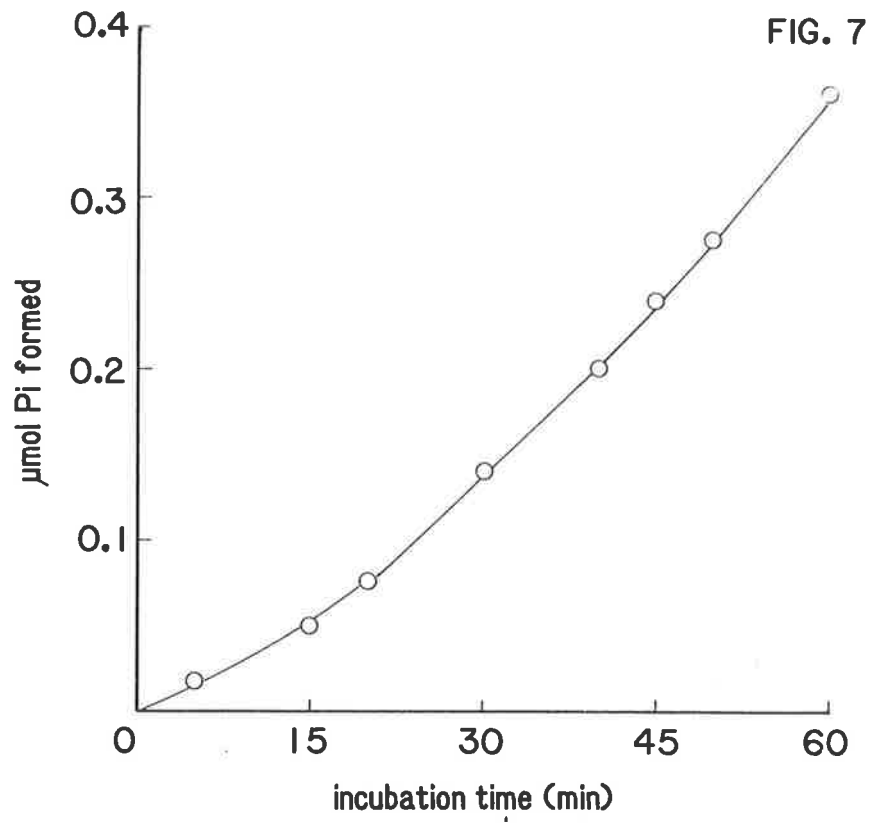
TABLE 9: EFFECTS OF SOME INHIBITORS ON ATPase ACTIVITY IN MEMBRANE
VESICLES.

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.
$(30 \text{ min})^{-1}$ $(\text{mg protein})^{-1}$. Except for sodium vanadate which
was dissolved in H_2O, all the inhibitors were dissolved in 95%
(v/v) ethanol.

Inhibitor	Concentration (μM)	% Inhibition
CCCP	10	0
	50	10
2,4.DNP	50	0
Oligomycin	50	0
DCCD	100	65
Sodium vanadate	10	50
Diethylstilbestrol	20	45
NBD chloride	100	92

(lysophosphatides + fatty acids) membranes $\xrightarrow{\text{serum albumin}}$ 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_2^- oxidase and ATPase enzymes. The effects of phospholipase A_2 treatment on the activities of two enzymes is shown in Fig.8. Phospholipase A_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_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_4^+ on the growth of *Nitrobacter agilis* cultures were grown in a nitrite medium supplemented with various concentrations of NH_4Cl. The optimum concentration for growth was found to be 2mM (Fig.9). Exponentially growing cultures of the bacterium (in 100ml medium supplemented wih 2mM NH_4Cl) utilized $75 \pm 5mg$ $NaNO_2$ $(24h)^{-1}$ as compared with cultures without NH_4Cl which utilized $50 \pm 5mg$ $(24h)^{-1}$. The lag period of growth, with or witout NH_4Cl, was about 36h. After 6 days, cells from NH_4Cl (2mM) supplemented cultures contained $0.55 \pm 0.1mg$ protein ml^{-1} culture as comparted with those without NH_4Cl $(0.29 \pm 0.1mg)$ (Fig.10).

3.2.2 *Inhibition of NO_2^- oxidation by NH_4^+ in washed cells*

Although the growth experiment indicated that *Nitrobacter* can utilize small amounts of NH_4^+ ($\simeq$2mM), high concentration resulted in an inhibition of growth . From O_2 electrode traces of NO_2^- oxidation (Fig.11) it is clear that NH_4^+ inhibits NO_2^- oxidation by washed cells of *Nitrobacter agilis*. The extent of inhibition increased with the increasing levels of NH_4^+ when NO_2^- concentration was kept constant (Fig.11a-c,e,f). Thus NH_4^+ at concentrations equal to those of NO_2^- inhibited NO_2^- oxidation by 20% (Fig.11b). The NH_4^+ inhibition could be reversed by increasing the NO_2^- concentrations (Fig.11d,g).

FIG.9: GROWTH OF *NITROBACTER AGILIS* IN NH_4Cl CONTAINING MEDIUM.

Cells were grown in 250 ml Erlenmeyer flasks containing 100 ml of culture medium (Section 2.2.1) and NH_4Cl 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_2^- utilization (Section 2.2.17.1) which was found to be proportional to the increase in total cell nitrogen.

No NH_4Cl (○); 0.1mM NH_4Cl (●); 0.5mM NH_4Cl (□);
2mM NH_4Cl (■).

FIG.10: CELL YIELDS FROM CULTURES OF *NITROBACTER AGILIS* GROWN WITH VARIOUS CONCENTRATIONS OF NH_4Cl.

After a 6 day incubation, cells grown with various concentrations of NH_4Cl, 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

FIG. 10
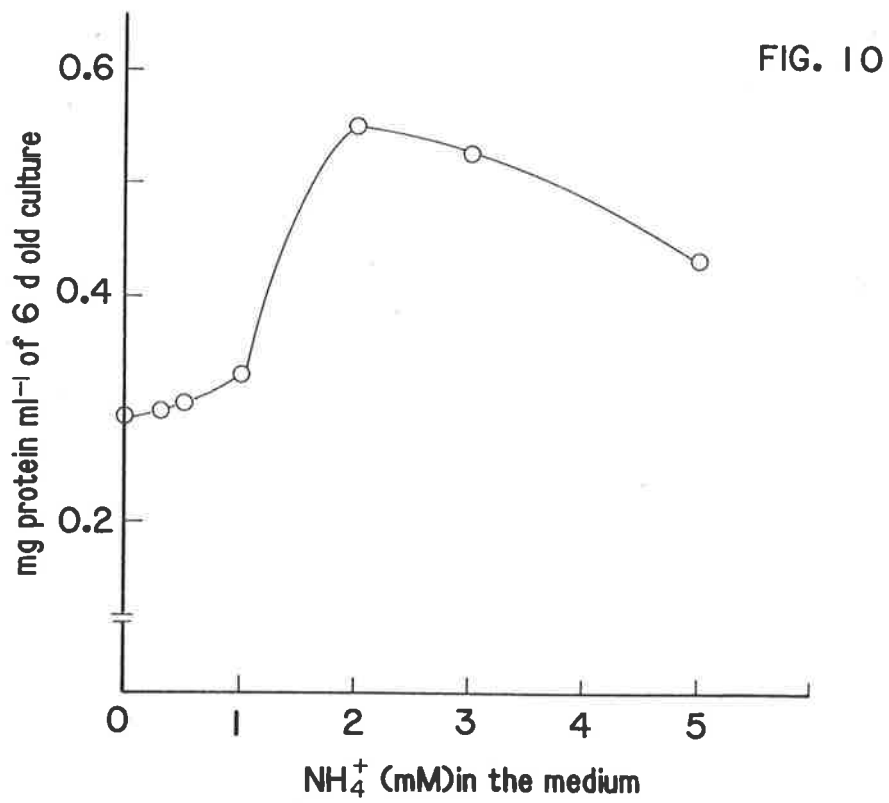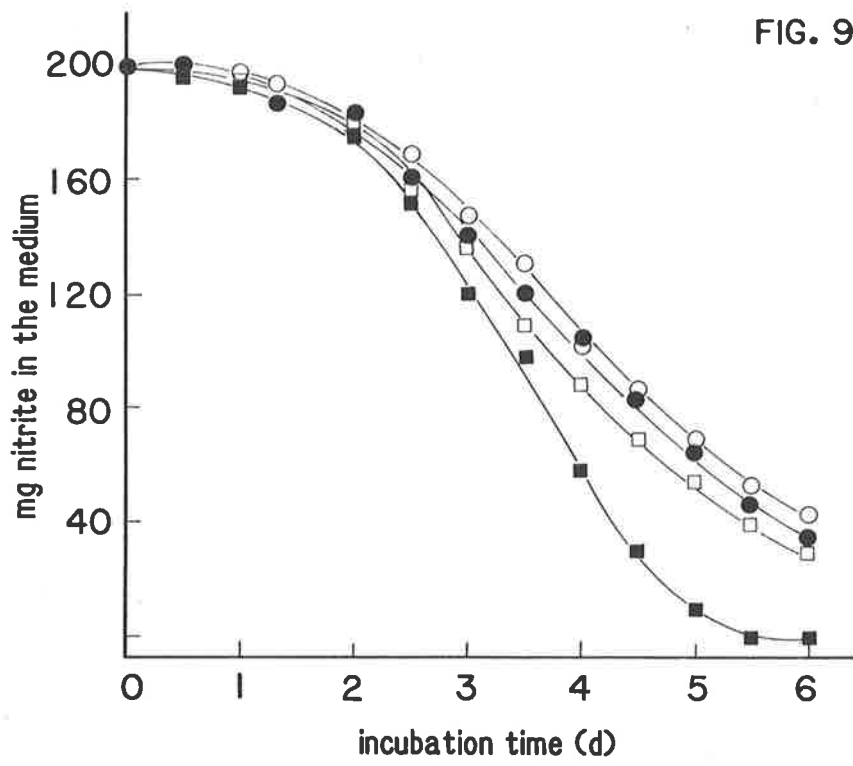
FIG.11: INHIBITION OF NO_2^- OXIDATION IN WASHED CELLS BY NH_4Cl.

100µl of washed cells (10 mg wct 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_4Cl 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_4Cl (µmol).

FIG. 11
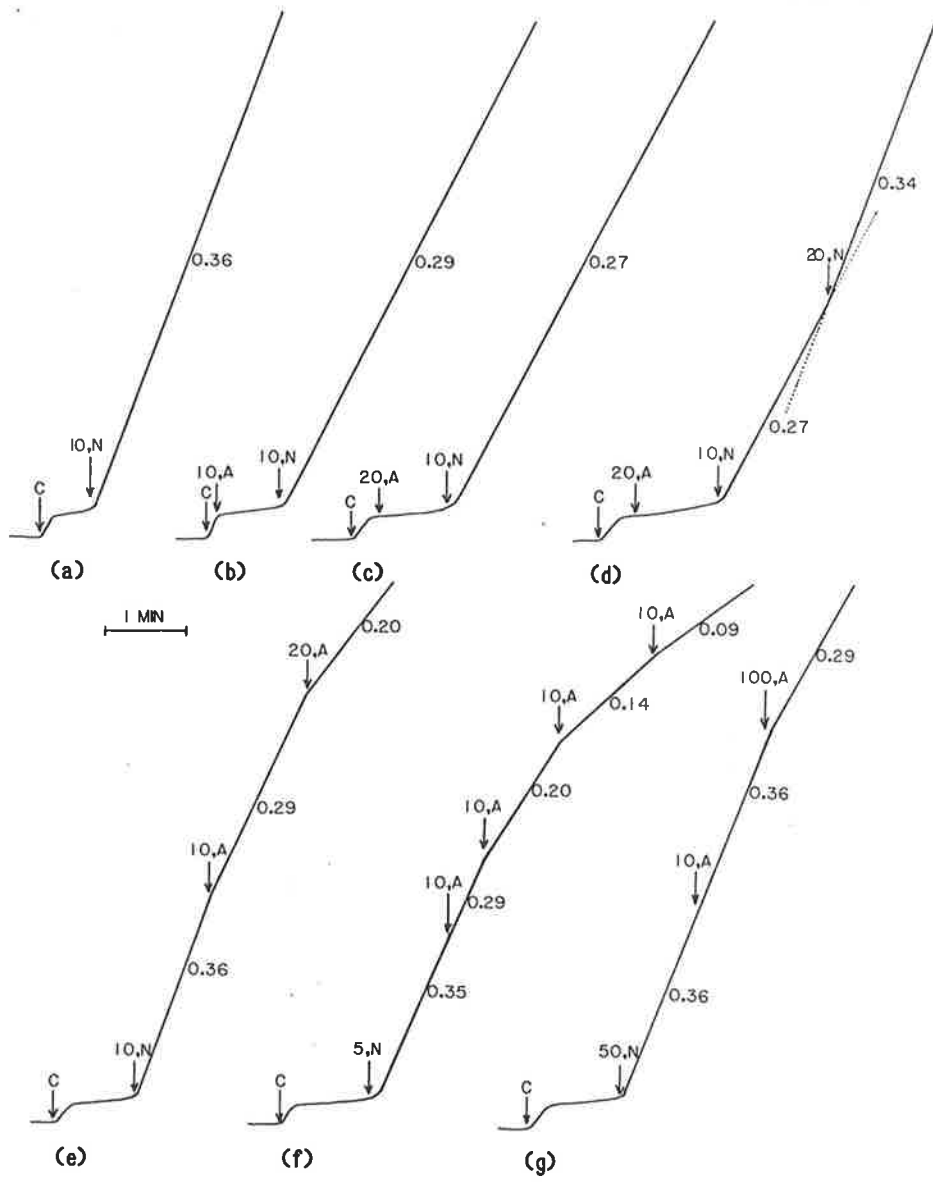
If the data from O_2 traces are plotted as % inhibition of NO_2^- oxidation vs molar ratio of NH_4^+ : NO_2^- (Fig.12), it is clear that when NH_4^+ concentration was increased keeping NO_2^- concentration constant, the extent of inhibition of NO_2^- oxidation increased rapidly until NH_4^+ : NO_2^- ratio was about 4 and increased slowly thereafter. To ascertain whether NH_4^+ at its saturating concentration resulted in a complete inhibition of NO_2^- 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 NH_4^+ was >1 (Fig.13), it only partially inhibited the oxidation of NO_2^-.

3.2.3 *Incorporation of ^{15}N labelled compounds into cell nitrogen*

The incorporation of $K^{15}NO_3$, $Na^{15}NO_2$, $^{15}NH_2OH$ and $^{15}NH_4Cl$ 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 $^{15}NH_4^+$ was approximately 80 fold greater than that of $^{15}NO_2^-$. The extent of incorporation was in the order $^{15}NH_4^+$ > $^{15}NH_2OH$ > $^{15}NO_2^-$ > $^{15}NO_3^-$. Time course for incorporation of ^{15}N compounds (Fig.14) indicated that the further incorporation of $^{15}NH_4^+$ and $^{15}NH_2OH$ ceased after about 4h of incubation, indicating that either ATP or NAD(P)H or both become rate-limiting. When unlabelled NO_2^- was included as an energy source, in addition to ^{15}N substrates, the extent of incorporation of $^{15}NO_3^-$, $^{15}NH_2OH$ or $^{15}NH_4^+$ was significantly increased (Table 10).

^{15}N 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 $^{15}NO_2^-$ into protein but not that of $^{15}NH_4^+$.

3.2.4 *Enzymes of NH_4^+ assimilation*

Enzymes of NH_4^+ 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

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FIG.12: COMPETITIVE INHIBITION OF NO_2^- OXIDATION BY NH_4Cl.

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_4Cl.

Rates of O_2 uptake by washed cells were determined as described
in Fig.11.   1/I is the reciprocal of NH_4Cl concentration and
1/i, reciprocal of fractional inhibition of NO_2^- oxidation by
NH_4Cl.

FIG. 12
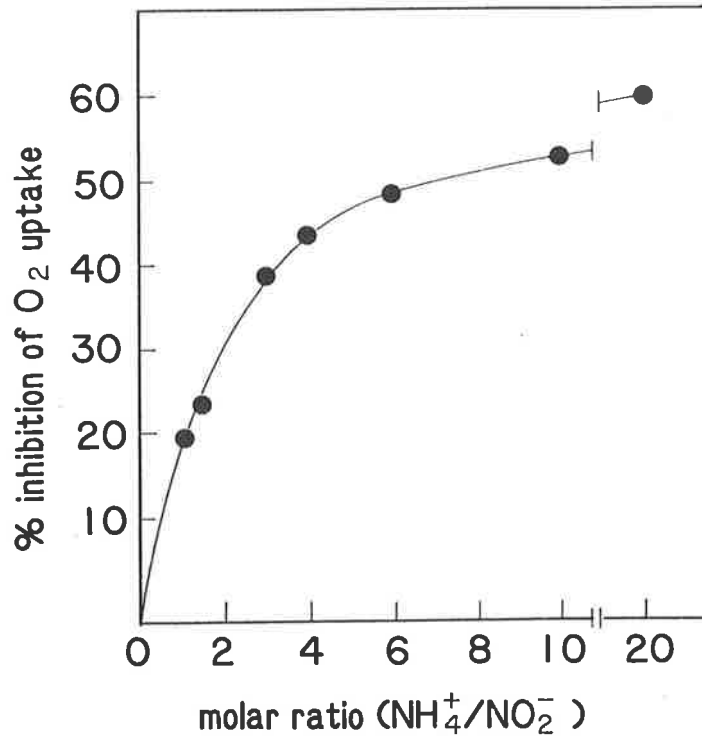
FIG. 13
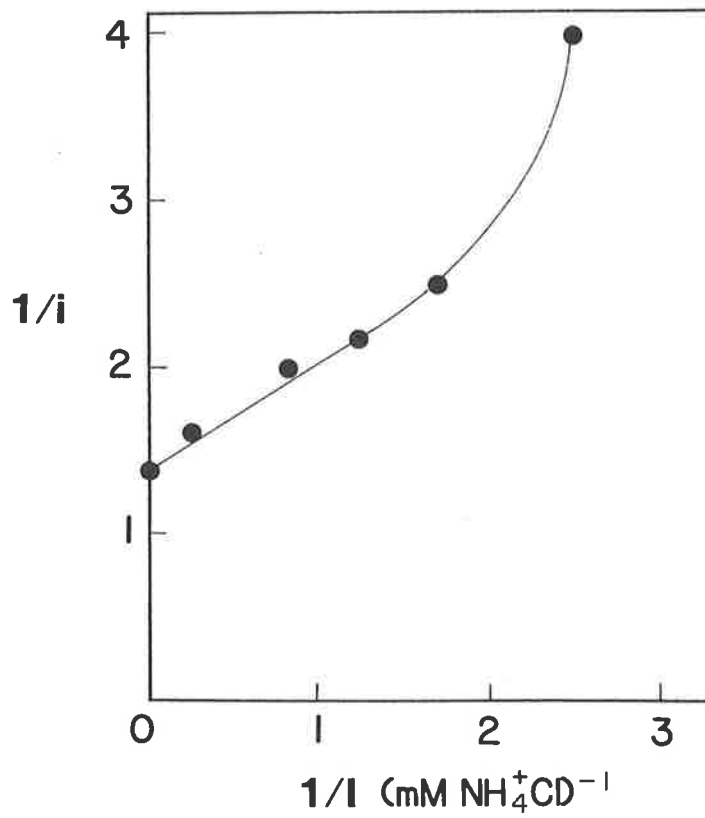
TABLE 10:   INCORPORATION OF ^{15}N-LABELLED[a] COMPOUNDS INTO WASHED CELLS.

Washed cells (25mg protein), suspended in 6ml 0.1M phosphate buffer (pH 7.5) supplemented with 1.5mM $KHCO_3$ 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.

Substrate	^{15}N incorporated [μg (mg protein)$^{-1}$]
$Na^{15}NO_2$	0.20 ± 0.02
$K^{15}NO_3$	0.10 ± 0.02
$K^{15}NO_3$ + $NaNO_2$	0.60 ± 0.02
$^{15}NH_2OH$	4.00 ± 0.50
$^{15}NH_2OH$ + $NaNO_2$	5.20 ± 0.50
$^{15}NH_4Cl$	8.30 ± 0.80
$^{15}NH_4Cl$ + $NaNO_2$	14.50 ± 0.80

[a]Initial enrichment of the ^{15}N compounds was as follows:

$^{15}NH_4Cl$ (30 atom % excess), $Na^{15}NO_2$ and $K^{15}NO_3$ (both 32.5 atom % excess) and $^{15}NH_2OH$ (97 atom % excess).

FIG.14: TIME COURSE FOR INCORPORATION OF $^{15}NO_2^-$, $^{15}NO_3^-$, $^{15}NH_2OH$ AND $^{15}NH_4^+$ 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.

$^{15}NH_4Cl$ (○); $^{15}NH_2OH$ (●); $^{15}NO_2^-$ (□); $^{15}NO_3^-$ (■).

FIG. 14
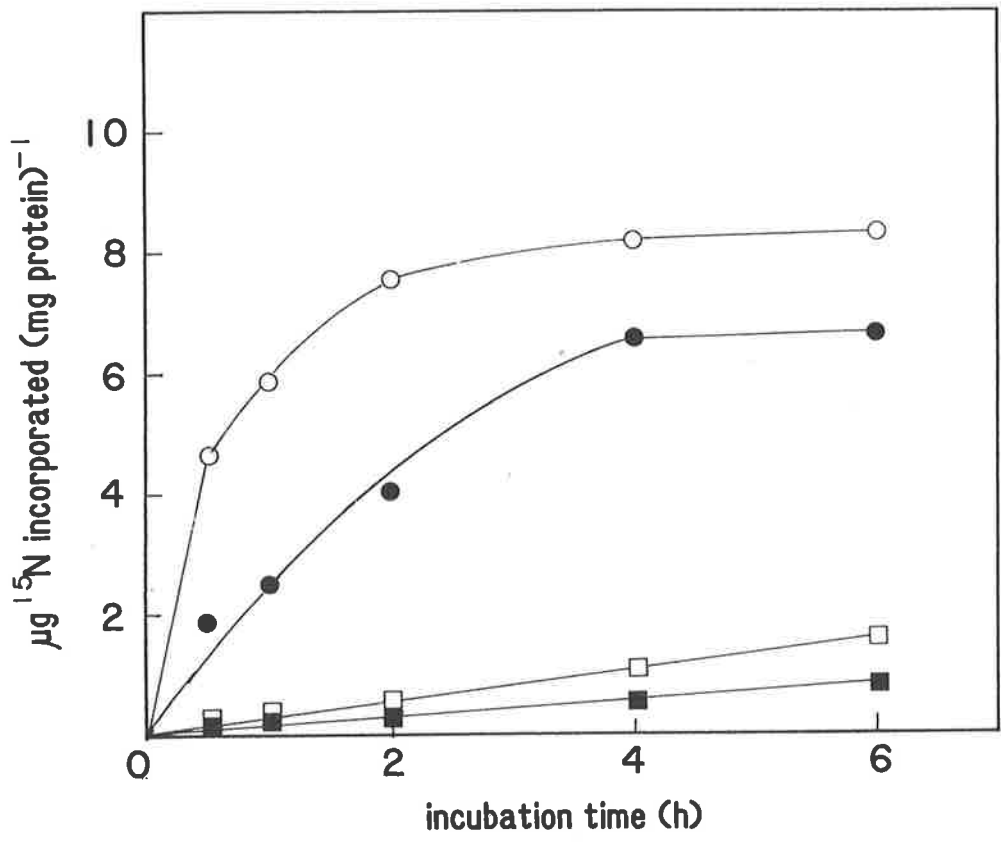
TABLE 11: INCORPORATION OF ^{15}N-LABELLED COMPOUNDS INTO PROTEIN OF CELL EXTRACTS (S_5).

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.

Substrate	^{15}N incorporated [µg (mg protein)$^{-1}$]
$^{15}NO_2^-$	0.10 ± 0.02
$^{15}NO_2^-$ + 0.5mM NADH	0.12 ± 0.02
$^{15}NO_2^-$ + 1.0mM NADH	0.24 ± 0.02
$^{15}NO_2^-$ + 1.0mM ATP	0.16 ± 0.02
$^{15}NO_2^-$ + 1.0mM ATP + 1.0mM NADH	0.27 ± 0.03
$^{15}NH_4^+$	0.49 ± 0.02
$^{15}NH_4^+$ + 0.5mM NADH	0.46 ± 0.02
$^{15}NH_4^+$ + 1.0mM NADH	0.47 ± 0.03
$^{15}NH_4^+$ + 1.0mM ATP	0.49 ± 0.03
$^{15}NH_4^+$ + 1.0mM NADH + 1.0mM ATP	0.50 ± 0.02

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_4Cl 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_4Cl. 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 activiy was depressed by about 95% in cells incubated with 250µM-MSX for 3h. Higher concentrations of MSX (up to 1mM) inhibited GS completely. The preincubation of cells with 200µM azaserine for 2h or 1mM azaserine for 30 min completly 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 (250µM) or azaserine (200µM) 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 (S_{144}).

Cell extracts (S_{144}) were prepared by centrifuging crude S_5 fraction at 144,000g for 1h at 4°C. Enzyme activities for GS, GOGAT and GDH were assayed in supernatant (S_{144}) as described in Section 2.2.6. Results are the mean values from 5 determinations with S_{144} from the same batch of cells ± SEM.

| Growth condition | Specific activity | | | |
| | GS^a | $GOGAT^b$ | GDH^c | |
			NADPH	NADH
Basal medium without NH_4Cl	121±6.0	8±0.5	17.5±1.5	6.0±1.0
Basal medium with 2mM NH_4Cl	110±5.0	0±0.5	38.5±1.5	12.5±1.0

[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
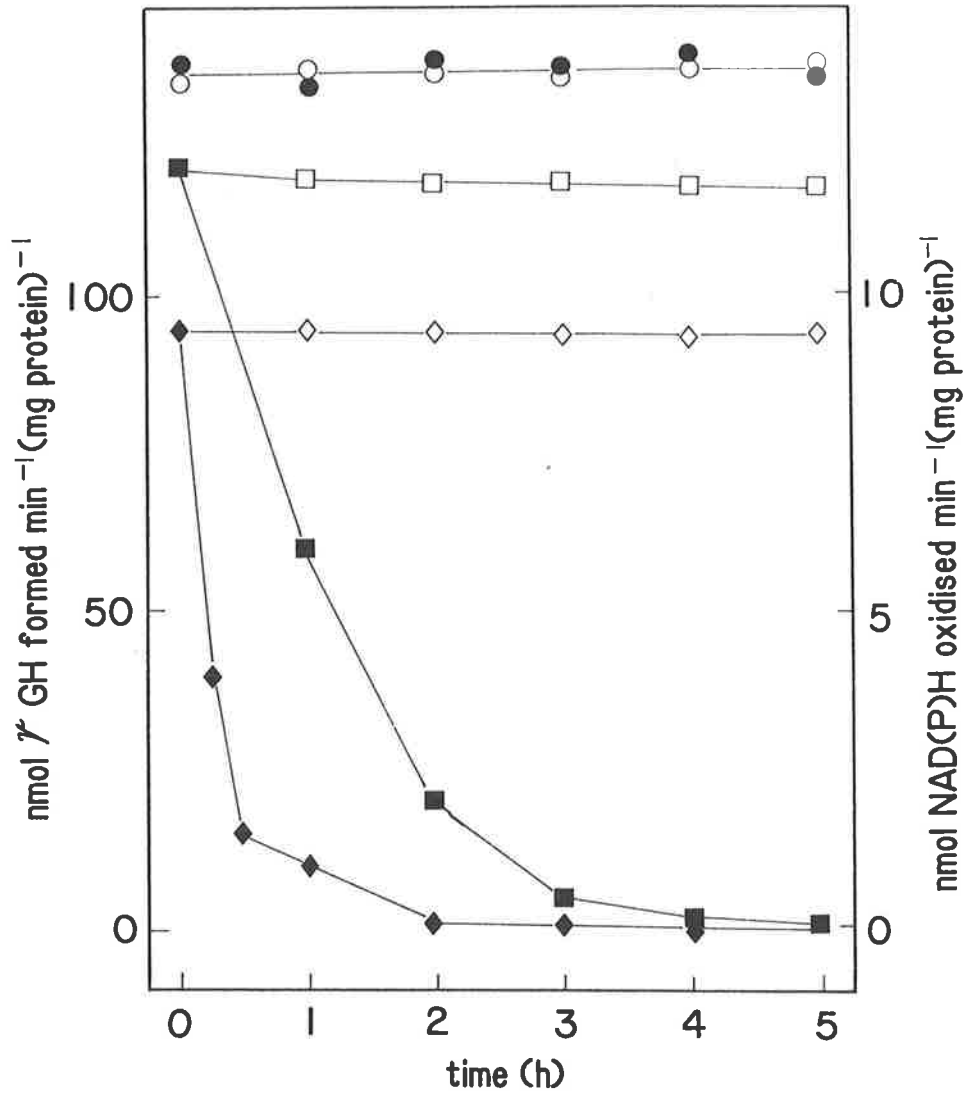
TABLE 13: INCORPORATION OF $^{15}NO_2^-$ and $^{15}NH_4^+$ INTO CELLS PRETREATED WITH MSX AND AZASERINE.

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 $^{15}NH_4^+$ incorporation experiments, reaction mixture was as described in Table 10. For $^{15}NO_2^-$ incorporation studies, cells (25mg protein) were suspended in 75ml culture medium (Section 2.2.1) in 125ml Erlenmeyer flasks and unlabelled $^{15}NO_2^-$ was replaced with $Na^{15}NO_2$ (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}N enrichment as described in Section 2.2.4. Results are the mean values of three determinations with the same batch of cells ± SEM.

Additions to cells	^{15}N incorporated [μg (mg protein)$^{-1}$]
$^{15}NO_2^-$	3.40 ± 0.20
Azaserine (200μM) + $^{15}NO_2^-$	3.00 ± 0.20
MSX (250μM) + $^{15}NO_2^-$	2.80 ± 0.20
Azaserine (200μM) + MSX (250μM) + $^{15}NO_2^-$	3.10 ± 0.30
$^{15}NH_4^+$	8.90 ± 0.50
Azaserine (200μM) + $^{15}NH_4^+$	8.80 ± 0.40
MSX (250μM) + $^{15}NH_4^+$	9.00 ± 0.20
Azaserine (200μM) + MSX (250μM) + $^{15}NH_4^+$	8.50 ± 0.30

$^{15}NO_2^-$ 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 *NITROSOMONAS EUROPAEA*

3.3.1   *Purification of GS*

The purification procedures for GS from *Nitrobacter agilis* and *Nitrosomonas europaea* are summarized in Tables 14 znd 15 respectively.   The purified enzymes from *Nitrobacter agilis* and *Nitrosomonas europaea* had specific activities of 220 and 4.26 μmol γGH 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, *Nitrosomonas* 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.   *Nitrosomonas 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 *Nitrosomonas 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 *Nitrosomonas 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 γ
GH from L-glutamine and NH_2OH at pH 7.2 as described in
Section 2.2.6.2. One unit is defined as μmol γ GH pro-
duced min^{-1}. Specific activity is defined as number of
units (mg proteins)$^{-1}$.

Purification step	Total Protein (mg)	Total units	Specific activity	Fold purifi- cation	% Recovery
Crude extract (S_{30})	148.56	75.81	0.51	1.0	100
Heat treatment	100.81	69.92	0.69	1.4	94
Pooled Blue Sepharose CL-6B fractions	2.10	45.33	21.59	42.3	60
Pooled Sepharose-4B fractions	0.20	44.0	220.0	431.0	58

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 γ GH from L-glutamine and NH_2OH at pH 7.2
(Section 2.2.6.2). One enzyme unit is defined as μmol γGH
produced min^{-1} and specific activity as numberof units (mg
protein)$^{-1}$.

Purification step	Total Protein (mg)	Total units	Specific activity	Fold purifi- tion	% Recovery
Crude extract (S_{30})	500.0	83.6	0.006	1.0	100
Heat treatment	399.0	91.2	0.008	1.3	109
pH precipitation	12.50	156.4	0.420	70.0	187
PEG precipitation	0.20	25.6	4.26	710.0	31

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 10mM Tris-HCl, 1mM $MnCl_2$ buffer (pH 7.2). The column
was then washed with buffer (flow rate 50ml.h^{-1}) until the
absorbance (A_{280}) was close to zero. The enzyme (GS) was
eluted from the column with 2mM ADP in the same buffer.
Enzyme activity was determined by following the production of
γ GH from glutamine and NH_2OH as described in Section 2.2.6.2.

A_{280} ($\circ$); enzyme activity ($\bullet$).

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 10mM Tris-HCl, 1mM $MnCl_2$ buffer (pH 7.2),
concentrated on an Amicon PM-10 membrane filter and loaded
onto a Sepharose-4B column (2 x 70cm) pre-equilibrated with
10mM Tris-HCl, 1mM $MnCl_2$ buffer (pH 7.2).The enzyme was eluted
with the same buffer (flows rate 12ml.h^{-1}). Transferase
activity was determined as described in Section 2.2.6.2

A_{280} ($\circ$); enzyme activity ($\bullet$).

FIG.16

FIG. 17
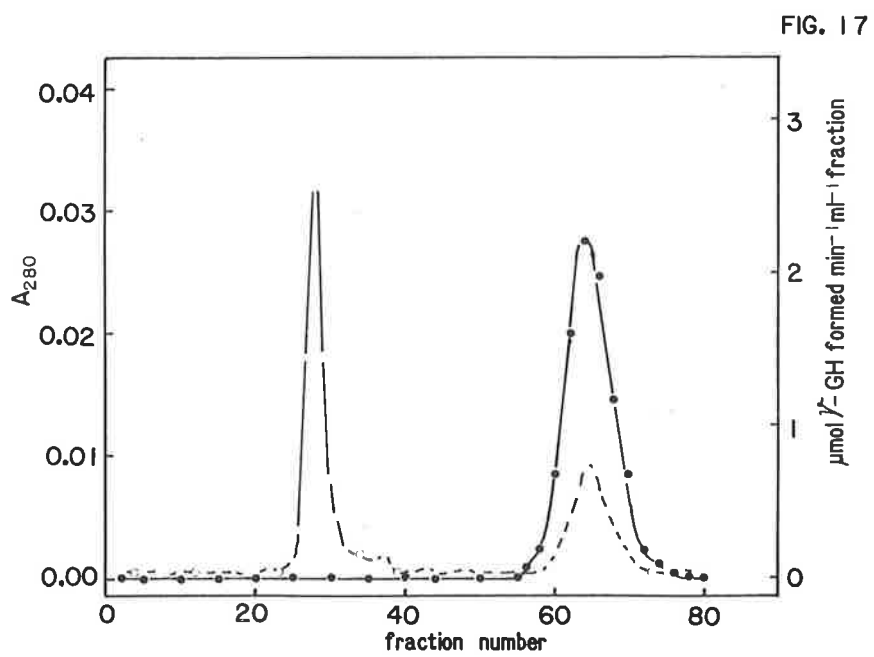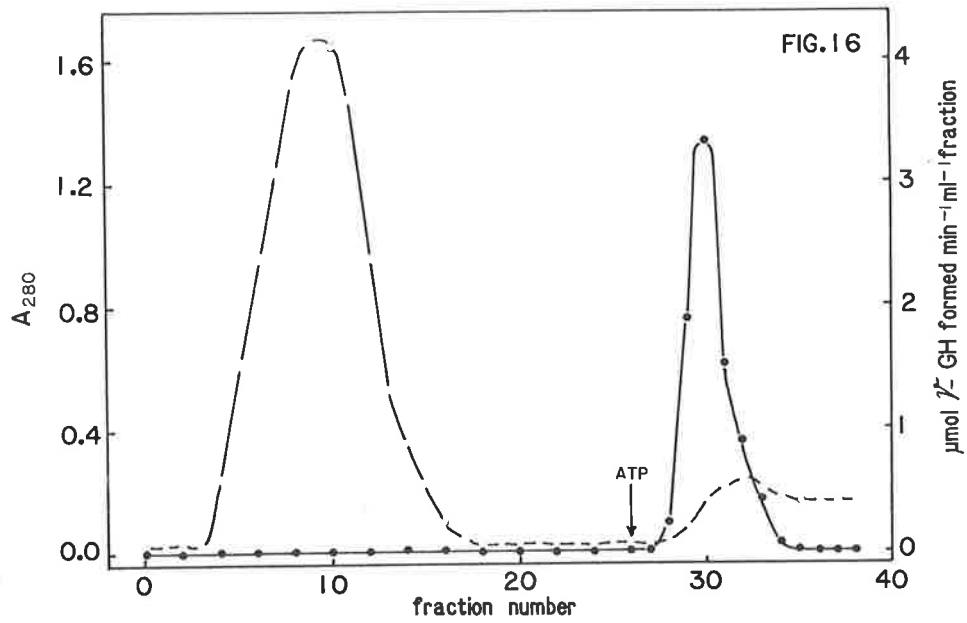
FIG.18: POLYACRYLAMIDE GEL ELECTROPHORESIS OF PURIFIED GS FROM *NITROBACTER AGILIS*.

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 ELECTRPHORESIS OF PURIFIED GS FROM *NITROSOMONAS EUROPAEA*.

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.

FIG. 18
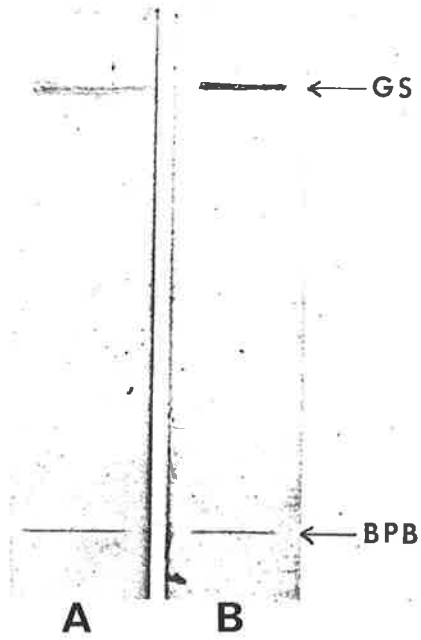
FIG. 19
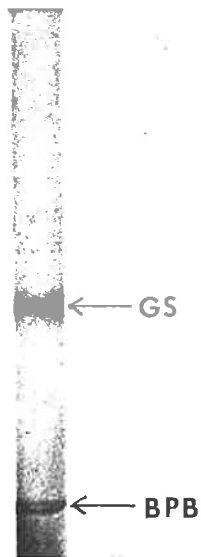
(Fig.20). The molecular weight of enzyme subunit was estimated to be 58,000 using standard SDS-treated protein markers (Fig.21). The molecular weight of the native enzyme determined by gel filtration using a Sepharose 6-B column (1.6 x 100cm) was estimated to be 700,000 (Fig.22). The results indicate 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 γ 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_2OH 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_4Cl 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 effectivness 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 contain-
ed 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. 20

FIG. 21
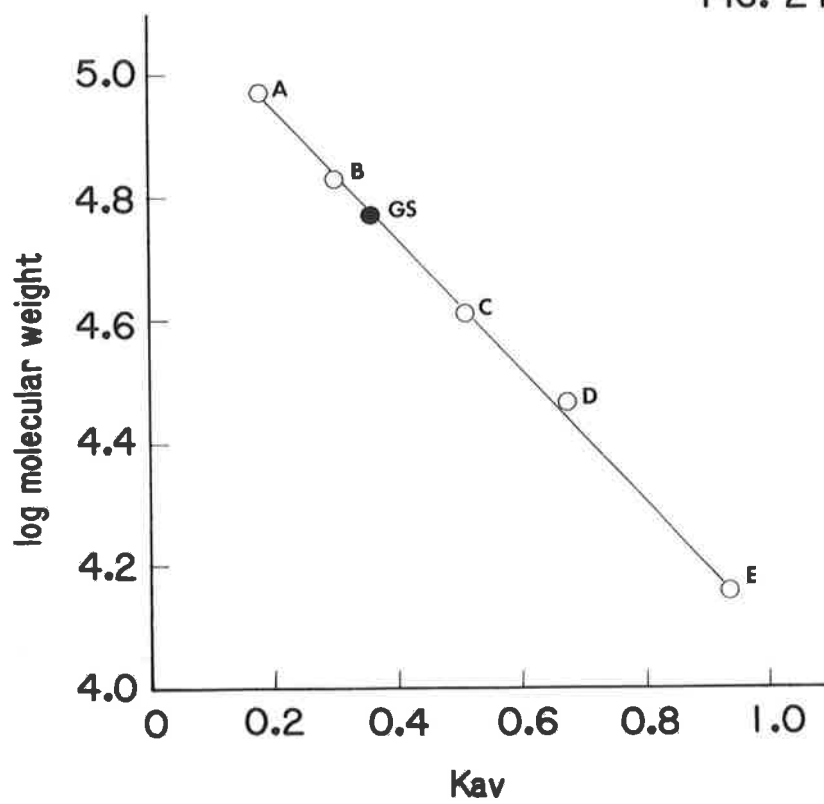
FIG.22: DETERMINATION OF NATIVE MOLECULAR WEIGHT OF PURIFIED GS
FROM *NITROBACTER AGILIS*.

The molecular weight of the native enzyme was determined by
gel-filtration in a Sepharose-6B column (1.6 x 100cm)
equilibriated 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
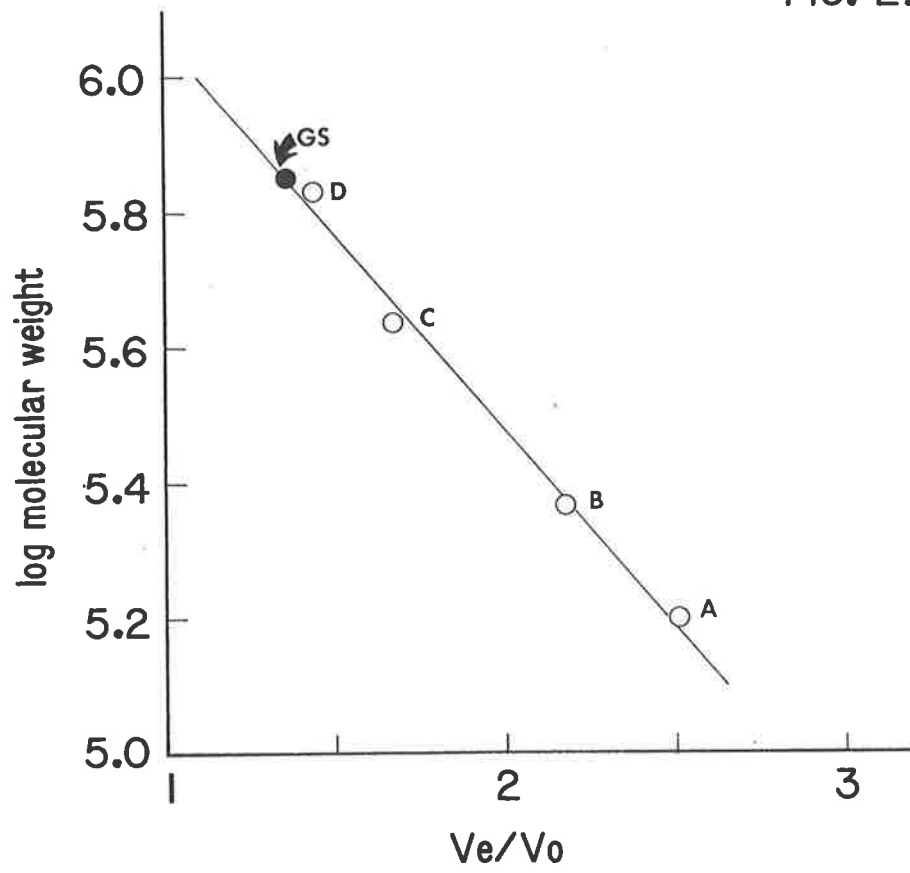
TABLE 16: SUBSTRATE REQUIREMENTS FOR THE γ GLUTAMYL TRANSFERASE
REACTION OF PURIFIED GS FROM *NITROBACTER AGILIS*.

The complete assay mixture in a final volume of 1ml contained
purified enzyme (5μg protein), L-glutamine (30mM), $NH_2OH.HCl$
(neutralized with 2M NaOH) (30mM), $MnCl_2.4H_2O$ (0.5mM), sodium
arsenate (20mM), ADP (0.4mM) and imidazole-HCl buffer (40mM)
pH 7.2. In other test-tubes Mn^{2+}, L-glutamine, NH_2OH, 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.

Reaction mixture	Activity %
Complete	100
Omit Mn^{2+}	0
Omit L-glutamine	0
Omit hydroxylamine	0
Omit sodium arsenate	5
Omit ADP	20

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), NH_4Cl (50mM), ATP (10mM), Mg^{2+} (5mM) and imidazole-HCl buffer (50mM, pH 7.0). In other test-tubes Mg^{2+}, L-glutamate, NH_4Cl 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.

Reaction mixture	% Activity
Complete	100
Omit Mg^{2+}	5
Omit glutamate	0
Omit NH_4Cl	0
Omit ATP	0

TABLE 18: EFFECTS OF VARIOUS DIVALENT CATIONS ON THE γ GLUTAMYL TRANS-
FERASE 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.

Metal ion	% activity at cation concentration (mM)		
	10	20	50
Mn^{2+}	100	77	40
Mg^{2+}	23	40	61
Ca^{2+}	0	0	0
Ni^{2+}	30	2	0
Zn^{2+}	0	0	Nd
Cu^{2+}	100	84	Nd
Fe^{2+}	0	2	Nd
Co^{2+}	5	45	Nd

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. Bio-
synthetic 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.

Metal ion	% activity of cation concentration (mM)		
	10	50	100
Mn^{2+}	73	73	79
Mg^{2+}	95	100	75
Ca^{2+}	Nd	42	48
Fe^{2+}	Nd	30	30
Co^{2+}	Nd	58	74
Ni^{2+}	Nd	52	55
Zn^{2+}	Nd	72	55
Cu^{2+}	Nd	30	74

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 Υ glutamyl-hydroxamate produced.

The effects of various concentrations of NH_2OH (0–40mM) on transferase activity are shown in Fig.24a. The enzyme activity increased up to 20mM NH_2OH. The K_m value for NH_2OH 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 recipocal plot (Fig.25b) of glutamate concentration against Pi produced gave a K_m value of 6.3 ± 1.6mM. The effects of NH_4Cl over a range of 0–5mM are shown in Fig.26a. The biosynthetic activity increased up to about 2mM NH_4Cl. The K_m value for NH_4Cl 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_2OH (30mM) accelerated its deactivation. The effects of NH_2OH were further investigated on both biosynthetic and transferase activities of the enzyme. The enzyme was preincubated for various periods with 10mM NH_2OH. The data indicate that both transferase and biosynthetic activities decreased; thus after 20 min preincubation with NH_2OH, 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
γ-GLUTAMYL TRANSFERASE ACTIVITY OF PURIFIED GS FROM
NITROBACTER AGILIS.

Aliquots of purified enzyme were added to the reaction
mixture (final volume 1ml) containing L-glutamine (0-50mM),
NH_2OH-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.   γGH produced was determined as described in
Section 2.2.6.2.

FIG.24: THE EFFECTS OF VARIOUS CONCENTRATIONS OF NH_2OH ON γ GLUTAMYL
TRANSFERASE ACTIVITY OF PURIFIED GS FROM *NITROBACTER AGILIS.*

Experimental details as in Fig.23, except that NH_2OH was
varied from 0-40mM at a fixed concentration of glutamine
(30mM).

FIG. 23
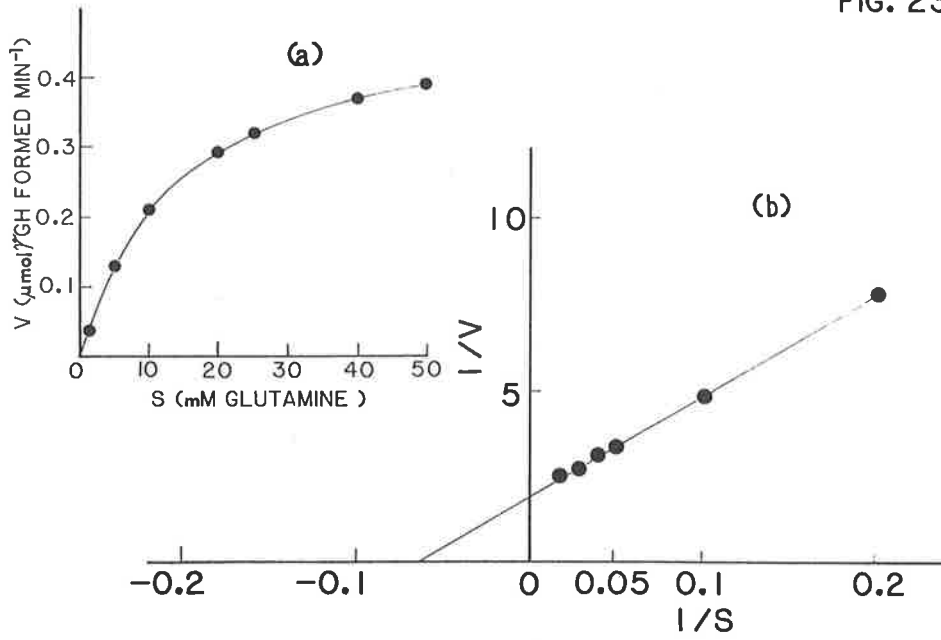
FIG. 24
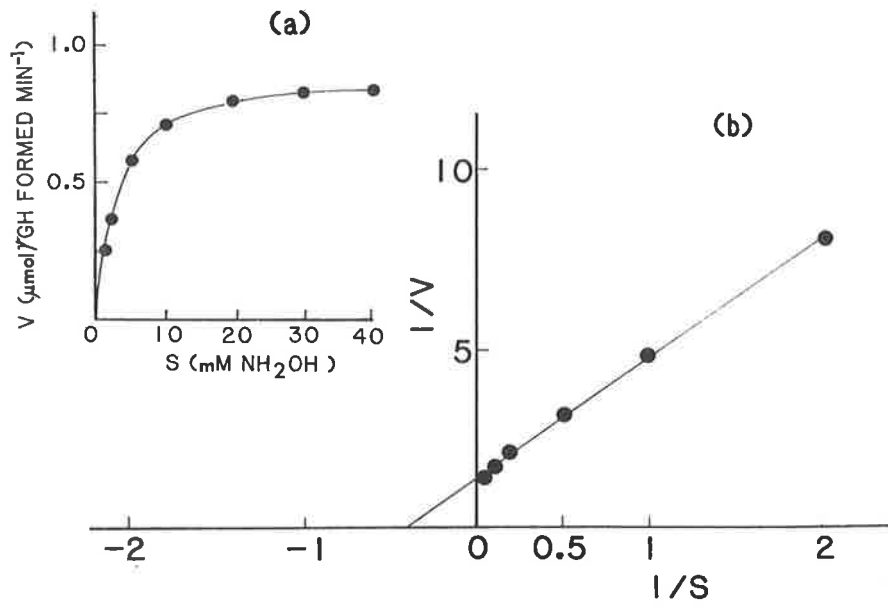
FIG.25: THE EFFECTS OF VARIOUS CONCENTRATIONS OF L-GLUTAMATE ON THE
BIOSYNTHETIC ACTIVITY OF PURIFIED GS FROM *NITROBACTER AGILIS*.

Aliquots of enzyme were added to the reaction mixture (0.2ml)
final volume) containing L-glutamate (0-50 mM), NH_4Cl (50mM),
ATP (10mM), Mg^{2+} (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 NH_4Cl ON THE BIO-
SYNTHETIC ACTIVITY OF PURIFIED GS FROM *NITROBACTER AGILIS*.

Experimental details as in Fig.25, except that NH_4Cl was
varied from 0-5mM at fixed glutamate concentration (100mM).

FIG. 25

(a) V (μmol Pi FORMED MIN⁻¹) vs S (mM GLUTAMATE)

(b) 1/V vs 1/S

FIG. 26

(a) V (μmolPi FORMED MIN⁻¹) vs S (mM NH₄Cl)

(b) 1/V vs 1/S
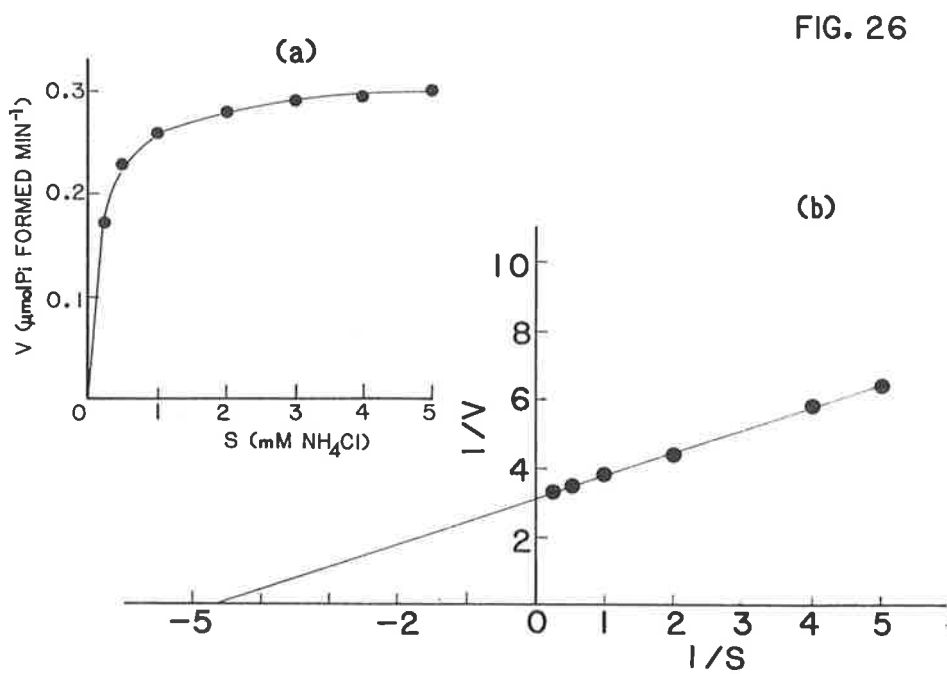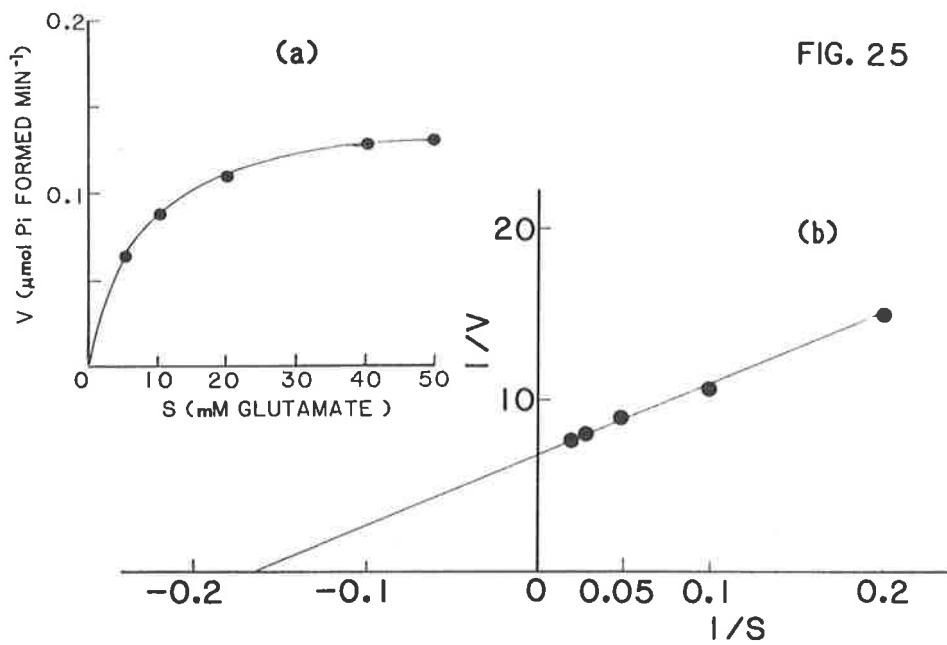
TABLE 20: PROPERTIES OF PURIFIED GS FROM *NITROBACTER AGILIS*.

Molecular weight of native enzyme	700,000
Subunit molecular weight in SDS gels	58,000
Number of subunits	12
K_m for glutamine	14.6 ± 1.5 mM
K_m for NH_2OH	2.6 ± 0.8 mM
K_m for glutamate	6.3 ± 1.6 mM
K_m for NH_4^+	0.2 ± 0.1 mM

FIG.27: THE COURSE FOR INACTIVATION BY NH_2OH OF γ GLUTAMYL
TRANSFERASE AND BIOSYNTHETIC ACTIVITIES OF PURIFIED GS
FROM *NITROBACTER AGILIS*.

Purified enzyme was preincubated with 10mM $NH_2OH.HCl$
(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 NH_2OH.   The
results are expressed as percent of activities without
preincubation with hydroxylamine.

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

FIG.28: NH_4^+ INHIBITION OF γ GLUTAMYL TRANSFERASE ACTIVITY OF PURIFIED
GS FROM *NITROBACTER AGILIS*.

Transferase activity of the purified enzyme was determined
as described in Section 2.2.6.2   except that NH_4Cl (0-50mM)
was also included in the reaction mixture.

FIG. 27
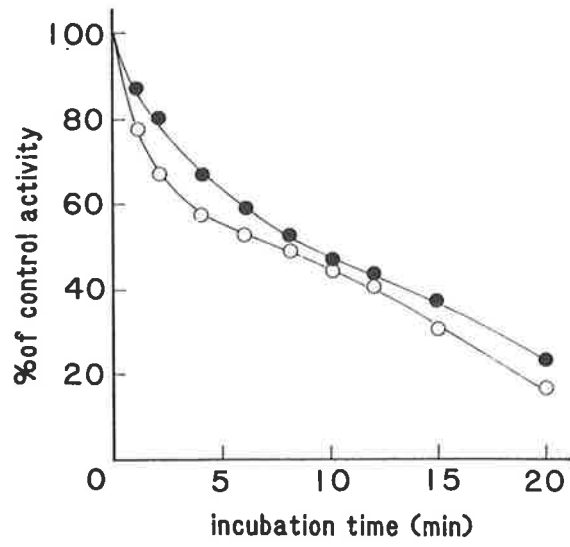
FIG. 28
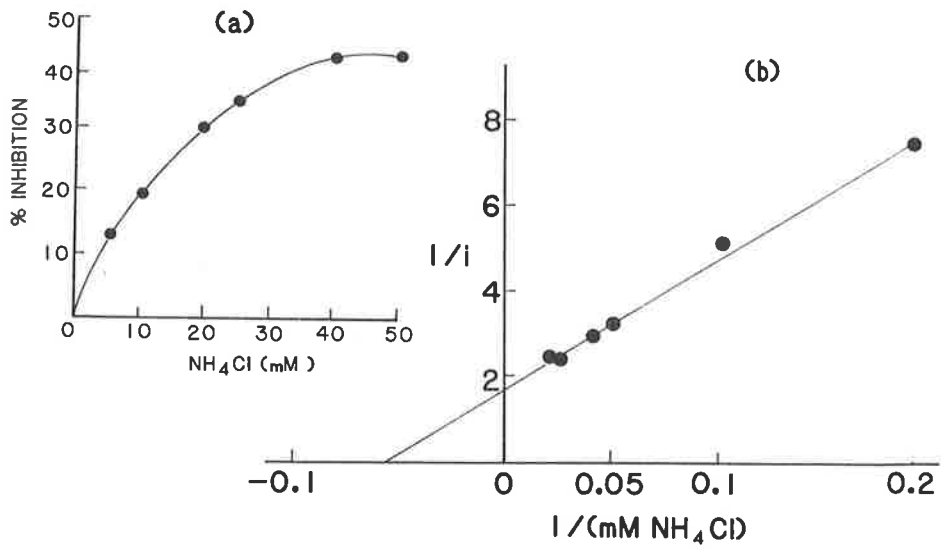
3.3.2.6 Inhibition of transferase activity by NH_4Cl

The inhibition of transferase activity by NH_4Cl (0-50mM) is shown in Fig.28 ; maximum inhibition (40%) was at 40mM NH_4Cl. The type of inhibition was further investigated. The inhibitory effects of NH_4Cl at various concentrations of glutamine and NH_2OH are shown in Fig.29 and 30 respectively. The kinetic data for double reciprocal plots indicate that NH_4Cl was a competitive inhibitor of glutamine (Fig.29) and a mixed type inhibitor of NH_2OH (Fig.30). Ammonium chloride reduced the affinity of glutamine and NH_2OH 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, alamine, serine, glycine and trytophan 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 NH_4Cl OF γ-GLUTAMYL TRANSFERASE ACTIVITY OF PURIFIED GS FROM *NITROBACTER AGILIS*.

Experimental details as in Fig.23 except that NH_4Cl (0-30mM) was also included in the assay mixture

No NH_4Cl (●); 10mM NH_4Cl (○); 20mM NH_4Cl (■); 30mM NH_4Cl (□).

FIG.30: MIXED TYPE INHIBITION BY NH_4Cl OF γ-GLUTAMYL TRANSFERASE ACTIVITY OF PURIFIED GS FROM *NITROBACTER AGILIS*.

Experimental details as in Fig.24 except that NH_4Cl (0-30mM) was also included in the assay mixture.

No NH_4Cl (●); 10mM NH_4Cl (○); 20mM NH_4Cl (■); 30mM NH_4Cl (□).

FIG. 29
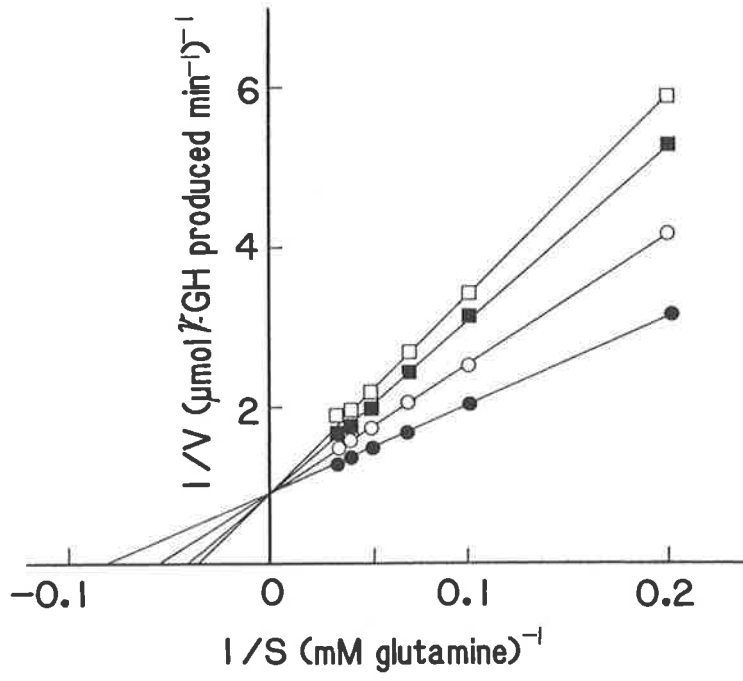
FIG. 30
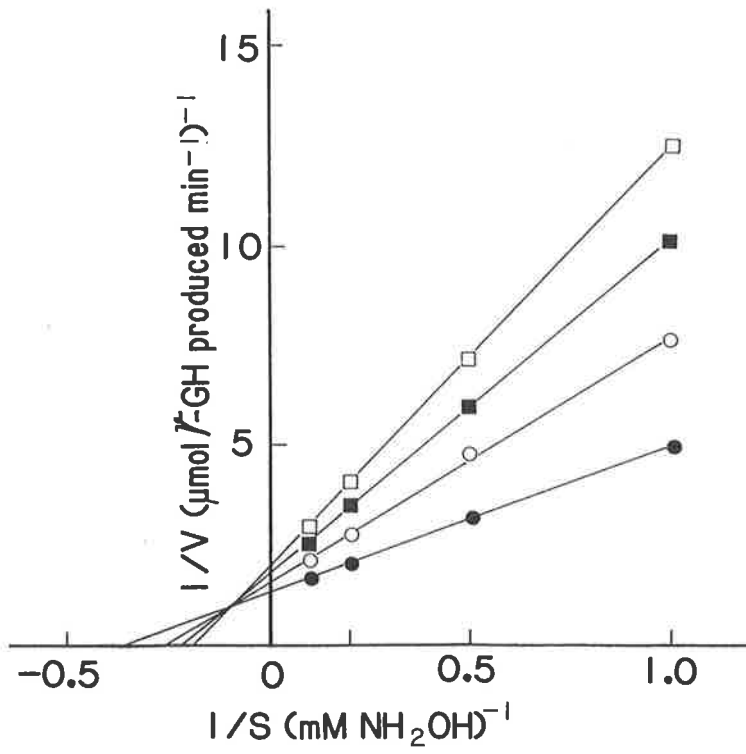
TABLE 21: EFFECT OF NH_4^+ ON K_m OF THE SUBSTRATES OF γ GLUTAMYL TRANS-
FERASE ACTIVITY OF PURIFIED GS FROM *NITROBACTER AGILIS*

Transferase activity of the enzyme was measured as described
in Section 2.2.6.2 except that the amounts of glutamine and
NH_2OH were varied between 0 to 50mM and NH_4Cl was included
in the assay mixture as indicated. K_m values were calcula-
ted from double reciprocal plots (Section 2.2.7).

NH_4^+ concentration (mM)	K_m (mM)	
	Glutamine	NH_2OH
0	14.6	2.6
10	18.2	4.0
20	23.8	5.1
30	27.8	5.6

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.

Amino acids	% Inhibition	
	Transferase activity	Biosynthetic activity
L–Glutamate	0	
L–Glycine	40	36
L–Aspartate	14	0
L–Histidine	4	6
L–Asparagine	4	0
L–Arginine	14	6
L–Leucine	0	0
L–Tyrosine	0	0
L–Serine	45	30
L–Valine	0	12
L–Proline	0	0
L–Lysine	10	12
L–Tryptophan	33	0
L–Phenylalanine	0	0
L–Isoleucine	0	0
L–Alanine	65	60

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
(0-10mM).   In Fig.31b, results of Fig.31a are plotted as
double reciprocal plots of inhibitor (amino acid) concentra-
tion (I) against fractional inhibition (i).

FIG. 31

(a)

(b)
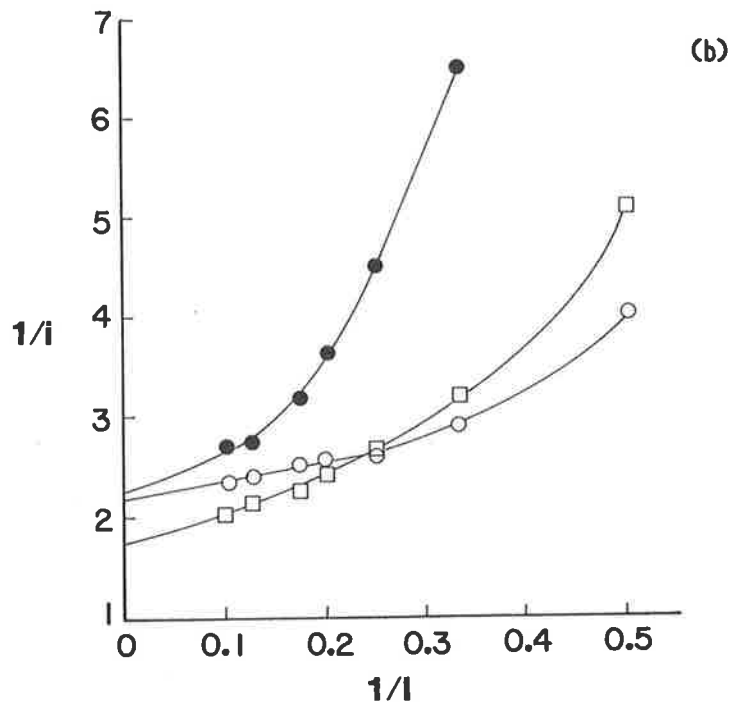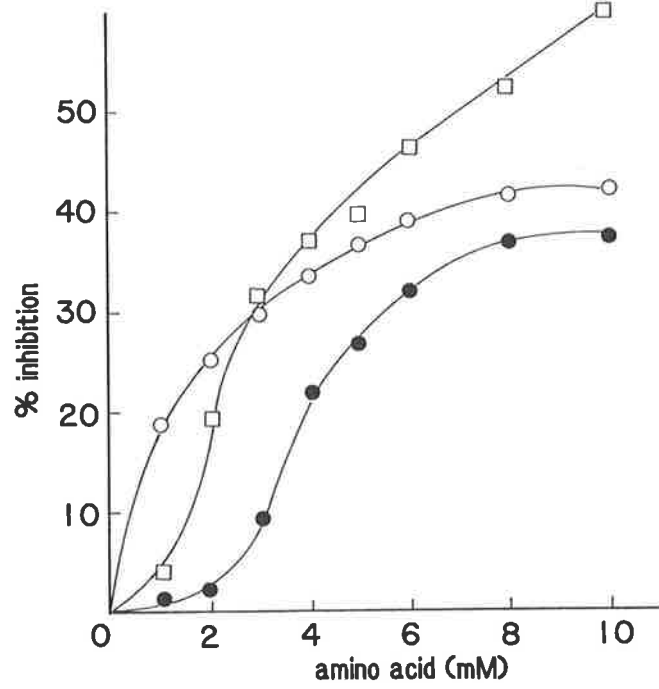
The combined effects of various amino acids on the transferase activity are shown in Table 23. The inhibition due to various combinations of amino acids was lower than the sum of the inhibitions resulting from an individual amino acid. Thus the inhibitory effects due to alanine and glycine were 40 and 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, ie. 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 (ecept 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 $MgCl_2$ has been used as an indication of the degree of adenylylation of GS. The fully adenylylated enzyme is inactive in the presence of Mg^{2+} 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.

Inhibitor	% Inhibition		
	Observed	Cumulative	Additive
Ala	40		
Gly	27		
Ser	36		
Ala + Gly	52	56	67
Ala + Ser	62	62	76
Ser + Gly	52	53	63
Ala + Gly + Ser	69	72	103

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.

Nucleotides	% Inhibition
IDP	45
ITP	63
CDP	50
CTP	55
TTP	57
UDP	53
AMP	65
ATP	56

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 (1970b) 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 ($2.5\mu g\ ml^{-1}$) the value decreased to 4. When cells grown at a low ammonia concentration (20mM) 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 $4^{\circ}C$, 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 120mM) (see Bhandari & Nicholas, 1979a) so that at the end of exponential phase, the residual NH_4^+ is about 40mM. Thus in these cells, glutamine synthetase would be highly adenylylated. The results of an experiment in which cells were grown with 20mM 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 γ-GLUTAMYL TRANSFERASE ACTIVITY IN CELL EXTRACTS (S_{25}) 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 ($2.5\mu g$ ml^{-1}.) before harvesting. Cells washed once with cold 10mM Tris-HCl, 1mM $MnCl_2$ 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 (S_{25}) was used to determine the transferase activity with and without 60mM $MgCl_2$, as described in Section 2.2.6.2. Transferase activity is defined as μmol γGH produced $(30\ min)^{-1}$ $(mg\ protein)^{-1}$.

Bacterium	CTAB treatment	Transferase activity $-Mg^{2+}$	$+60mM\ Mg^{2+}$	$+Mg^{2+}/-Mg^{2+}$ Ratio
Nitrobacter agilis				
growing on 40mM NO_2^-	−	10.50	2.10	0.20
	+	9.30	1.50	0.16
Nitrosomonas europaea				
growing on 30mM NH_4^+	−	0.16	0.04	0.25
	+	0.16	0.11	0.69
NH_4^+ depleted cultures	−	0.13	0.04	0.31
	+	0.08	0.11	1.38

FIG.32: EFFECTS OF NH_4^+ CONCENTRATION IN CULTURE MEDIUM ON γ-GLUTAMYL TRANSFERASE ACTIVITY OF GS IN CRUDE EXTRACTS OF *NITROSOMONAS EUROPAEA* AT VARIOUS STAGES OF GROWTH.

Cultures were grown in 10 ℓ batches (2 ℓ inoculum from a culture completely depleted of NH_4^+) at 28°C with vigorous aeration. Aliquots (0.5-1 ℓ) 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 (2.5 μg 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 (O); glutamine synthetase specific activity (●); $+Mg^{2+}/-Mg^{2+}$ ratio (□).

FIG. 32
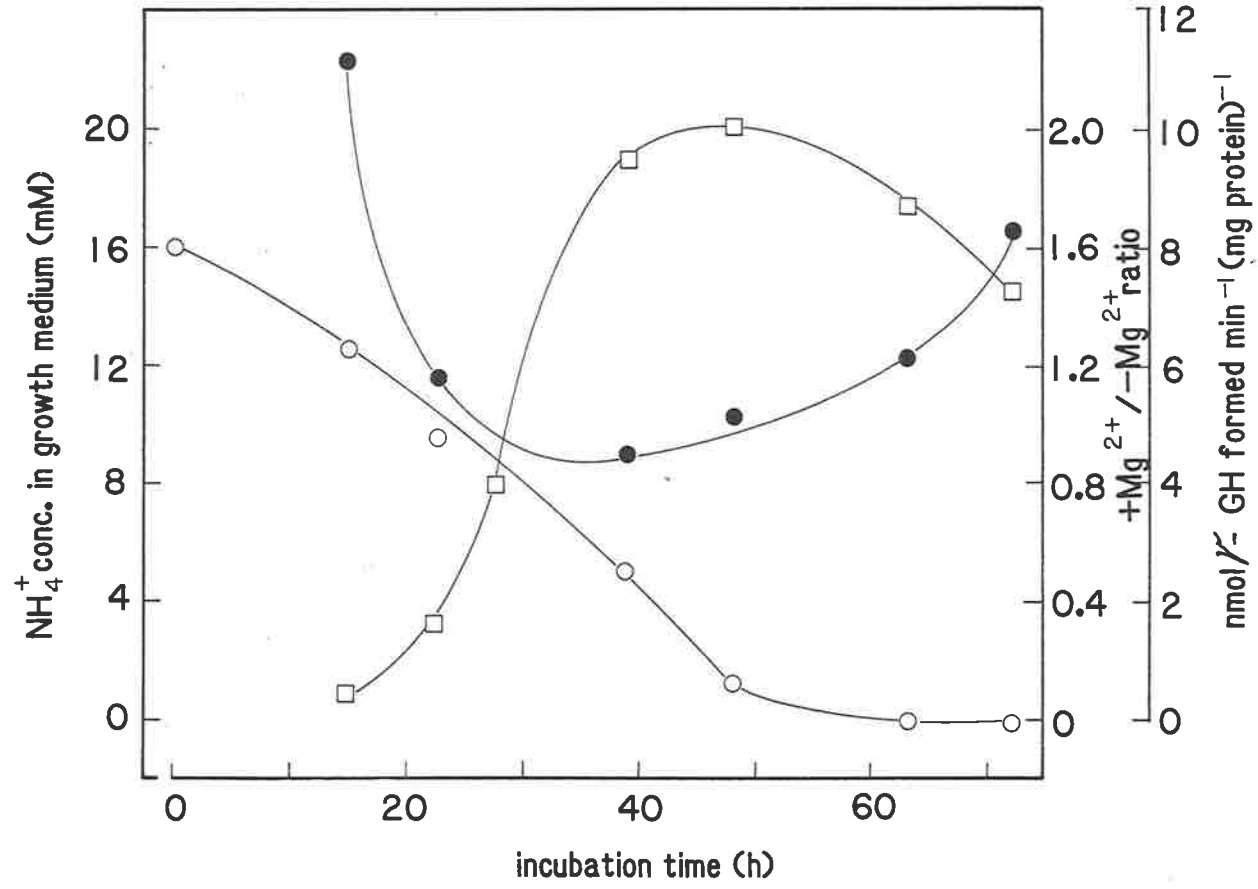
The medium (8ℓ) was inoculated with 2ℓ of a culture which had been depleted of NH_4^+ (the ratio of transferase activity with and without Mg^{2+} was 1.6). The oxidation of ammonia started immediately and after 55h all the NH_4^+ was utilized. The specific activity of GS decreased from about 0.011 $\mu mol\ min^{-1}$ (mg protein)$^{-1}$ at 15h to 0.004 $\mu mol\ min^{-1}$ (mg protein)$^{-1}$ at 40h and then increased thereafter. The $+Mg^{2+}/-Mg^{2+}$ ratio increased from 0.1 at 15h to about 2.0 at 48h when the cultures contained only 1mM residual ammonia indicating that Mg^{2+} effect reflected the adenylylation state of the enzyme.

The effects of snake venom phosphodiesterase treatment on transferase activity of GS with and without 60mM $MgCl_2$, are shown in Fig.33. In these experiments transferase assays were carried out at pH 7.4 in the presence of 0.3mM $MnCl_2$. The Mg^{2+} 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 Mg^{2+} inhibited transferase activity of *Nitrosomonas* enzyme (Fig.33b). Moreover, longer incubation times (up to 4h) and higher phosphodiesterase concentrations (up to 200$\mu g\ ml^{-1}$) did not affect the extent of Mg^{2+} 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 Mg^{2+} (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 grater 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 ($50\mu g$ 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 $MgCl_2$ at pH 7.4. Enzyme activity is expressed as A_{540}. The enzyme preparation was diluted initially so that $50\mu l$ of enzyme produced enough γGH in 15 min to give an absorbance of approx. 0.15 (without added Mg^{2+}).

Untreated enzyme with (●) and without (○) Mg^{2+}, treated enzyme with (□) and without (■) Mg^{2+}.

FIG. 33
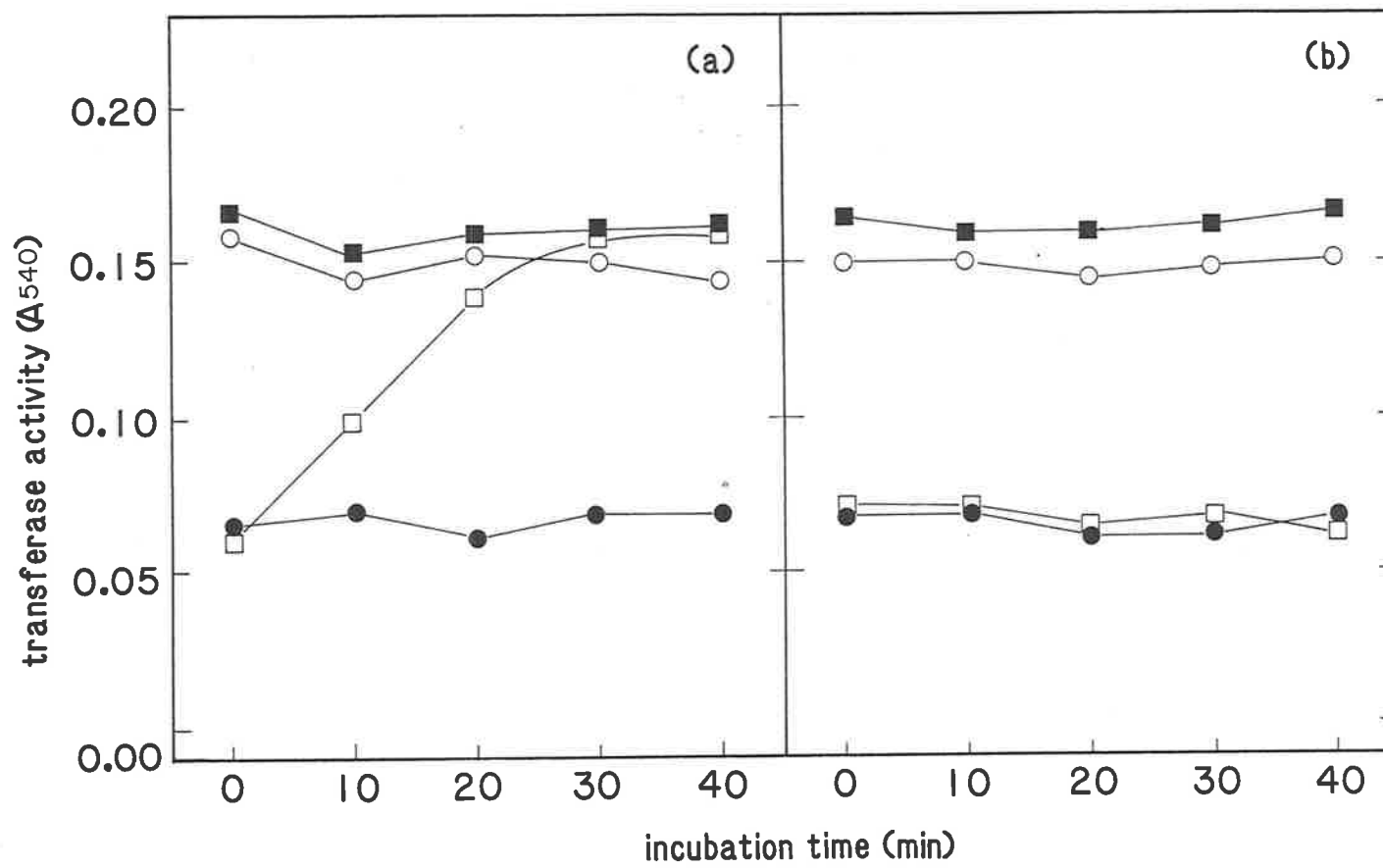
FIG.34: DETERMINATION OF ISOACTIVITY pH FOR PURIFIED GS FROM
NITROBACTER AGILIS.

Phosphodiesterase treatment ($20\mu g\ ml^{-1}$) of purified
glutamine synthetase was as described in Fig.33. The
transferase activity of treated and untreated prepara-
tions was determined at various pH values as
described in Section 2.2.6.2.

Untreated glutamine synthetase (O);
phosphodiesterase treated for 20 min (●);
40 min (□) and 60 min (■) respectively.

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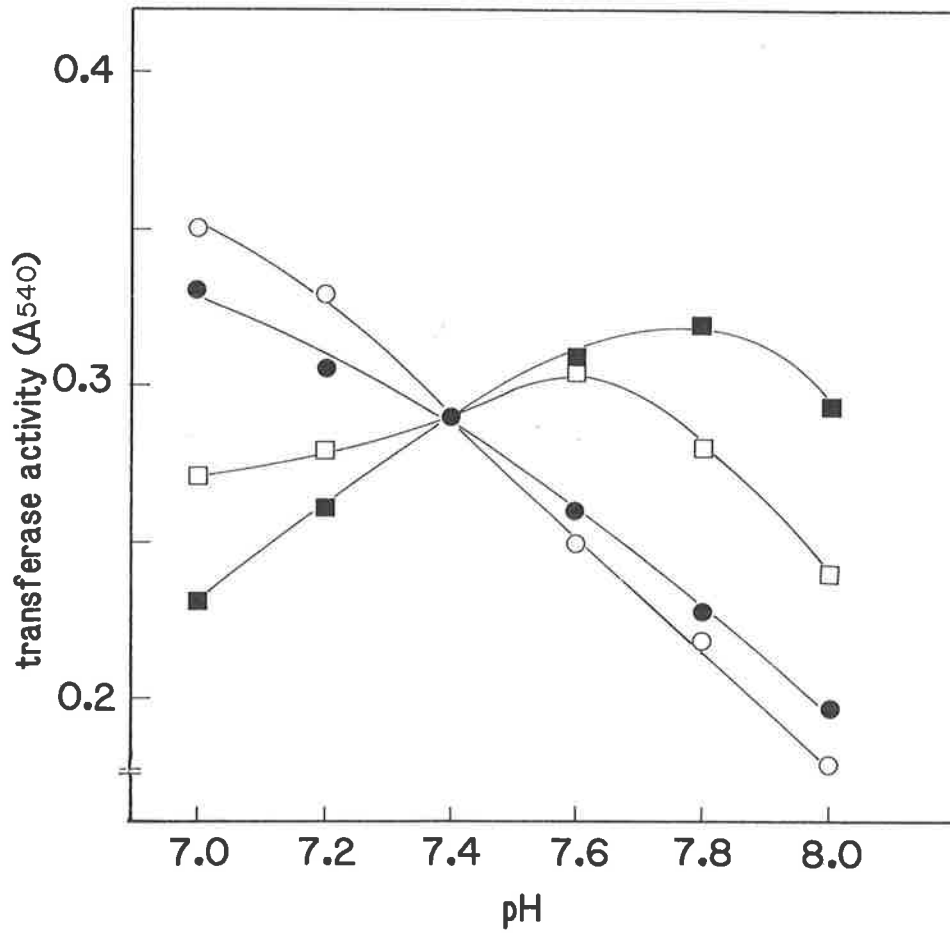
TABLE 26:   EFFECTS OF SOME AMINO ACIDS AND 5'-AMP ON ADENYLYLATED AND
DEADENYLYLATED FORMS OF THE PURIFIES GS FROM *NITROBACTER AGILIS*.

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 γGH produced min^{-1} (mg protein)$^{-1}$
respectively.

| Additions | % Inhibition of transferase activity | |
	GS-AMP	GS
L-Alanine	70	85
L-Glycine	35	60
L-Serine	40	55
5'-AMP	65	36

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 NAD^+ or $NADP^+$ or both (Fig.35).   The activity bands stained in gels A $(NAD)^+$ and B $(NADP^+)$ had different electrophoretic mobilities and those in gel C $(NAD^+ + NADP^+)$ also had two distinct bands.   It is known that 2'-5' ADP Sepharose-4B binds enzymes that require $NADP^+$.   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 NAD^+-GDH only (gel D).   When the affinity column was eluted with 2mM NADPH, the eluate did not contain any NAD^+-GDH (gel E) but $NADP^+$ 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 $NADP^+$-GDH (see Smith *et al.*, 1975).   Only the $NADP^+$ 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 NAD^+ and the other on $NADP^+$ while in *Nitrosomonas europaea* one single $NADP^+$ dependent GDH is present.

3.4.2 *Purification of $NADP^+$-GDH*

The $NADP^+$-GDH from *Nitrobacter agilis* was partially purified by affinity chromatography (Table 27).   The purified enzyme from *Nitrosomonas europaea* reported to be dependent on $NADP^+$ only (Hooper *et al.*, 1967) was also prepared (Table 28).   The crude extracts (S_{30}) of *Nitrobacter agilis* contained both NAD^+ and $NADP^+$ dependent activities (Table 27) while those of *Nitrosomonas europaea* had $NADP^+$-GDH with only negligible NAD^+-dependent activity (Table 28).   In S_{110} fractions the specific activity of $NADP^+$-GDH of *Nitrosomonas europaea* was about 13 fold greater than that of the $NADP^+$ enzyme from *Nitrobacter agilis*.   The $NADP^+$ 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 NAD^+-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$^+$).

FIG.35
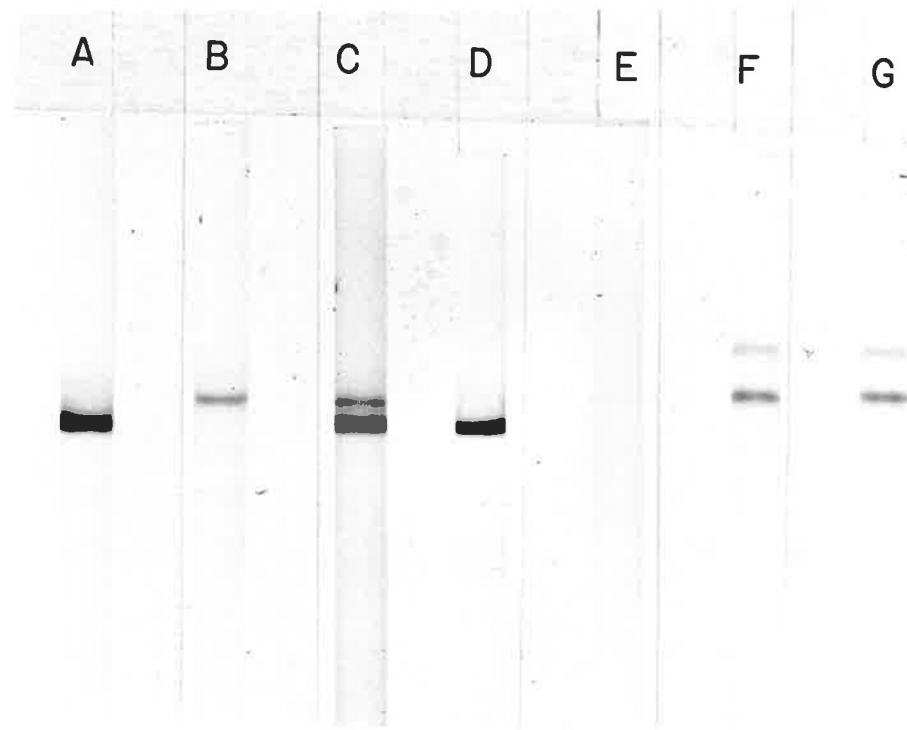
TABLE 27: PURIFICATION OF $NADP^+$-GDH FROM *NITROBACTER AGILIS*.

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 $(mg\ protein)^{-1}$.

Purification step	Total Protein (mg)	Total Units		Specific Activity		Fold Purification	
		$NADP^+$	NAD^+	$NADP^+$	NAD^+	$NADP^+$	NAD^+
S_{30}	121	3111	2133	26	18	1	1
S_{110}	59.5	2609	1649	44	28	1.7	1.5
Pooled 2'5'ADP Sepharose-4B fractions	1	1353	3	1353	–	52	–

TABLE 28: PURIFICATION OF NADP$^+$-GDH FROM *NITROSOMONAS EUROPAEA*.

All purification steps and assay of enzyme activity as described in Sectins 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)$^{-1}$.

Purification step	Total Protein (mg)	Total Units		Specific Activity		Fold Purification	
		NADP$^+$	NAD$^+$	NADP$^+$	NAD$^+$	NADP$^+$	NAD$^+$
S$_{30}$	1360	448000	131	330	0.1	1	1
S$_{110}$	975	402000	0	412	0	1.3	–
Pooled 2'5'-ADP Sepharose-4B fractions	4.95	231000	0	46670	0	142	–

3.4.3 *Amination and deamination reactions in S$_{110}$ fraction*

The NADP$^+$-GDH from *Nitrosomonas europaea* functions in either direction ie. 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_{110}) OF
NITROBACTER AGILIS.

Crude extract of *Nitrobacter agilis* prepared as described in
Section 2.2.3 was further centrifuged at 110,000g and super-
natant (S_{110}) used for enzyme assays.   Amination and de-
amination 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^{-1} (mg protein)$^{-1}$.

Reaction		Specific Activity
Amination:	$NADPH \rightarrow NADP^+$	48.6
	$NADH \rightarrow NAD^+$	25.0
Deamination:	$NADP^+ \rightarrow NADPH$	≤ 2.0
	$NAD^+ \rightarrow NADH$	22.5

FIG.36: DETERMINATION OF pH OPTIMA FOR AMINATION AND DEAMINATION ACTIVITIES OF PARTIALLY PURIFIED $NADP^+$-GDH FROM *NITROBACTER AGILIS*.

Amination ($\bullet$) and deamination ($\bigcirc$) 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. 36

FIG. 37

(a)

(b)
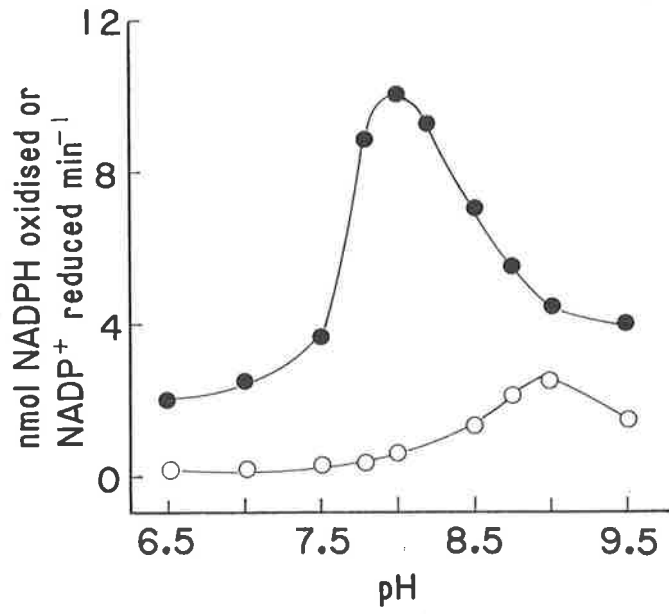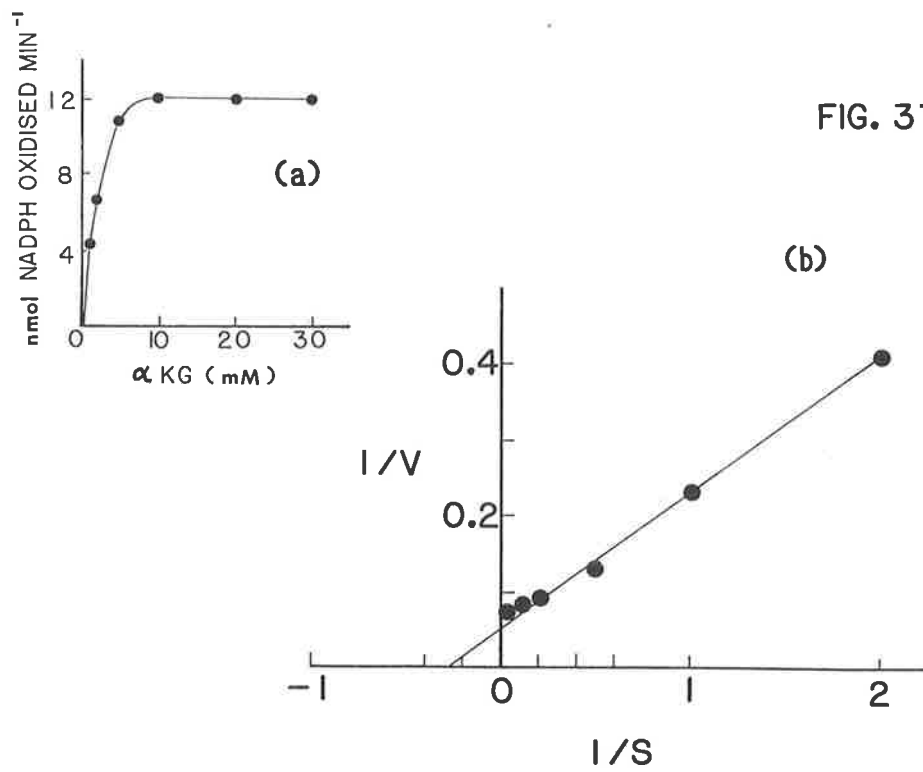
FIG.38: THE EFFECTS OF VARIOUS CONCENTRATIONS OF NH_4Cl ON THE AMINATION REACTION OF PARTIALLY PURIFIED $NADP^+$-GDH FROM *NITROBACTER AGILIS*.

Amination reaction of partially purified $NADP^+$-GDH (Table 27) was determined as described in Section 2.2.6.4 except that NH_4Cl 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_4Cl 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).

FIG. 38

(a)

(b)

FIG. 39

(a)

(b)
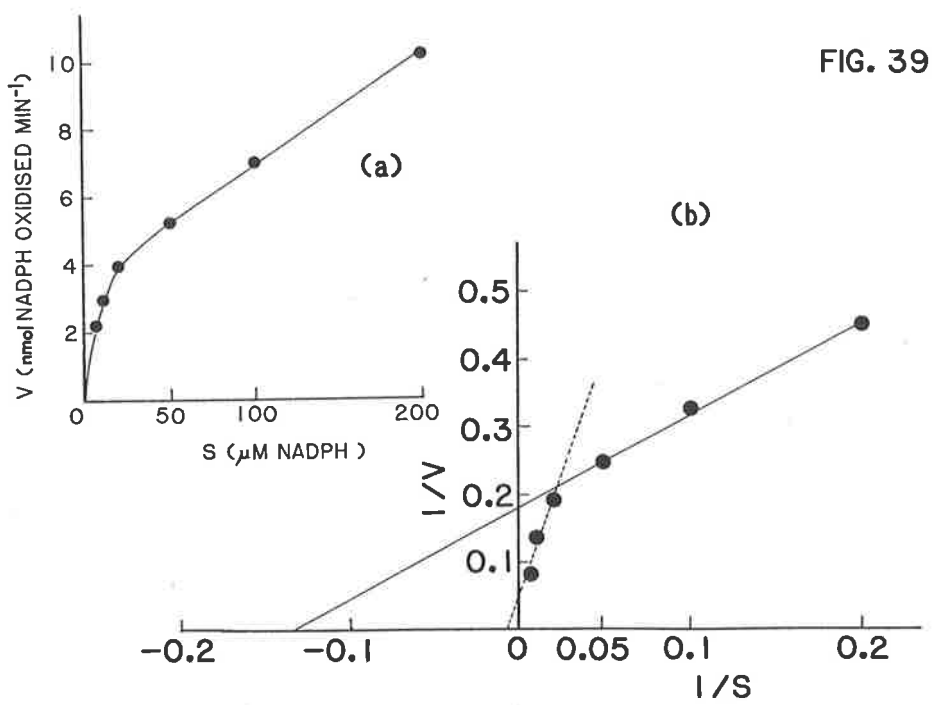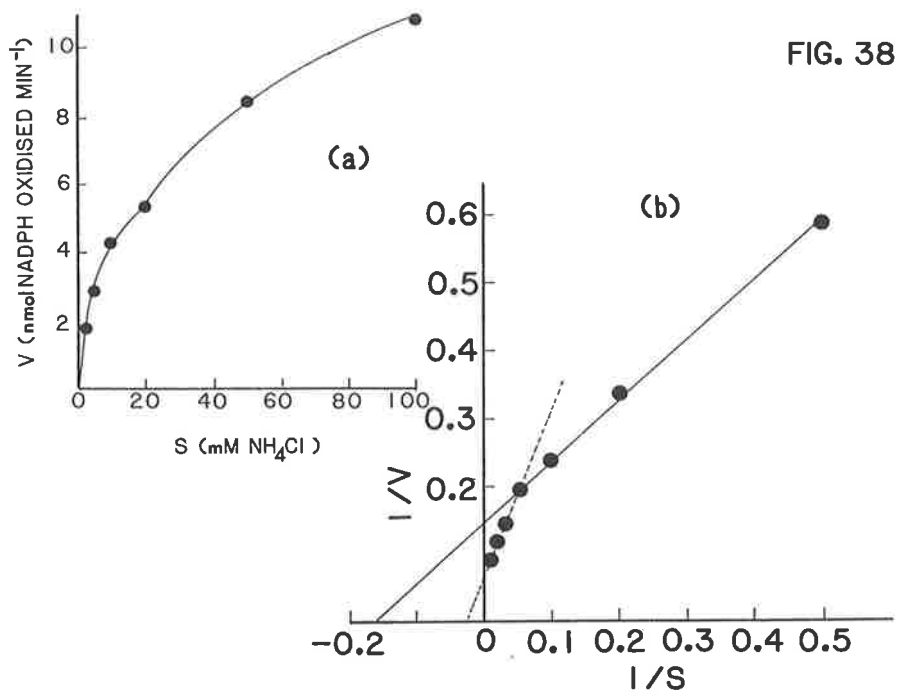
TABLE 30: EFFECTS OF ORGANIC ACIDS ON THE AMINATION ACTIVITY OF PARTIALLY PURIFIED $NADP^+$-GDH FROM *NITROBACTER AGILIS*.

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)$^{-1}$.

Additions[a]	Final concentration (mM)	% Inhibition (−) or stimulation (+)
Fumaric acid	10	−28
	20	−45
Cis oxaloacetic acid	5	+18
	10	+13
	20	+13
α−ketoglutaric acid	100	0
DL−malic acid	20	−13
Pyruvic acid	10	−5
	20	−10

[a]All 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_2OH 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_2OH 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 µmol) 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 1.32μmol NADPH oxidised min^{-1} (mg protein)$^{-1}$.

Additions	Final concentration (mM)	% Inhibition (−) or stimulation (+)
NADPH	0.50	+5
NADP$^+$	0.17	−5
	0.35	−10
NAD$^+$	0.25	0
	0.50	−10
ATP	0.50	0

FIG.40: PROTON EXTRUSION IN WASHED CELLS OF *NITROSOMONAS EUROPAEA*
AND *NITROBACTER AGILIS* DETERMINED BY FLUORESCENCE METHOD.

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 NH_4Cl (0.5mM) or NH_2OH (0.5mM) were
the substrates for *Nitrosomonas europaea* and $NaNO_2$ (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
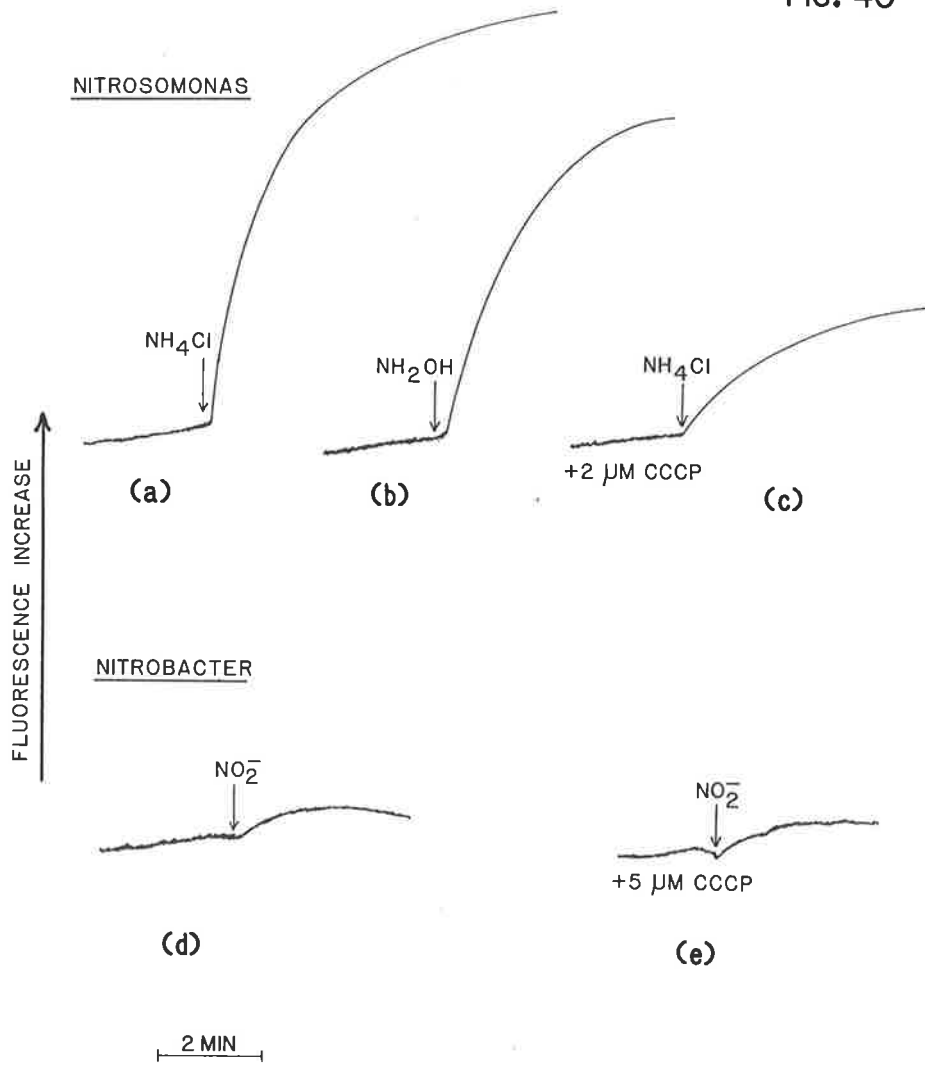
TABLE 32: KINETIC PARAMETERS OF RESPIRATION IN WASHED CELLS OF *NITROSOMONAS EUROPAEA*.

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)$^{-1}$.

Substrate[a]	Respiration rate	K_m	
		Substrate (mM)	O_2 (µM)
Endogenous	20–60		10–20
NH_4Cl	800–1510	1.0	5–10
$NH_2OH.HCl$	400–690	0.3	15–20
$N_2H_4.H_2SO_4$	130–250	1.0	15–20

[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)$^{-1}$.

Preparation	Substrate[a]	Respiration rate	K_m	
			NO_2^- (mM)	O_2 (μM)
Washed cells	Endogenous	20–40		
	NaNO$_2$	400–800	0.8	15–20
Spheroplasts	Endogenous	20–30		
	NaNO$_2$	350–700	0.8	15–20
Vesicles	Endogenous	0		
	NaNO$_2$	30–40	0.8	15–20

[a]The given respiration rates are for 4mM NaNO$_2$.

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_2OH 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 reproducable.
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 ($NaCl_3C_2O_2$), KI, tetraphenyl boron
($NaB(C_6H_5)_4$) and triphenyl-methyl phosphosphonium bromide ($TPMP^+Br^-$)
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 ($NaB(C_6H_5)_4$)
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 $NaCl_3C_2O_2$
produced similar and maximal $\rightarrow H^+/O$ 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 *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 for cells.

Bacterium	Substrate	% Inhibition of respiration				
		KSCN	NaClO$_4$		Na-trichloroacetate	
		0.15M	0.15M	0.30M	0.1M	0.2M
Nitrosomonas europaea	Endogenous	60	80	100		
	2mM NH$_4$Cl	100	34	59		
	2mM NH$_2$OH–HCl	75	23	39		
	2mM N$_2$H$_4$–H$_2$SO$_4$	+240[a]				
Nitrobacter agilis	Endogenous	75		100		
	4mM NaNO$_2$	41		36	35	50

[a]Stimulation of respiration.

of $N_2H_5^+$ oxidation. $NaClO_4$ was an effective inhibitor of endogenous respiration in both bacteria but otherwise was a weak inhibitor. $NaCl_3C_2O_2$ was used only in oxygen pulse studies with *Nitrobacter agilis* and so inhibition studies were carried out only with this organism. Effects of lipophilic cation $TPMP^+$ on NH_4^+ and NH_2OH oxidation by *Nitrosomonas europaea* and NO_2^- oxidation by *Nitrobacter agilis* are shown in Table 35. $TPMP^+$ was a potent inhibitor of oxidation of endogenous substrates and of added NH_4^+ in *Nitrosomonas europaea* at low concentrations (5mM), but was much less effective for NH_2OH oxidation. However $TPMP^+$ even at very high concentrations (100 to 300mM) only partially inhibited NO_2^- oxidation (15 to 20%) by *Nitrobacter agilis*. Thus $TPMP^+$ was suitable for O_2 pulse experiments with *Nitrosomonas europaea* when NH_2OH was the oxidizable substrate and with *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 5µM 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 NH_4^+, NH_2OH and $N_2H_5^+$ oxidation by *Nitrosomonas europaea* and NO_2^- oxidation by *Nitrobacter agilis* are 2,1,1 and 0 respectively for each mole of substrate oxidised:

(i) $\quad\quad NH_4^+ + 3O \longrightarrow NO_2^- + H_2O + 2H^+$

(ii) $\quad\quad NH_2OH + 2O \longrightarrow NO_2^- + H_2O + H^+$

(iii) $\quad\quad N_2H_5^+ + 2O \longrightarrow N_2 + 2H_2O + H^+$

(iv) $\quad\quad NO_2^- + O \longrightarrow NO_3^-$
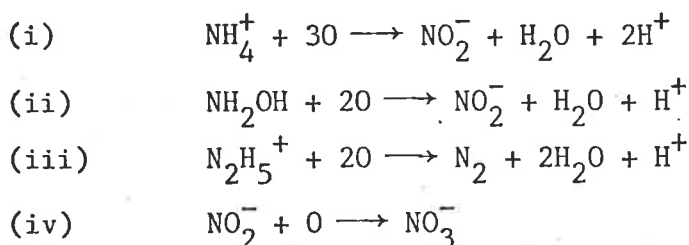
The results shown in Table 36 for the oxidation of NH_4^+, NH_2OH and $N_2H_5^+$ by *Nitrosomonas europaea* at pH 7.4 and of NO_2^- by *Nitrobacter agilis* at pH 7.8 are in reasonable agreement with the values expected from equations (i) to (iv) and the following pK values (Jencks and Rogenstein, 1968):

NH_4^+, 9.25; NH_3OH^+, 5.96; $N_2H_5^+$, 8.1; HNO_2, 3.29.
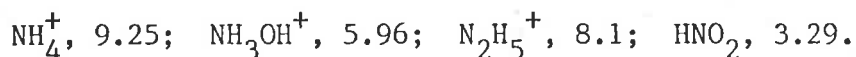
TABLE 35: EFFECTS OF LIPOPHILIC CATION TPMP$^+$ ON AEROBIC OXIDATION OF
SUBSTRATES BY WASHED CELLS OF *NITROSOMONAS EUROPAEA* AND
NITROBACTER AGILIS.

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.

Bacterium	TPMP$^+$ concentration (mM)	Substrate[a]	% Inhibition of respiration
Nitrosomonas europaea	10	Endogenous	100
	5	NH_4Cl	80
	10	NH_4Cl	100
	5	$NH_2OH.HCl$	5
	15	$NH_2OH.HCl$	50
Nitrobacter agilis	50	KNO_2	5
	100	KNO_2	12
	200	KNO_2	25
	300	KNO_2	50

[a]Concentration of substrates, NH_4Cl and NH_2OH were 5mM and for KNO_2 2mM.

TABLE 36:   PRODUCTION OF STOICHIOMETRIC PROTONS BY *NITROSOMONAS EUROPAEA* AND *NITROBACTER AGILIS*.

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.

Bacterium	Substrate	$\rightarrow H^+/O$ ratio in presence of CCCP	$\rightarrow H^+$/substrate ratio in reductant pulse experiments
Nitrosomonas europaea	Endogenous	0 – 0.15	
	NH_4Cl	0.68 ± 0.04 (0.66)	1.9 ± 0.1 (1.99)
	$NH_2OH.HCl$	0.48 ± 0.05 (0.52)	1.02 ± 0.06 (1.04)
	$N_2H_4-H_2SO_4$	0.46 ± 0.05 (0.42)	0.89 ± 0.07 (0.83)
Nitrobacter agilis	Endogenous	0	
	$NaNO_2$	0.0 ± 0.06 (0.0)	undetermined

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 $\rightarrow H^+/O$ 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 200ng atom) pulses of O_2. In one batch of cells out of 13 the endogenous substrate became permanently exhausted following oxygen pulses. The $\rightarrow H^+/O$ ratios for endogenous substrate oxidation were independent of the amount of O_2 per pulse from 5 to 40ng 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 $\rightarrow H^+/O$ ratios obtained with the amine-like substrates at concentrations >2mM referred largely or entirely to the oxidation of these substrates and not to endogenous sub-strates. This result was consistent with the data in Table 32 which show that the rates of oxidation of endogenous substates 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 $\rightarrow H^+/O$ ratio was essentially independent of the size of the oxygen pulse up to about 30ng 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 con-centration $< K_m$ (O_2). The relevant $\rightarrow H^+/O$ 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 $\rightarrow H^+/O$ ratios in turn depended on the concentrations of NH_4^+, NH_2OH and $N_2H_5^+$ (Fig.43). At high concentrations of these substrates (20-40mM) the $\rightarrow H^+/O$ ratios approached the stoichiometric value (Table 36) for the reaction, eg. 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 O_2 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 NH_2OH oxidation in cells depleted of endogenous substrates and with 150mM KSCN as permeant ion. Fig.41d illustrates the fast equilibriation of protons after addition of 4μM CCCP to the reaction mixture.

FIG. 41

10µl KCl/O₂

10µl KCl/O₂

CCCP
4µM

1 min.

d
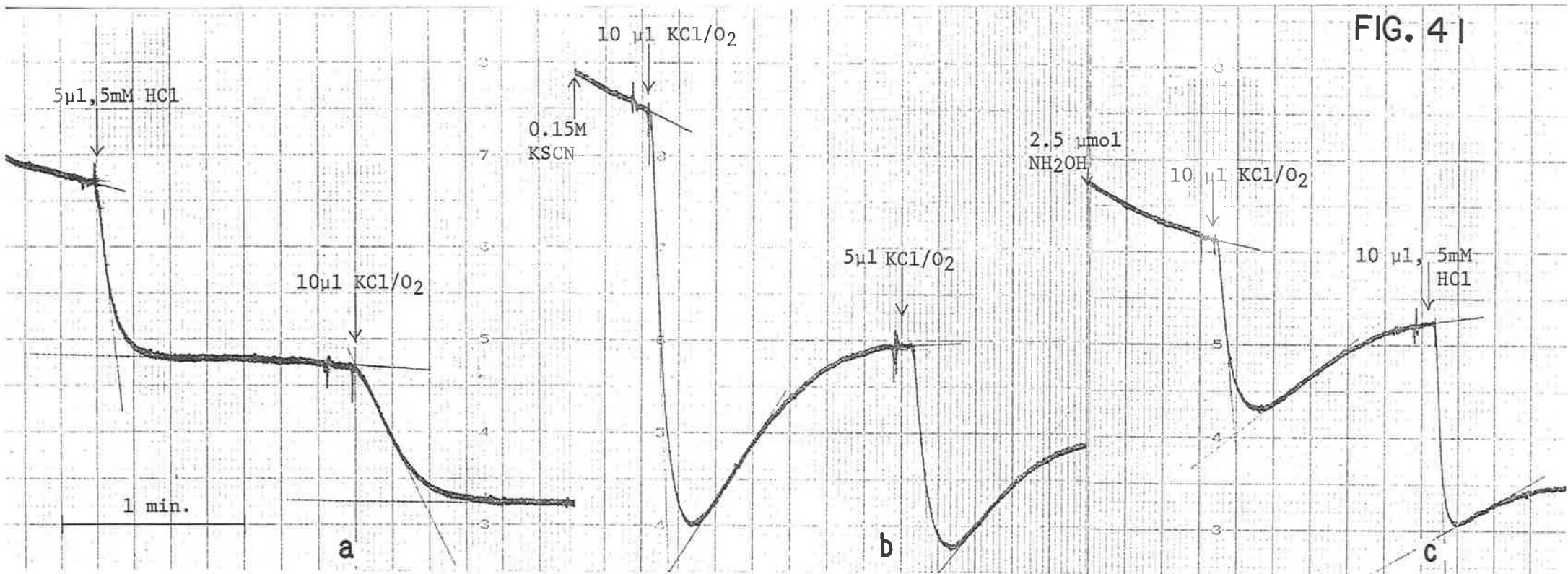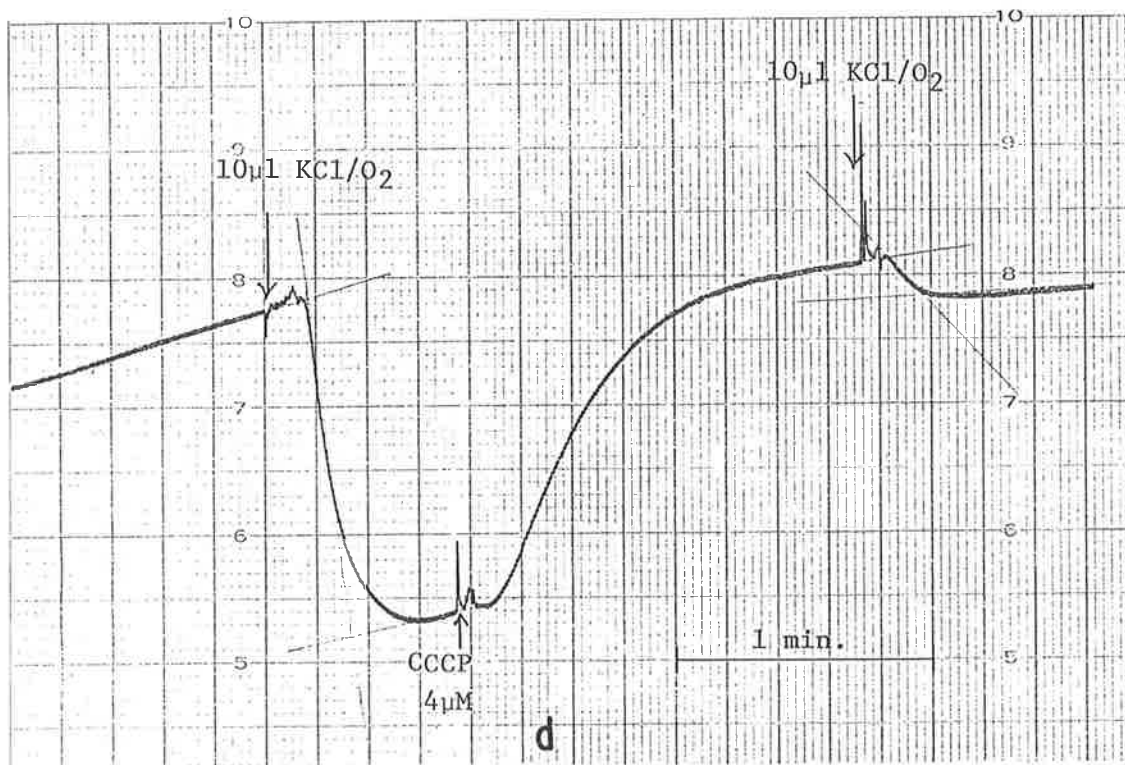
FIG.42: DEPENDENCE OF $\rightarrow H^+/O$ RATIO ON THE SIZE OF THE OXYGEN PULSE FOR OXIDATION OF NH_2OH BY *NITROSOMONAS EUROPAEA*.

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 (△).

FIG.43: DEPENDENCE OF $\rightarrow H^+/O$ RATIO ON THE SUBSTRATE CONCENTRATION FOR OXIDATION OF AMINE-LIKE SUBSTRATES BY *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 $N_2H_5^+$(△). Each point on the curves represents the asymptotic limit or plateau inferred from an experiment of the type illustrated in Fig.42.

FIG. 42
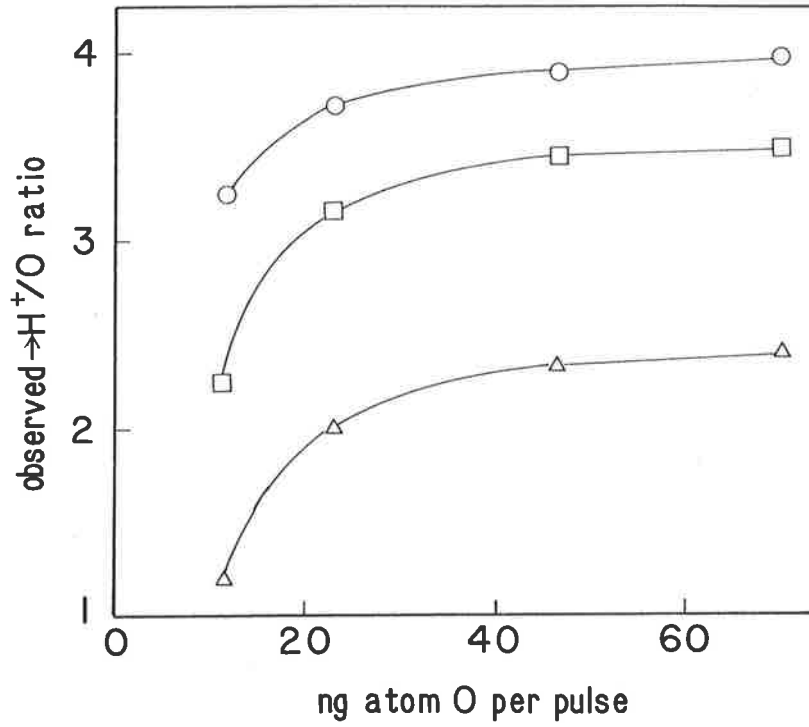
FIG. 43
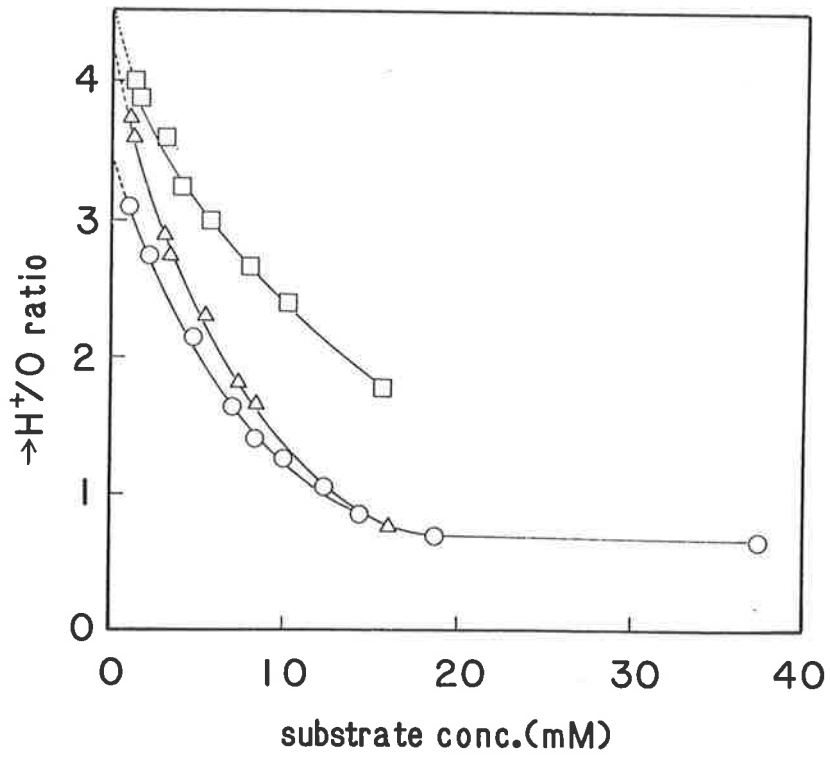
of respiration.   The respiratory proton pump would appear to be inhibited
at high concentrations of these amine-like substrates. · In this regard methyl-
ammonium $(CH_3NH_3^+)$ which is not oxidised by *Nitrosomonas europaea* mimicked the
effect of the amine-like substrate in diminishing H^+/O ratios.   For example,
20mM $CH_3NH_3^+$ lowered the $\rightarrow H^+/O$ 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 $\rightarrow H^+/O$ 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_2OH and $N_2H_5^+$.   The data for NH_2OH and $N_2H_5^+$ (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_2OH 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:

$$NH_2OH + NO_2^- \longrightarrow N_2O + H_2O + OH^-$$
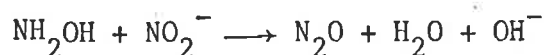
Such a reaction could, if rapid, provide a different overall reaction and possi-
bly a different H^+/O ratio:

$$NH_2OH + O \longrightarrow \tfrac{1}{2} N_2O + 1 \tfrac{1}{2} H_2O$$
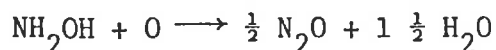
Pulse experiments carried out with NO_2^- instead of O_2 with NH_4^+ and NH_2OH as
substrates indicated that no proton translocation occurred.   Moreover the
rate of anaerobic reduction of NO_2^- by NH_2OH in *Nitrosomonas europaea* was very
low.   Thus these reactions were not considered to be kinetically 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 tripenyl 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 *NITROSOMONAS 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.

Bacterium	TPMP$^+$ concentration (mM)	Substrate[a]	Apparent $\rightarrow$H$^+$/O ratios
Nitrosomonas europaea	5	endogenous	b
	5	NH$_4$Cl	b
	5	NH$_2$OH	3.5-4.0
	15	NH$_2$OH	3.5-4.0
Nitrobacter agilis	50	KNO$_2$	$\leq$0.1
	100-300	KNO$_2$	$\leq$0.05

[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 $\rightarrow$H$^+$/O 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 $\rightarrow$H$^+$/O 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 4μM 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
(50μg 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 $\rightarrow$H$^+$/O 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 (50μg 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
$\rightarrow$H$^+$/O ratio of about -0.2 (Fig.45a).   With 2 and 4mM NO_2^- the initial
alkalinization was smaller ($\rightarrow$H$^+$/O = 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 ΔpH
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 compart-
ment 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. 44
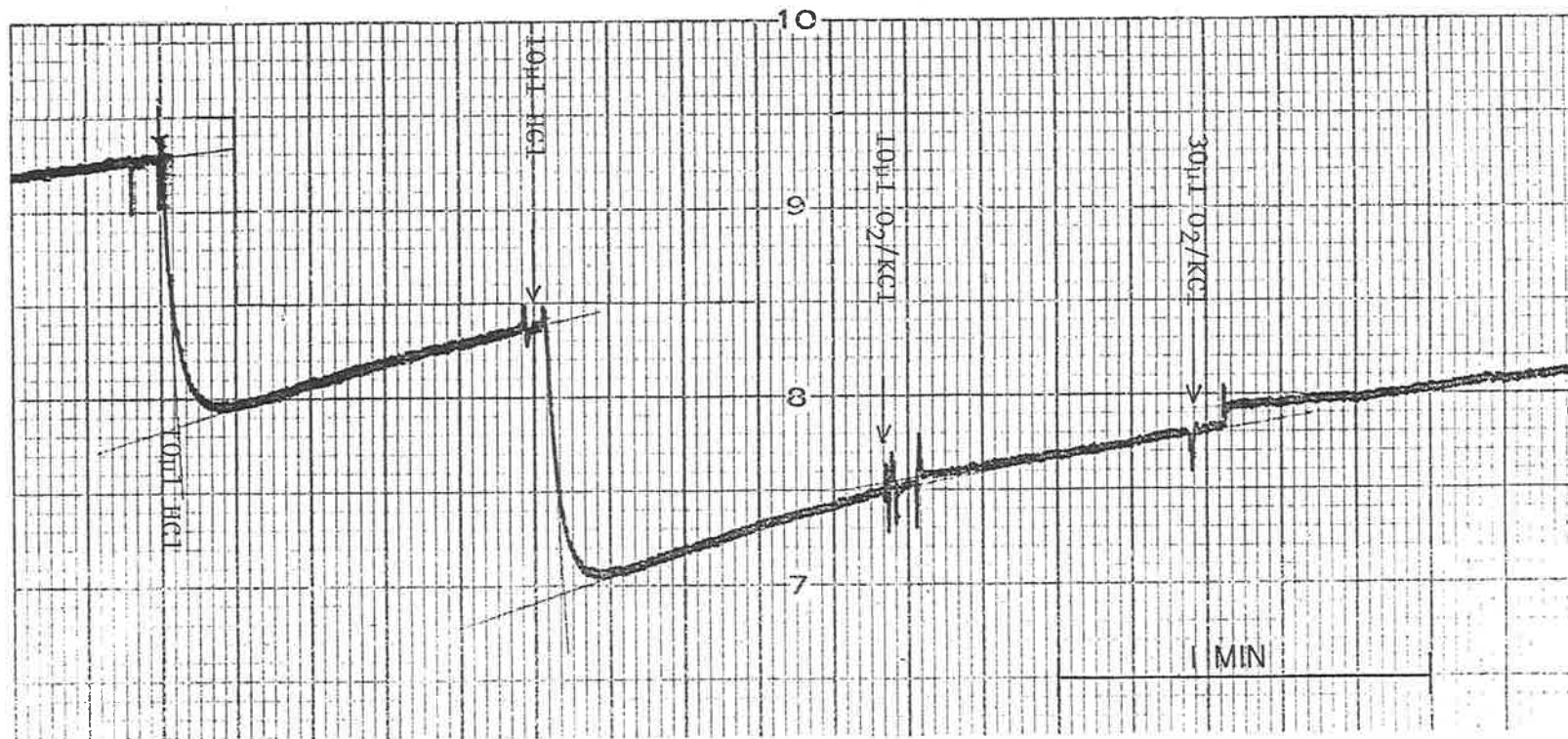
FIG.45: OXYGEN PULSE RESPONSES IN MEMBRANE VESICLES OF *NITROBACTER AGILIS*.

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 ($\approx$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.

FIG. 45

(a) endogenous substrate val/K$^+$

10μl HCl (5mM)

10μl KCl/O$_2$

(b) NO$_2^-$ substrate −KSCN

10μl KCl/O$_2$

10μl KCl/O$_2$

6μmol NO$_2^-$

170μl 1.5M KSCN

10μl KCl/O$_2$

30μl KCl/O$_2$

(c) NO$_2^-$ substrate +KSCN

1 min.
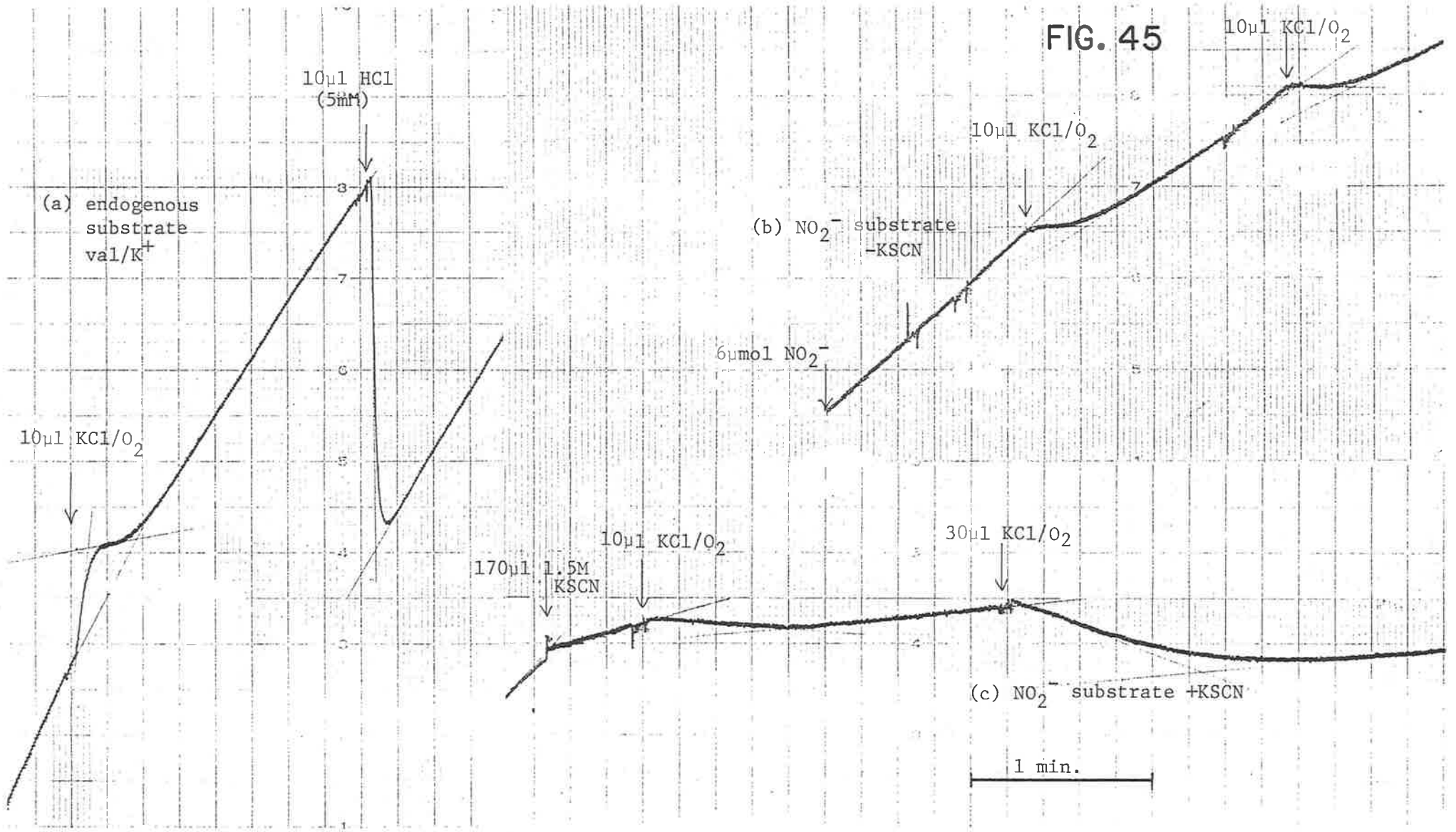
3.5.1.6 Duration of respiration after an oxygen pulse

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 O 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 O 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 (ΔpH) 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 ΔpH 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 ΔpH 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 Δψ AND ΔpH IN WASHED CELLS OF *NITROSOMONAS EUROPAEA* AND *NITROBACTER AGILIS*.

Washed cell suspensions in 50mM Na-phosphate buffer (pH 6.0) were used to determine ΔpH and Δψ 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).   ΔpH and Δψ are the mean values of 10 determinations with the same batch of cells.

| Bacteria | Probe | ΔpH ± SEM | | Δψ ± SEM (mV) | |
		Untreated cells	EDTA-treated cells	Untreated cells	EDTA-treated cells
Nitrosomonas europaea	[^{14}C] Benzoic Acid	0.22 ± 0.12	0.33 ± 0.03		
	[^{14}C] Acetyl salicylic acid	0.20 ± 0.12	0.28 ± 0.03		
	[^{14}C] Methylamine-HCl	0.0 ± 0.01	0.0 ± 0.01		
	[^{3}H] TPP^{+}			100 ± 15	125 ± 5
Nitrobacter agilis	[^{14}C] Benzoic Acid	0.80 ± 0.10	1.36 ± 0.05		
	[^{14}C] Acetyl salicylic acid	0.72 ± 0.12	1.22 ± 0.06		
	[^{14}C] Methylamine-HCl	0.00 ± 0.00	0.0 ± 0.0		
	[^{3}H] TPP^{+}			82 ± 8	105 ± 5

FIG.46: UPTAKE OF [^{14}C] BENZOIC ACID, [^{14}C] ACETYL SALICYLIC ACID
AND [^{14}C] METHYLAMINE AS A FUNCTION OF pHe 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}C] benzoic acid (O);   [^{14}C] acetyl salicylic acid (●);
and [^{14}C] methylamine (□).

FIG. 46
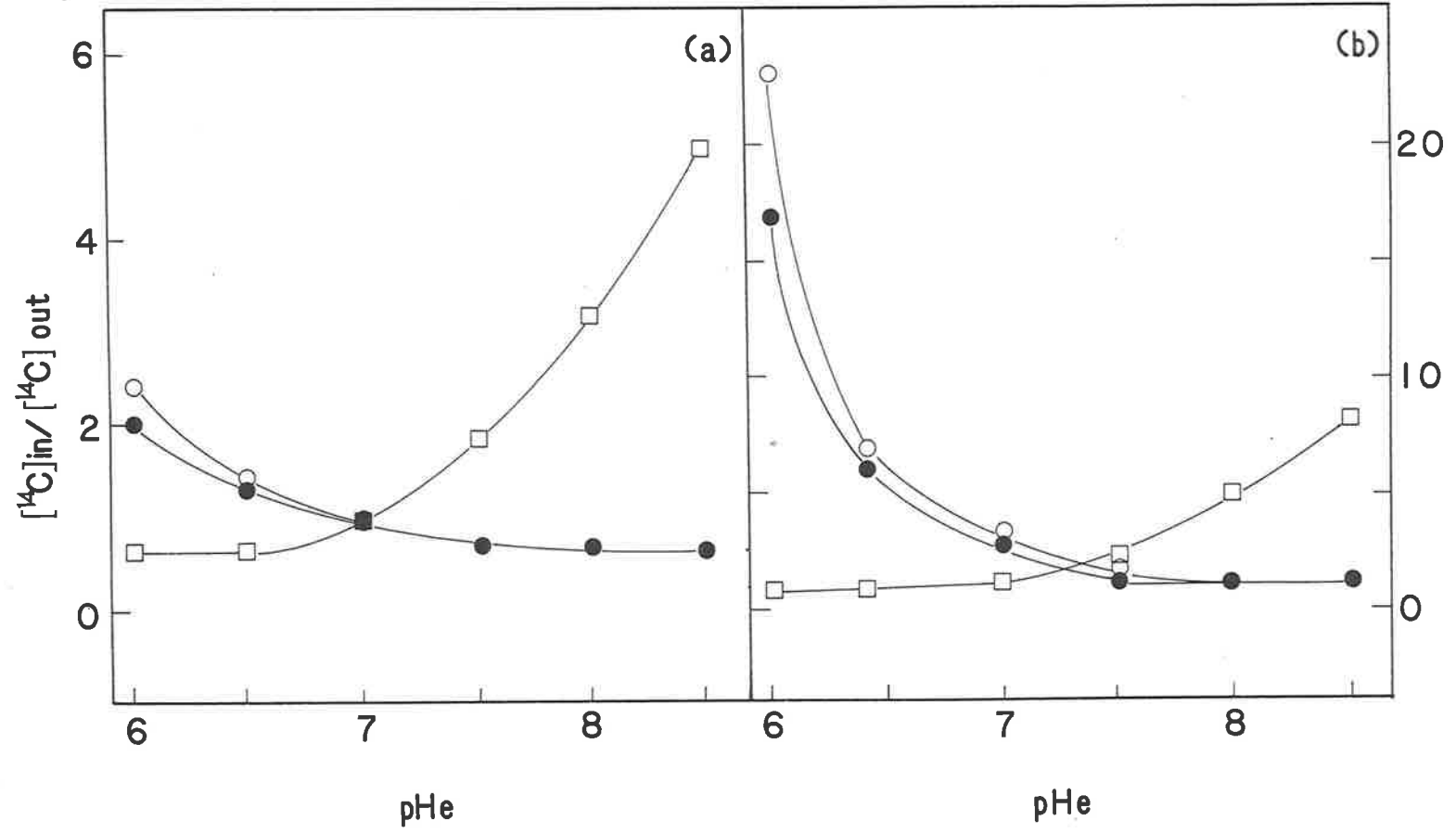
the pHe at which neither of the two weak acids nor weak base was taken up by the cells, was about 7.5. Benzoic and acetyl salicylic acids were not metabolized by either bacterium. *Nitrosomonas europaea* however slowly utilized the weak base methylamine when the external pH was greater than 7.5. Because methylamine 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 pHi and thus it increased from 6.3 to 7.8 when the pHe was varied over the range 6.0 to 8.5 (Fig.47a). On the other hand in *Nitrobacter agilis* (Fig.47b) pHi increased from 7.3 to 7.8 when the pHe was increased from 6.0 to 8.5. Thus at pHe 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 pHi are either similar to pHe or more acidic (inside acid). Over a range of pHe (6.0 to 8.5), the pHi 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 $\Delta\psi$ as a function of pHe

The variations in $\Delta\psi$ as determined by $[^3H]TPP^+$ uptake over a pHe 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 $\Delta\psi$ increased with increasing pHe. Thus in *Nitrosomonas europaea* $\Delta\psi$ increased from 125mV at pHe 6.0 to 178 at pHe 8.5. In *Nitrobacter agilis* the effect of pHe on $\Delta\psi$ was less pronounced than in *Nitrosomonas europaea*, thus it increased from 105mV at pHe 6.0 to 135mV at pHe 8.5, an increase of approximately 10mV for each pH unit. The increase in $\Delta\psi$ 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 $\Delta\psi$ and ΔpH, it is clear from Fig.47a that Δp remained almost constant (135-145mV) in *Nitrosomonas europaea*

FIG.47: EFFECTS OF pHe ON pHi, ΔpH, $\Delta\psi$ 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
($\blacktriangle$) represented in terms of mV (59 x ΔpH) were determined
from the uptake of [^{14}C] benzoic acid and [^{14}C] methylamine.
$\Delta\psi$ values (O) were calculated from the uptake of [^{3}H] TPP^{+}.
Δp ($\bullet$) was calculated from ΔpH and $\Delta\psi$ as described in
Section 2.2.13.4.

FIG.47
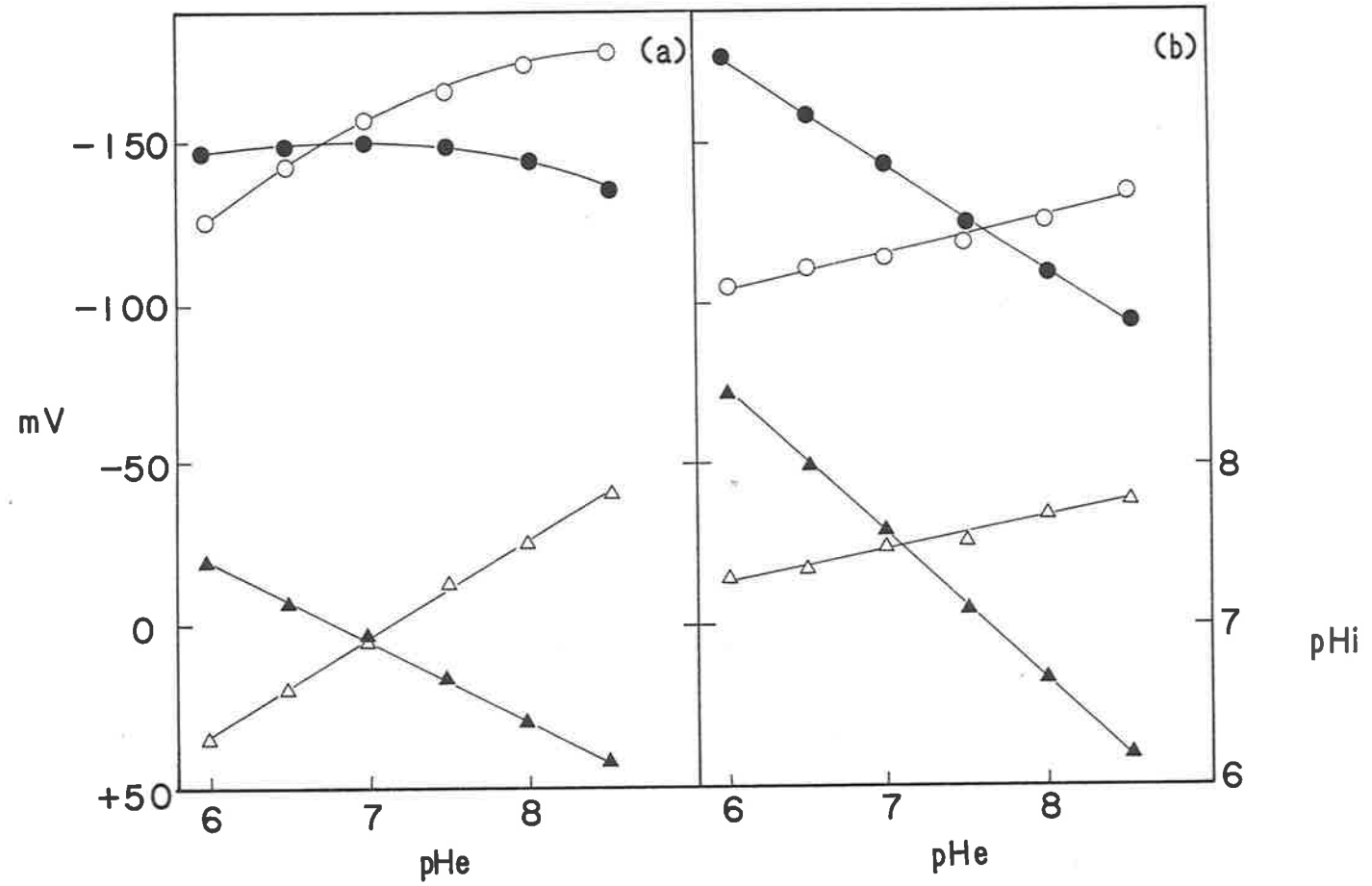
over a range of external pH. This was largely the result of an increase in $\Delta\psi$ and a decrease in ΔpH when the pHe was increased from 6.0 to 8.5, thus a decrease in ΔpH was compensated by an increase in $\Delta\psi$. In *Nitrobacter agilis* (Fig.47b), however, the contribution of ΔpH decreased rapidly when the pHe was increased (-73mV at pH 6.0 to + 40mV at pH 8.5), while $\Delta\psi$ increased by 30mV only from pHe 6 to 8.5, thus decreasing the total proton-motive force from 177mV at pHe 6.0 to 95mV at pHe 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 ΔpH 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 ΔpH and $\Delta\psi$ at various stages of growth, cultures (1ℓ) were harvested at various times as shown in Fig.48a and b. Thus ΔpH and $\Delta\psi$ were determined at two pHe 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 pHi (Fig.48a,b). Thus at pHe 6.0, $\Delta\psi$ was approximately 122mV and ΔpH 0.3 units (inside alkaline) and at pHe 8.0, $\Delta\psi$ was approximately 165mV and ΔpH 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 Δp

To determine the relevance of respiratory potential to Δp maintenance, the effects of respiratory inhibitors on ΔpH 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 Δp (Table 39). In *Nitrobacter agilis* (Table 40), sodium azide at 50µM completely

FIG.48: INTRACELLULAR pH AND $\Delta\psi$ IN CELLS OF *NITROSOMONAS EUROPAEA* AT VARIOUS STAGES OF GROWTH DETERMINED AT pHe VALUES OF (a) 6.0 AND (b) 8.0.

Cultures (18ℓ) were grown in 20ℓ 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 NH_4^+ oxidation and by determining the protein contents of the cells. At the times indicated, 1-to 2ℓ 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. $\Delta\psi$ (O) was calculated from the uptake of [^{3}H] TPP$^+$. Intracellular pH (□) was determined by the uptake of [^{14}C] benzoic acid and [^{14}C] methylamine at pHe 6.0 and 8.0 respectively.

FIG. 48
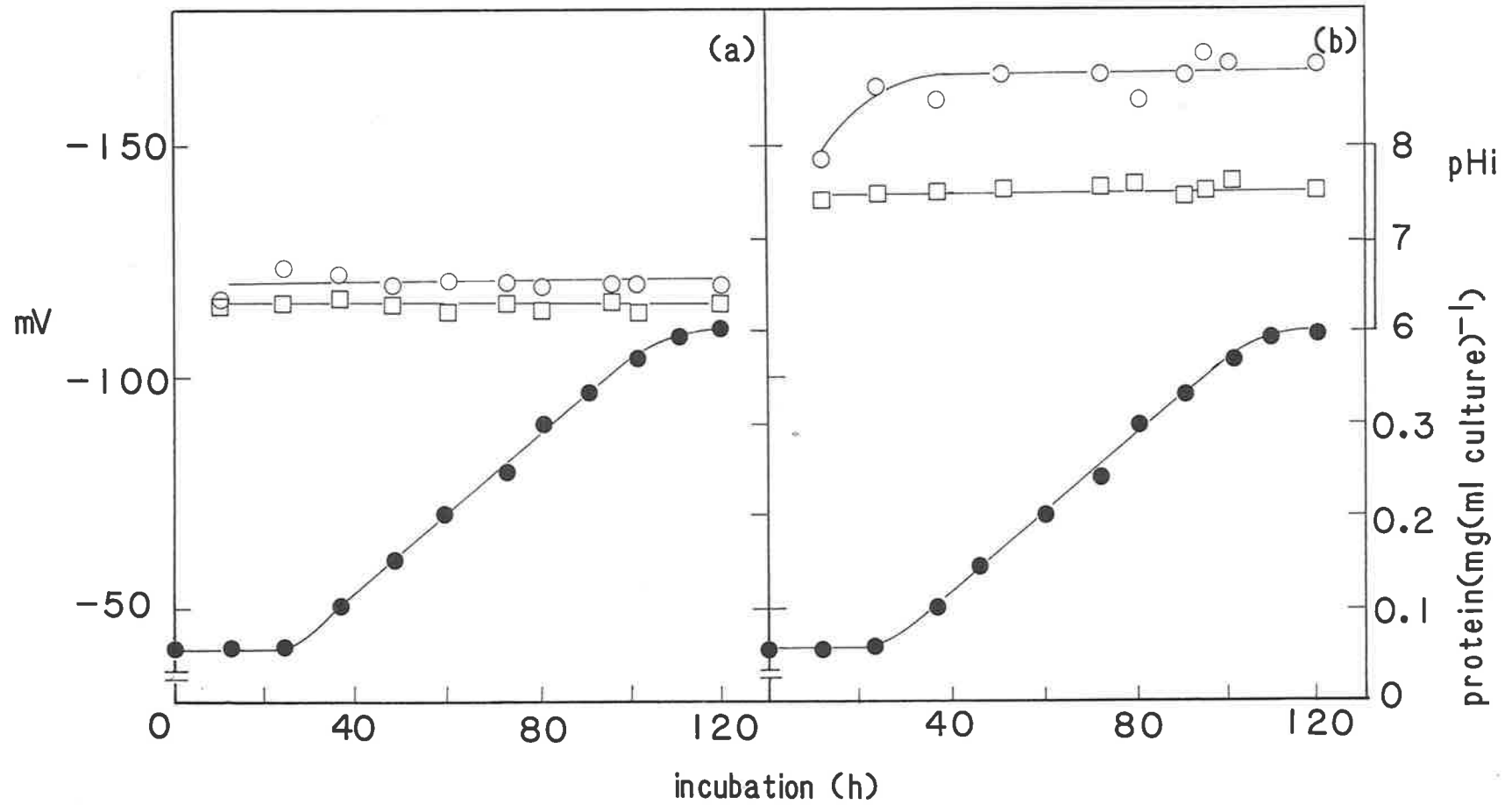
TABLE 39: EFFECTS OF SOME INHIBITORS ON RESPIRATION AND PROTON-MOTIVE FORCE IN *NITROSOMONAS EUROPAEA*.

Washed cell suspensions in 50mM Tris-HCl at pH 6.9 were employed for $\Delta\psi$ and ΔpH determinations. $\Delta\psi$ and ΔpH were calculated from the uptake of [^{3}H] TPP$^+$ and [^{14}C] benzoic acid repectively 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)$^{-1}$.

Inhibitor	Concentration (μM)	% Inhibition of (NH_4^+) respiration	$\Delta\psi$[a] (mV)	ΔpH[b]	Δp[c] (mV)
None			147	0.18	158
DIECA	20	100	148	0.00	148
CCCP	10	80	110	0.10	116
	100	100	80	0.00	80
DCCD	200	50	117	0.16	128
DESB	50	25	187	0.50	216

[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 $\Delta\psi$ and ΔpH determinations. $\Delta\psi$ and ΔpH were calculated from
the uptake of $[^3H]$ TPP$^+$ and $[^{14}C]$ 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 650 ng atom O min^{-1}
(mg protein)$^{-1}$.

Inhibitor	Concentration (µM)	% Inhibition of respiration	$\Delta\psi^a$ (mV)	ΔpH^b	Δp^c (mV)
None			115	0.34	138
Sodium azide	50	100	114	0.05	117
CCCP	10	70	82	0.20	94
	50	100	75	0.12	82
DCCD	100	55	116	0.38	138
	250	100	118	0.08	113
DESB	20	45	124	0.31	142
	50	85	133	0.09	139

[a]Inside negative

[b]Inside alkaline

[c]Inside 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 ΔpH completely, thus lowering Δp 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 Δp in both Nitrosomonas europaea (Table 39) and Nitrobacter agilis (Table 40) were investigated. The respiration of both nitrifiers was completely inhibited by 50μM CCCP but Δp 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 Δp, two ATPase inhibitors, DCCD and DESB, were used in studies on $\Delta\psi$ and ΔpH.   DCCD at high concentrations (>200μM) affected Δp in both nitrifiers by lowering $\Delta\psi$ in Nitrosomonas europaea (Table 39) and ΔpH in Nitrobacter agilis (Table 40).   DESB had little or no effect on Δp in Nitrobacter agilis (Table 40) but at 50μM it elevated Δp by about 60mV (negative inside) in Nitrosomonas europaea.

3.5.2.7 Effects of NH_4^+ and NH_2OH on $\Delta\psi$ and ΔpH

Permeant amines and amine-like compounds have a tendency to re-distribute 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_2OH or $N_2H_5^+$) in Nitrosomonas europaea tended to diminish proton pumping (Section 3.5.1.4).   As shown in Fig.49, both NH_4^+ and NH_2OH at high concentrations diminished the small ΔpH across the cell membranes of Nitrosomonas europaea completely. Moreover, NH_4^+ also decreased $\Delta\psi$ (170mV to 140mV at 100mM NH_4^+) but relatively small concentrations of NH_2OH ($\approx$20mM) rapidly dissipated $\Delta\psi$ by about 60mV.   Increasing the NH_2OH beyond 20mM did not dissipate $\Delta\psi$ any further.   Nitrobacter agilis does not oxidise NH_4^+ or NH_2OH, but it can assimilate small amount of NH_4Cl (Section 3.2.1).   High concentraions of NH_4^+ (>10mM) dissipated ΔpH completely and $\Delta\psi$ partially (20mV at 100mM NH_4^+).

FIG.49: EFFECTS OF NH_4^+ AND NH_2OH ON $\Delta\psi$ AND ΔpH 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 treat-
ment of the suspensions with either NH_4Cl (O) or NH_2OH (●)
at indicated concentrations. ΔpH was calculated from the
distribution of $[^{14}C]$ benzoic acid in the presence of either
NH_4Cl (▼) or NH_2OH (△). For details of uptake studies see
Section 2.2.13.3.

FIG.49
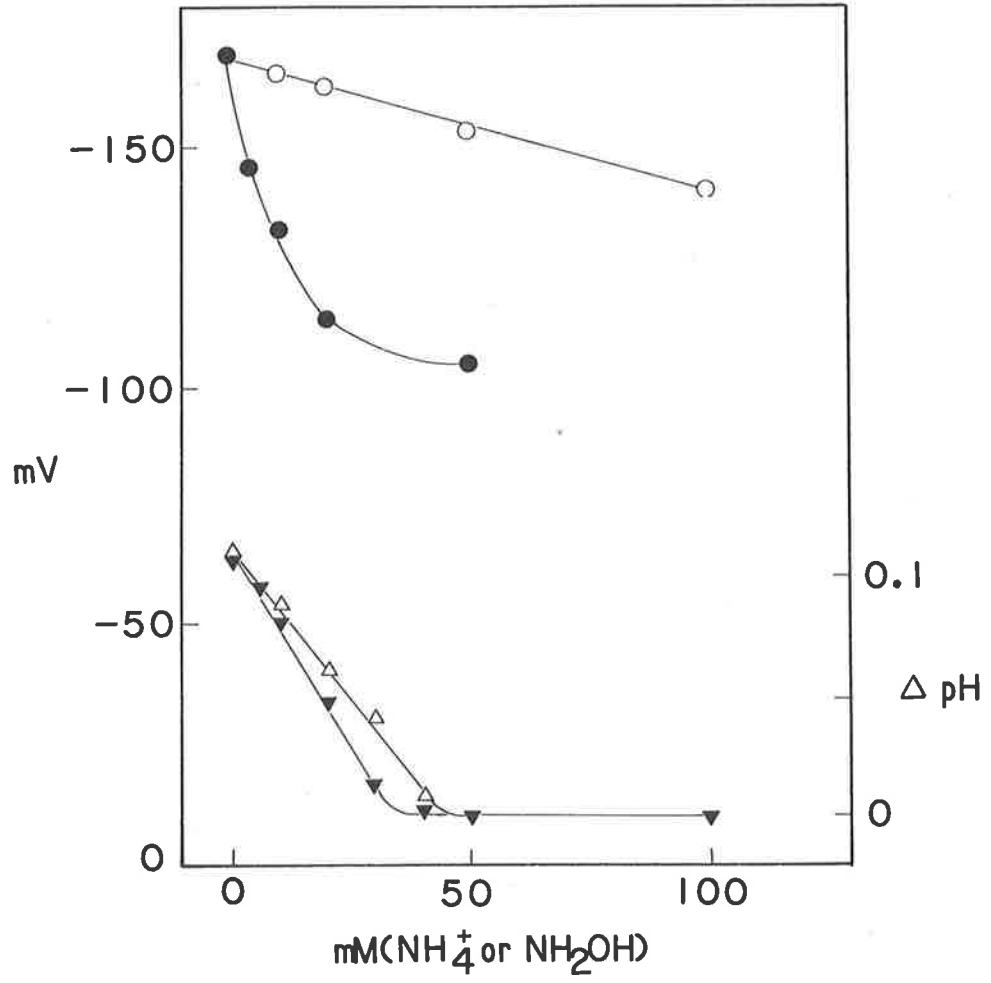
3.5.3 *ATP biosynthesis driven by artificially induced proton motive force*

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 substate oxidation is completely inhibited. The addition of valinomycin (100μg) 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 DIECA (50μM), 2-chloro-6-trichloromethyl pyridine (N-serve) (100μM) 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
NH_4Cl 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 NH_4^+ 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 sus-
pensions (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 NH_4Cl (●) or 100µg valino-
mycin (O) was added.   At the times indicated 0.4ml aliquots
were removed for ATP assay by firely method as described in
Section 2.2.17.5.   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 sus-
pended in 100ml of 0.1M sodium phosphate containing 0.2M
sucrose and 1mM $MgCl_2$.   For NH_4^+ 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 NH_4Cl (●) or 100µg valinomycin (O) was added.
Further details are given in Fig.50.

FIG.50
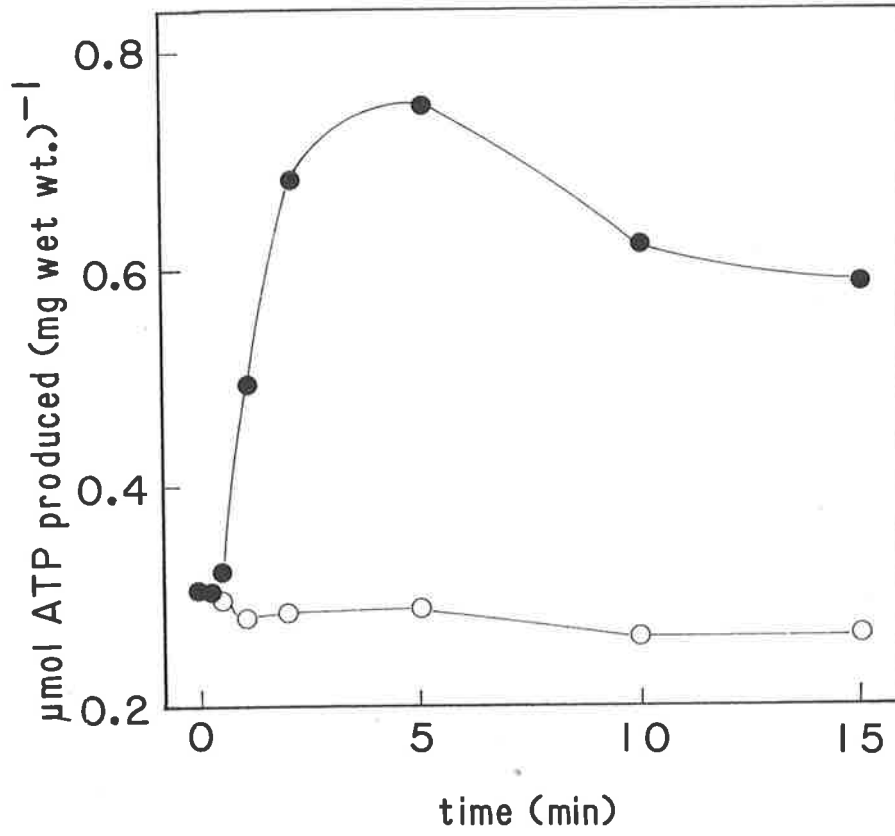
FIG.51
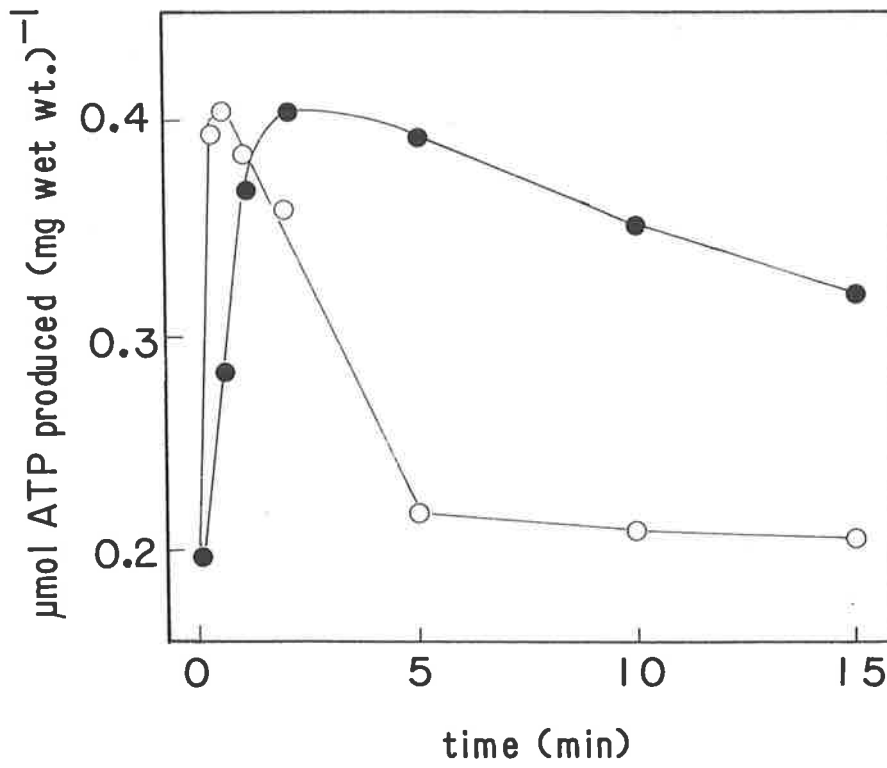
FIG.52: EFFECT OF DCCD ON VALINOMYCIN INDUCED ATP SYNTHESIS IN
SPHEROPLASTS OF *NITROSOMONAS EUROPAEA*.

Spheroplasts were suspended (4mg wet wt. ml^{-1}) in a reaction
mixture described in Fig.51, supplemented with DCCD (50-100μM).
Valinomycin (100μg) 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 10mM KCl.   Reaction was started by the addition of
100μg valinomycin at zero time.   Experimental details as in
Fig.50 and 51.

no KCl (●);   2mM KCl (○);   5mM KCl (▲);   7mM KCl (△);
10mM KCl (▼).

FIG. 52
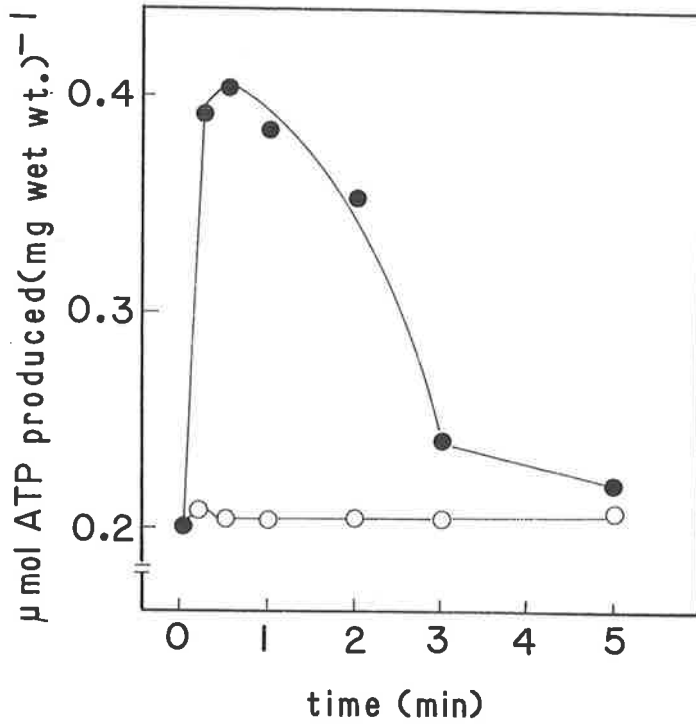
FIG. 53
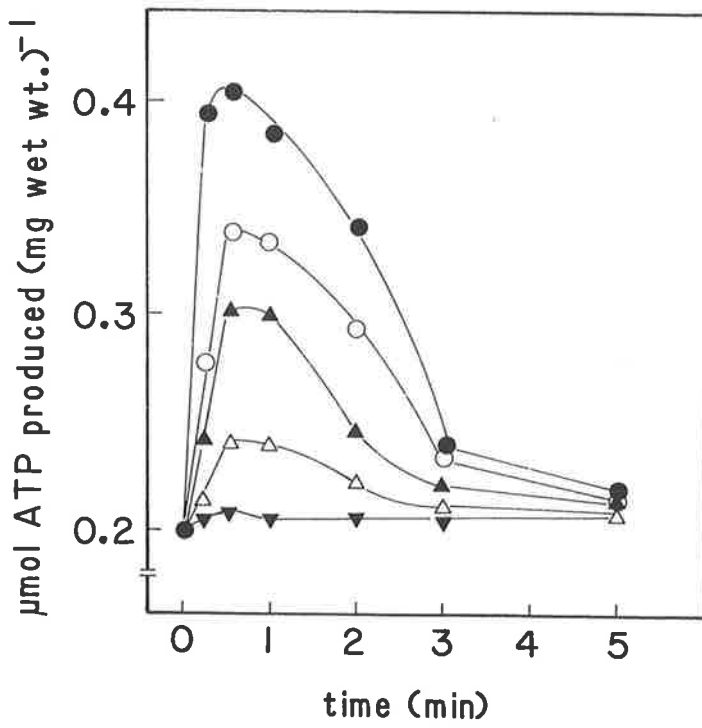
ATP biosynthesis in *Nitrosomonas europaea*.

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 in-hibited it at 10mM concentration.

As these experiments were conducted at pH 6.0 the cells would also have a ΔpH (inside alkaline) to add to total Δp.

Nigericin, another K^+ ionophore which results in K^+/H^+ exchange across the membranes, would create a ΔpH (inside acid) if added to cell suspension in K^+ free medium. Thus theoretically the addition of niger-icin to cells should result in a decrease in intracellular ATP because inside acid ΔpH 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 un-expected 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_4Cl 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 $MgCl_2$ in a 1cm cuvette
were added 0.1μmol quinacrine hydrochloride and 0.1ml sphero-
plasts (50mg wet weight). The following additions (↓) were
made:

NH$_4$Cl (70μmol), nigericin (NIG) (50μg) or KCl (200μmol).
Fluorescence emission was determined (arbitrary units) in
a Fluorispec model SF-1 fluorimeter (Baird Atomic,
Massachussetts, 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
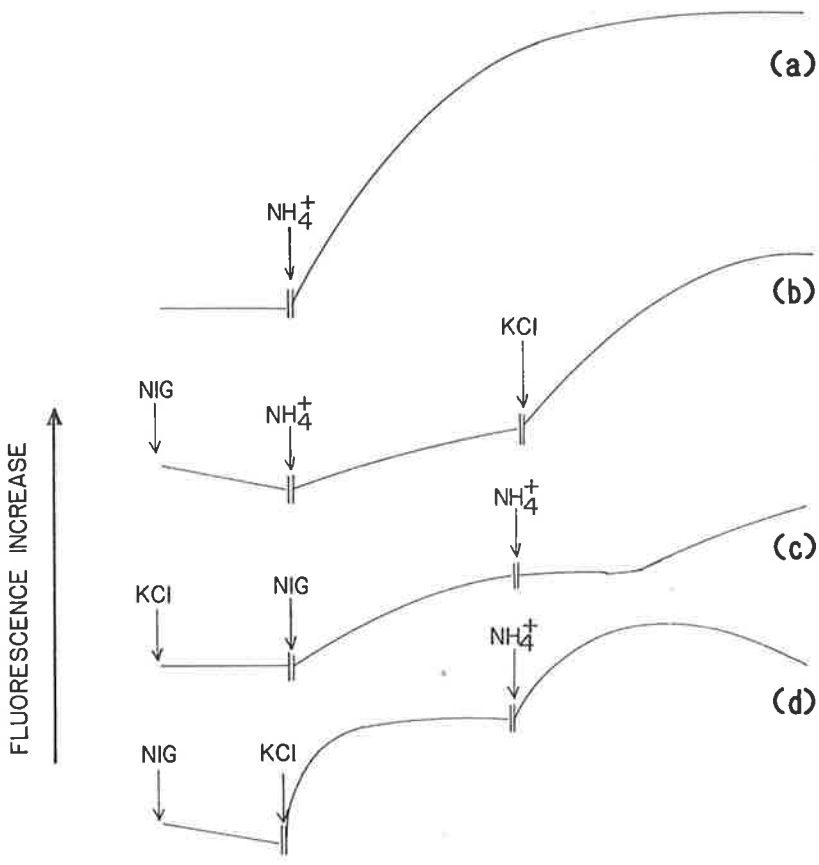
FIG. 55
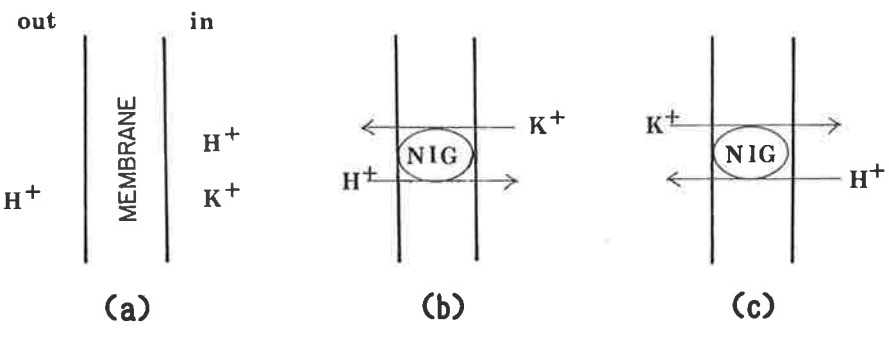
ment are shown in Fig.55. In K^+ free medium, the addition of nigericin would result in loss of intracellular K^+ and its replacement by H^+ (Fig.55b) resulting in decrease in quinacrine fluorescence. However in K^+ containing medium, nigericin addition would result in K^+ uptake associated with H^+ 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 eithr 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 Δp 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 50mM Tris-HCl (pH 7.5 - 9.0) containing 150mM NaCl, a slow extrusion of intracellulor K^+ occurred (Fig.56). In the presence of 50mM DEA-HCl (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 150mM NaCl in addition to 50mM DEA. The intracellular concentration of Na^+ in both bacteria remained constant during DEA treatment. The DEA trated cells contained <5mM 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*

TABLE 41: INTRACELLULAR CONCENTRATIONS OF Na^+ and K^+ IN WASHED CELLS OF *NITROSOMONAS EUROPAEA* AND *NITROBACTER AGILIS*.

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.

| Batch | Intracellular concentration (mM) | | | |
| | *Nitrosomonas europaea* | | *Nitrobacter agilis* | |
	Na^+	K^+	Na^+	K^+
1	80	85	25	160
2	59	146	37	290
3	73	170	30	270
4	62	99	45	310
5	65	135	Nd	Nd

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 21ml of 50mM DEA-HCl, 150mM NaCl (pH 9.2) containng 4 μCi ml^{-1} ^{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 ^{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 PPO and 0.03% w/v POPOP in toluene). Radioactivity (^{22}Na$^+$) was measured in a Packard Tricarb 460 CD scintillation spectrometer.

FIG. 56
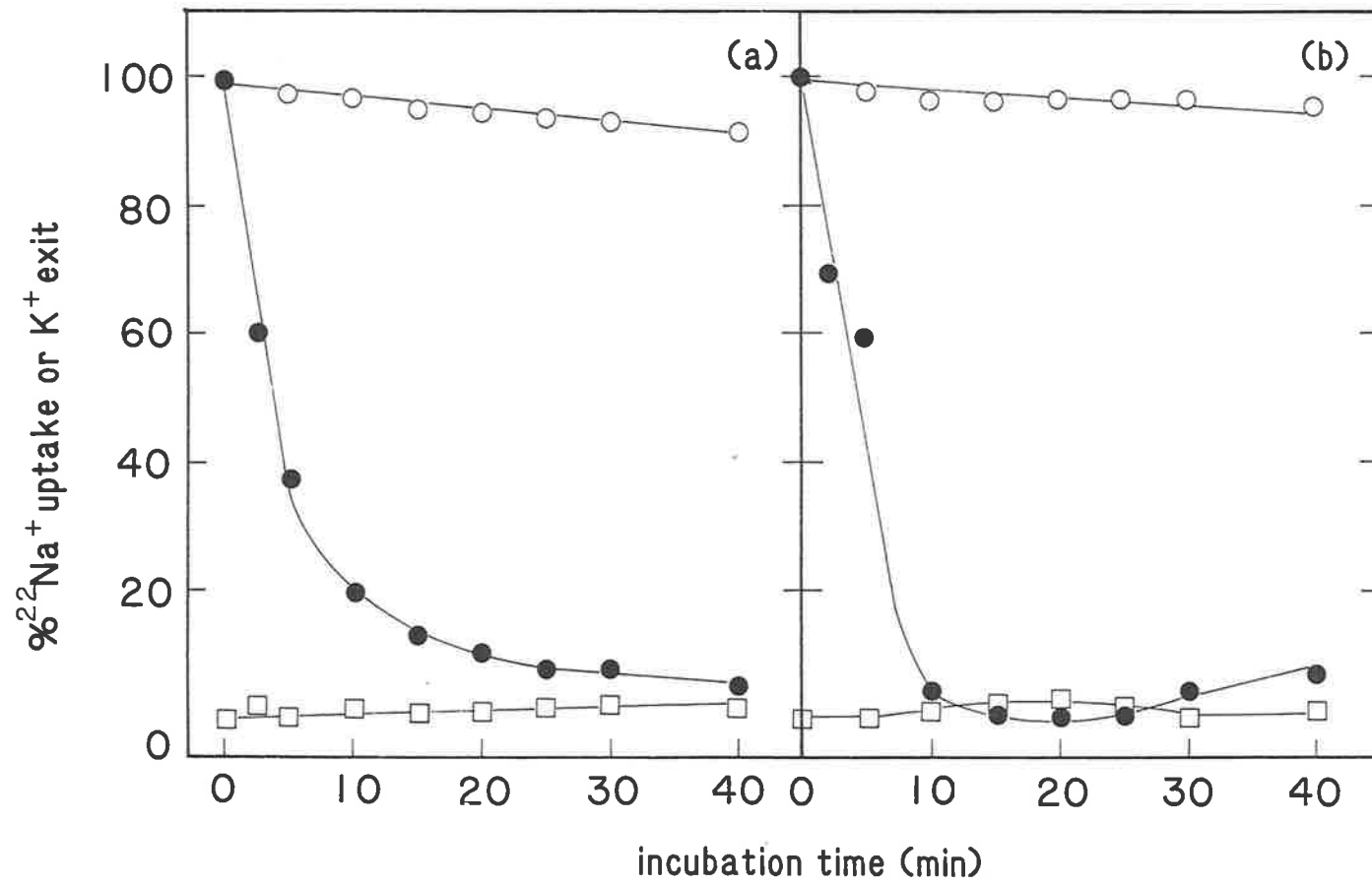
TABLE 42: EFFECTS OF K^+ DEPLETION AND K^+ ADDITION ON RESPIRATION IN *NITROSOMONAS EUROPAEA* AND *NITROBACTER AGILIS*.

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)$^{-1}$.

Bacterium	DEA Treatment	Substrate[a]	K^+ addition[b]	Respiration rate
Nitrosomonas europaea	-	NH_4Cl	-	240
	-	NH_4Cl	+	160
	-	NH_2OH	-	760
	-	NH_2OH	+	740
	+	NH_4Cl	-	200
	+	NH_4Cl	+	140
	+	NH_2OH	-	560
	+	NH_2OH	+	540
Nitrobacter agilis	-	$NaNO_2$	-	120
	-	$NaNO_2$	+	120
	+	$NaNO_2$	-	110
	+	$NaNO_2$	+	110

[a]The concentration of each substrate was 2.5mM
[b]K^+ was added as KCl (25mM).

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 Δp, viz. ΔpH 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 ΔpH 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, ΔpH 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 ΔpH (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 ΔpH 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 ΔpH, 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, $\Delta\psi$ 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 ie. 59 x ΔpH($\bigcirc\bullet$)was determined with $[^{14}C]$ benzoic acid; $\Delta\psi$ values($\triangle\blacktriangle$)were calculated from the uptake of $[^3H]TPP^+$; Δp($\square\blacksquare$)was determined from the ΔpH and $\Delta\psi$ values (Δp = $\Delta\psi$-59ΔpH). Open symbols, without KCl; closed symbols, + 20mM KCl.

FIG. 57
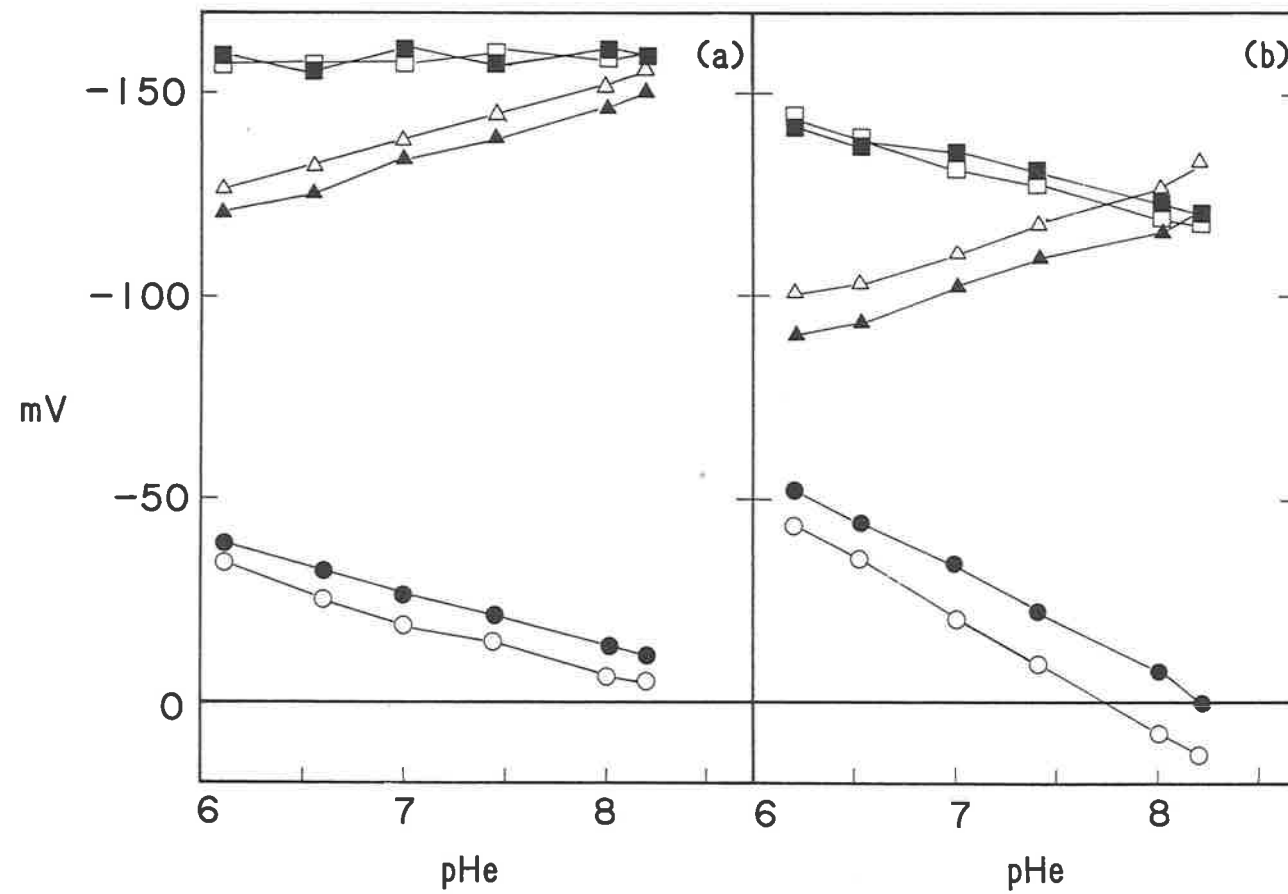
Nitrobacter agilis during DEA treatment. If DEA is removed from the reaction mixture by washing with a Na^+ containing buffer (eg. Tris, Hepes or phosphate) the cells readily take up Na^+. The time course of uptake of $^{22}Na^+$ by DEA treated cells of *Nitrosomonas europaea* and *Nitrobacter agilis* respectively are shown in Fig.58. Thus when the amine loaded cells of either bacterium were exposed to $^{22}Na^+$ there was an immediate uptake of $^{22}Na^+$. The extent of accumulation of $^{22}Na^+$ was dependent on the concentration of DEA in the external medium. Thus with 50mM DEA, no net entry of ^{22}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 $^{22}Na^+$ ($+Na^+$) cells reached an equilibrium state and contained 150-200mM intracellular Na^+.

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

$^{22}Na^+$ loaded cells were prepared as described above (Fig.58) and an active extrusion of $^{22}Na^+$ from the cells was determined by a filtration method at 25^oC (Fig.59). In *Nitrosomonas europaea* only about 20% $^{22}Na^+$ was extruded from cells when $K^+$was added to $^{22}Na^+$ loaded cells (Fig.59a). In the presence of CCCP, the addition of K^+ to $^{22}Na^+$ loaded cells of *Nitrosomonas europaea* did not result in an extrusion of $^{22}Na^+$. In *Nitrobacter agilis* $^{22}Na^+$ loaded cells, the extrusion of $^{22}Na^+$ (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 $^{22}Na^+$ 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 NH_4^+ on *Nitrosomonas europaea* and NO_2^- in *Nitrobacter agilis* on the $^{22}Na^+$ system were studied. The addition of NH_4Cl to $^{22}Na^+$ loaded cells of either *Nitrosomonas europaea* or *Nitrobacter agilis*, resulted in a rapid extrusion of $^{22}Na^+$ (Fig.59a,b). However this loss was not respiration dependent because the addition of diethyldithiocarbamate, an inhibitor of NH_4^+ oxidation by *Nitrosomonas europaea* (Table 39), prior to NH_4Cl did not prevent the extrusion of $^{22}Na^+$ in *Nitrosomonas europaea* . This was also confirmed by replacing NH_4^+ with CH_3NH_2 (which is not oxidised by the cells), which

FIG.58:   UPTAKE OF ^{22}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}Na$^+$ uptake studies 100µl of this cell suspension was diluted to 1ml with 100mM, Na-HEPES buffer (pH 7.5) containing ^{22}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}Na$^+$.

FIG. 58

154
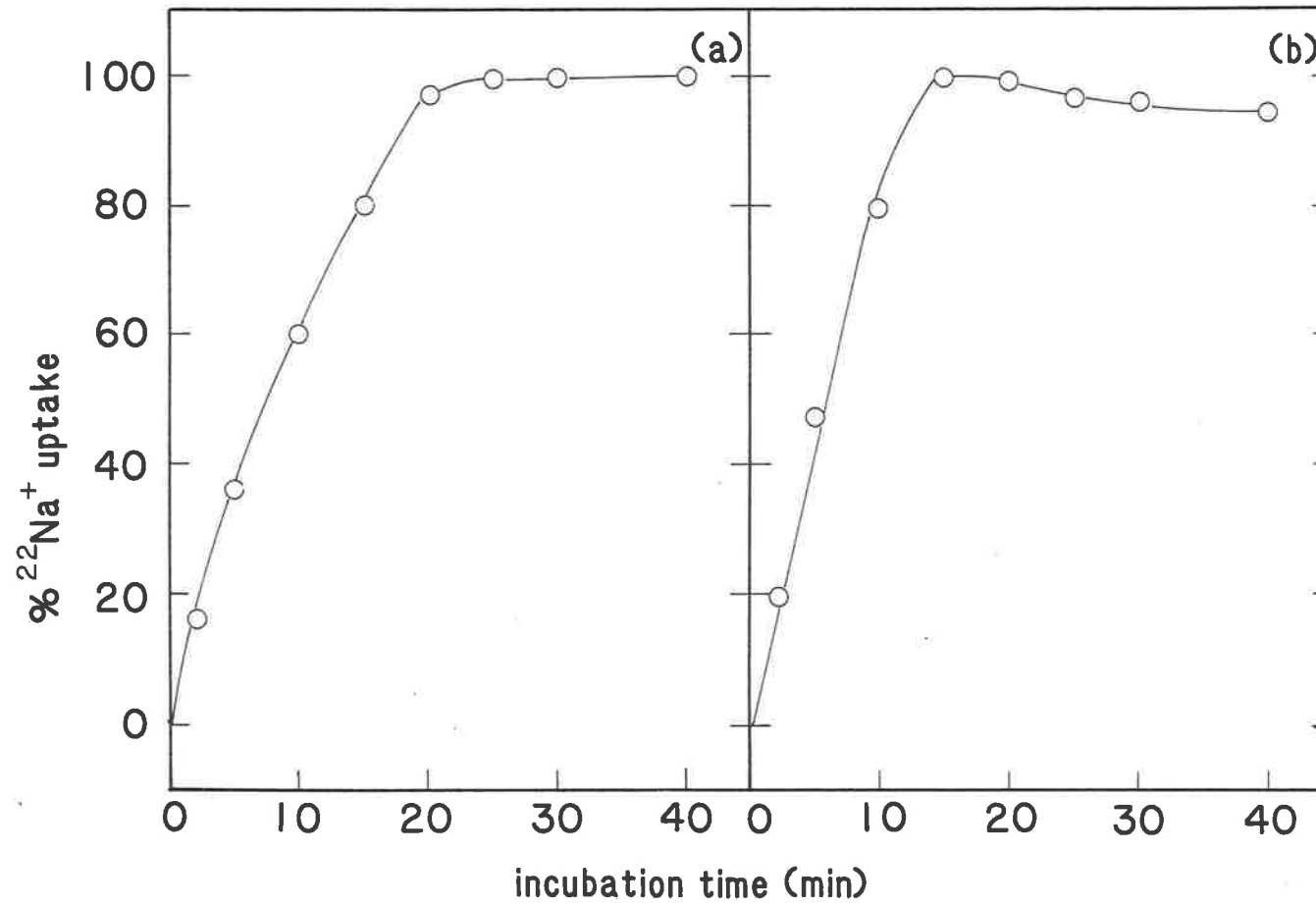
FIG.59: $^{22}Na^+$ EXTRUSION FROM $^{22}Na^+$ LOADED CELLS OF (a) *NITROSOMONAS EUROPAEA* AND (b) *NITROBACTER AGILIS*.

$^{22}Na^+$ 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_4Cl (O);   5mM $NaNO_2$ (●);   10mM NH_4Cl + 20μM CCCP (▼);

10mM NH_4Cl + 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
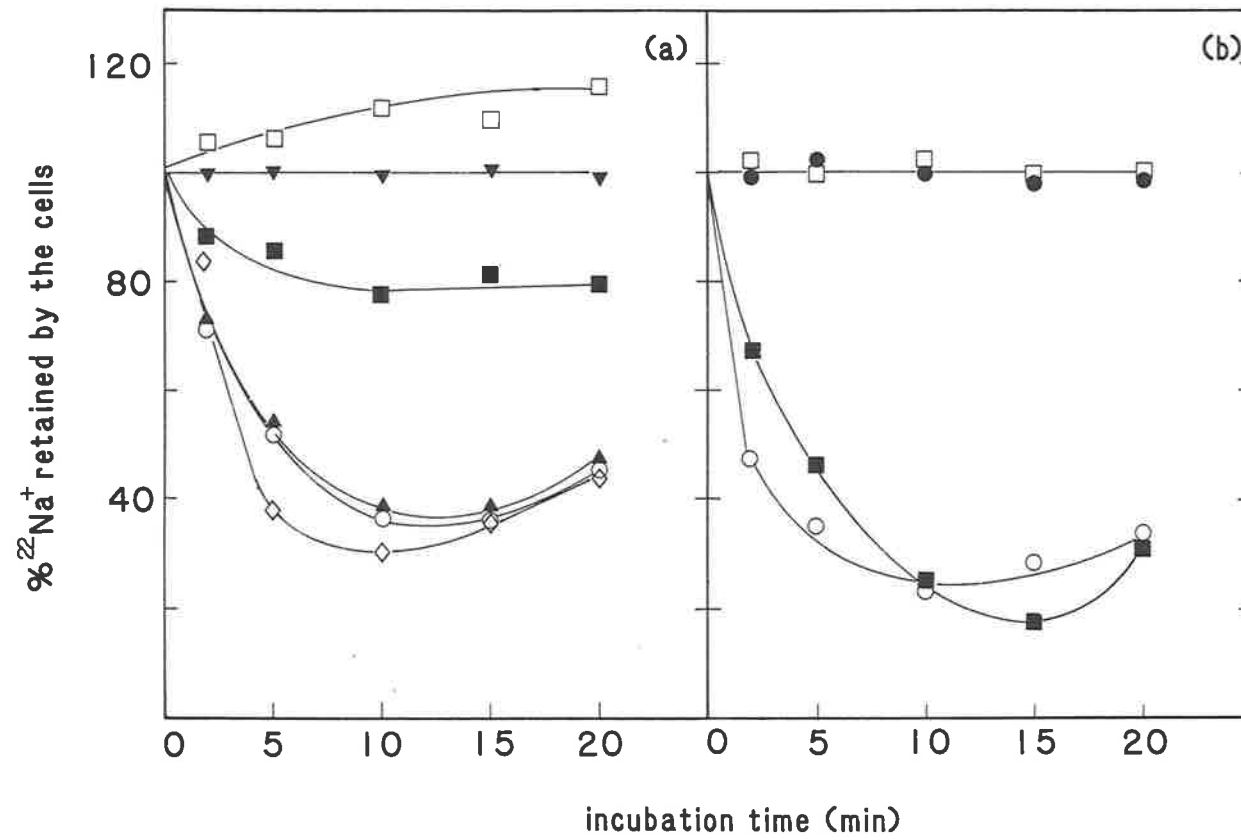
also resulted in $^{22}Na^+$ extrusion from the cells. This, together with NH_4Cl dependent $^{22}Na^+$ loss from *Nitrobacter agilis* cells indicates that NH_4^+ acts as a permeant amine like diethanolamine and methylamine and is transported into the cells of both *Nitrosomonas europaea* and *Nitrobacter 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 $^{22}Na^+$ loaded cells did not result in the extrusion of $^{22}Na^+$ (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}N and ^{18}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

$$O_3P-\overset{*}{O}^- + \begin{bmatrix} O \\ \| \\ N^+ \\ \| \\ O \end{bmatrix} \longrightarrow O_3P-\overset{*}{O}-N\begin{matrix} //O \\ \\ \diagdown O^- \end{matrix} \longrightarrow ATP + \overset{*}{O}-NO_2^-$$
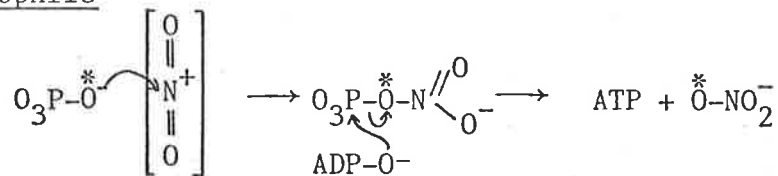
ADP-O-

ADP oxygen as nucleophile

$$ADP-O^- + NO_2^+ \longrightarrow ADP-\overset{}{O}-N\overset{+}{\diagup}\overset{O^-}{\diagdown O} \longrightarrow ADP-\overset{*}{O}-PO_3 + NO_3^-$$

$$O_3P\overset{*}{O}$$

$$ADP-\overset{*}{O}-PO_3 \xrightarrow[\text{hydrolysis}]{\text{Cellular ATP}} ADP-\overset{*}{O}^- + Pi$$

$$ADP-\overset{*}{O}^- + NO_2^+ \longrightarrow ADP-\overset{*}{O}-N\overset{+}{\diagup}\overset{O^-}{\diagdown O} \longrightarrow ADP\overset{*}{O}PO_3 + \overset{*}{O}NO_2^-$$

$$O_3P\overset{*}{O}$$
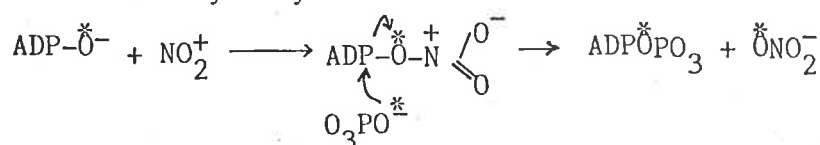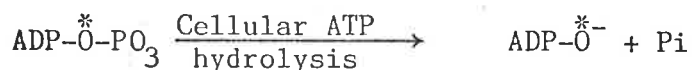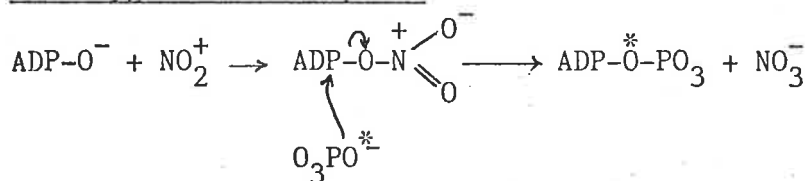
Thus this mechanism was suggested to involve NO_2^+ as an intermediate during NO_2^- oxidation. It has been shown that the source of oxygen during NO_2^- oxidation by *Nitrobacter* is H_2O (Aleem *et al.*, 1965), based on the incorporation of 0.044 to 0.078 atom % ^{18}O into NO_3^- from 82 atom % $H_2^{18}O$. The following experiments were designed to check the source of 'O' in NO_3^- produced as a result of NO_2^- oxidation by *Nitrobacter agilis*. Cells were incubated with nitrite (NO_2^- or $^{15}NO_2^-$) and one of the following ^{18}O compounds : $H_2^{18}O$ (90-97 atom %), $^{18}O_2$ (99 atom %) and $P^{18}O_4^{2-}$ (approximately 90 atom %). Two distinct methods (GC/MS and ^{15}N-NMR) were used for isotope analysis of the product of NO_2^- oxidation (nitrate). For GC/MS studies, nitrate was converted to N_2O and phosphate to trimethyl phosphate which were then analysed as described in detail in Section 2.2.15.1. For ^{15}N-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 NO_2^- oxidation

3.5.5.1.1 Changes in ^{18}O contents of phosphate during NO_2^- oxidation

The mass spectra of a commercially available sample of trimethyl phosphate and one prepared by the methylation of $H_3P^{18}O_4$ are shown in Fig.60A and B respectively. Fig.60C illustrates the spectrum of trimethyl phosphate prepared from $P^{18}O_4^{2-}$ which had previously been incubated with NO_2^- and the cells of *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 NO_2^- oxidation by *Nitrobacter*.

3.5.5.1.2 $^{15}N^{18}O$ 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}N and ^{18}O following its conversion to N_2O gas (Section 2.2.15.1) is given in Table 43. When unlabelled NO_2^- was oxidised by cells of *Nitrobacter agilis* the only source of ^{18}O which was significantly incorporated with NO_3^- was $H_2^{18}O$ (Fig. 61). In this experiment, the normalized integral at 46 amu($N_2^{18}O$) of 7.48 was in reasonable agreement with the value of 7.88 expected if the $H_2^{18}O$ 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 pre-
pared by the methylation of $H_3P^{18}O_4$ 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.

FIG. 60

(A)

(B)

(C)
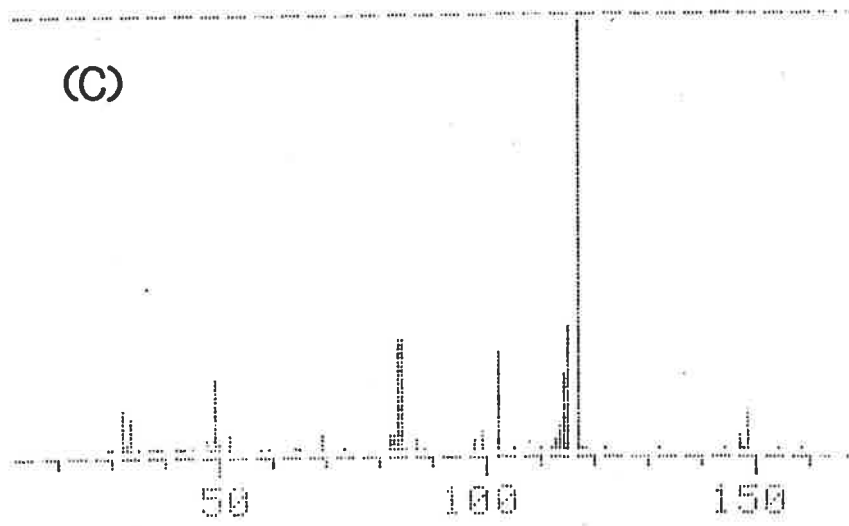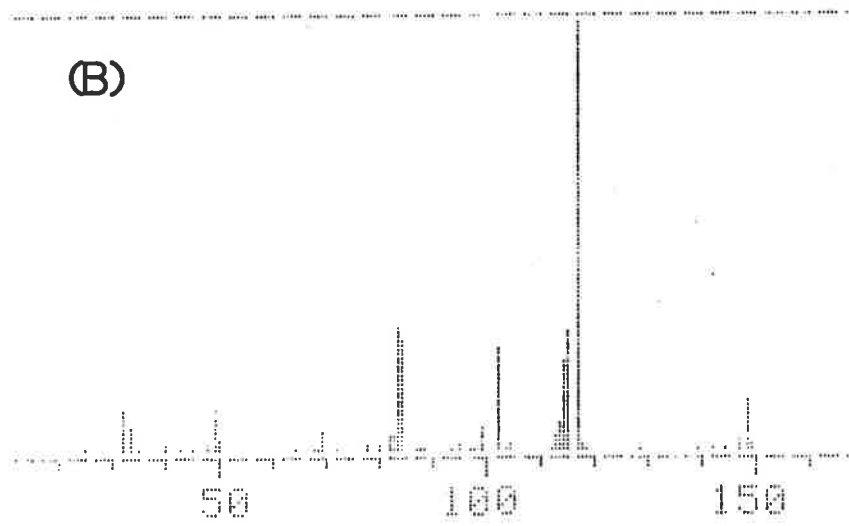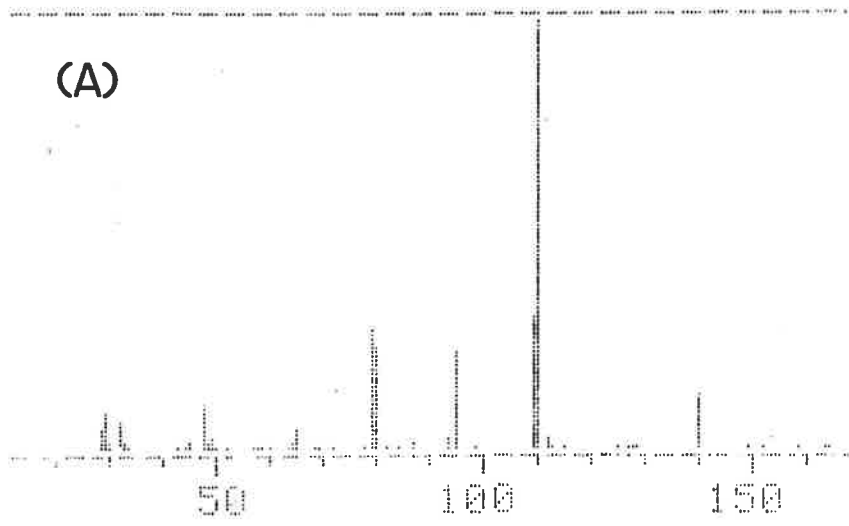
TABLE 43: STABLE ISOTOPE EXPERIMENTS WITH WASHED CELLS OF *NITROBACTER AGILIS*.

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_2^-, the NO_3^- was recovered and converted to N_2O as described in Section 2.2.15.1. The details of GC/MS 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_2^- and that of stocks of $^{15}NO_2^-$ and $H_2^{18}O$ 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.

Incubation mixture	Normalized integrals for N_2O species at the indicated amu values				
	44	45	46	47	48
	N_2O	$N^{15}NO$	$N_2^{18}O + {}^{15}N_2O$	$N^{15}N^{18}O$	$^{15}N^{15}N^{18}O$
$NO_2^- + H_2O + PO_4^{2-} + air$	100	0.20 (0.73)	0.15 (0.20)	0.0 (0)	0.0 (0)
$NO_2^- + H_2^{18}O + PO_4^{2-} + air$	100	0.20 (0.72)	7.48 (7.88)	0.0 (0.05)	0.2 (0)
$NO_2^- + H_2O + P^{18}O_4^{2-} + air$	100	0.80 (0.73)	0.21 (0.20)	0.0 (0)	0.0 (0)
$NO_2^- + H_2O + PO_4^- + {}^{18}O_2$	100	0.42 (0.73)	0.18 (0.20)	0.0 (0)	0.0 (0)
$^{15}NO_2^- + H_2O + PO_4^{2-} + air$	0.82 (1.00)	100	0.20 (0.37)	0.0 (0.20)	0.0 (0)
$^{15}NO_2^- + H_2^{18}O + PO_4^{2-} + air$	0.79 (1.02)	100	3.50[a](0.44)	7.80(7.95)	0.5 (0.03)
$^{15}NO_2^- + H_2O + P^{18}O_4^{2-} + air$	0.82 (1.00)	100	1.20[a](0.37)	0.41(0.20)	0.0 (0)
$^{15}NO_2^- + H_2O + PO_4^{2-} + {}^{18}O_2$	0.83 (1.00)	100	1.10[a](0.37)	0.31(0.20)	0.0 (0)

[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 N_2O GAS

(A) a commercial sample (B) and (C) prepared by the pyrolysis of ammonium nitrate obtained from a NO_2^- oxidising system with H_2O and $H_2^{18}O$ 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 N_2O ie. NO^+ (ion 30) and N_2^+ (ion 28) are absent in these spectra. Details of the experiments and GC/MS technique are described in Section 2.2.15.1.

FIG. 61

(A)   (B)   (C)
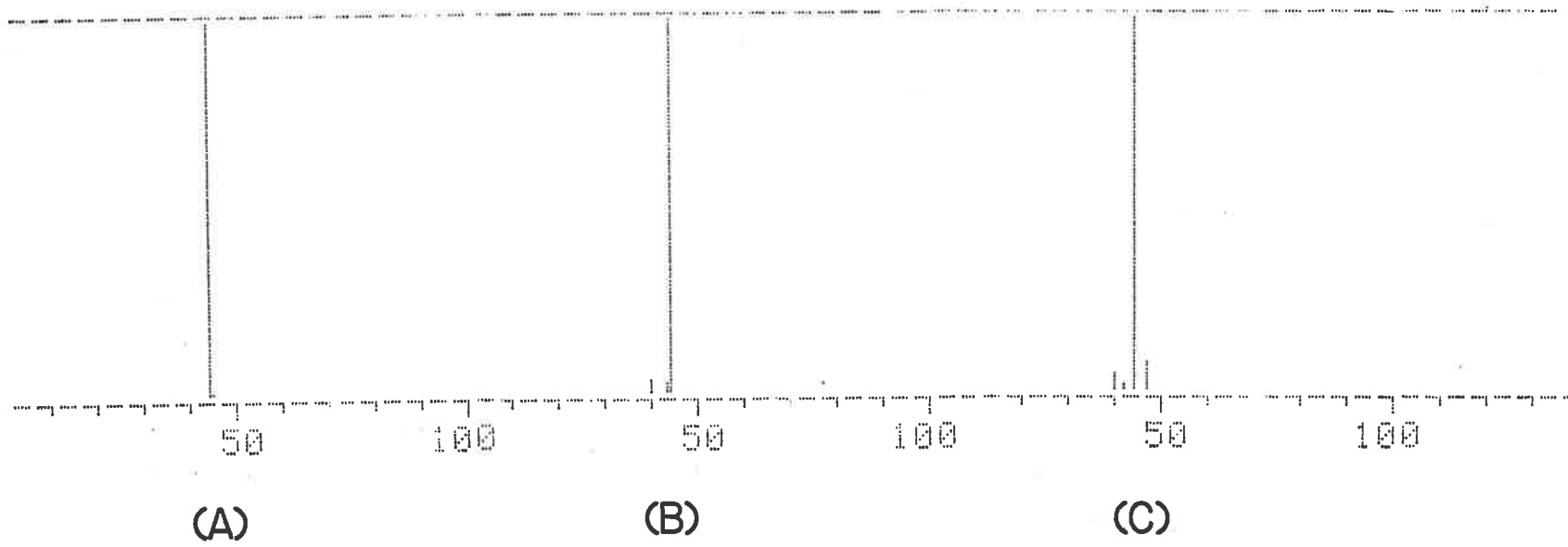
the experiment had contained 95 atom % ^{18}O, assuming that only one oxygen atom of NO_3^- had been derived from water and that the NO_2^- stock contained 10% (w/w) NO_3^- initially. Similarly in experiments, when 99 atom % $^{15}NO_2^-$ was used instead of unlabelled NO_2^-, only ^{18}O from $H_2^{18}O$ was significantly incorporated in $^{15}NO_3^-$. 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 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 $^{15}NO_3^-$ was more than 40mM. Smaller concentrations required longer accumulation time.

When the cells were incubated with $^{15}NO_2^-$ with $H_2^{16}O$ in 100mM $P^{16}O_4^{2-}$ buffer, only one resonance was observed which corresponded to $^{15}N^{16}O_3^-$. In Fig.62B, the NMR spectrum of the product of $^{15}NO_2^-$ 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 'O' in nitrate produced by $^{15}NO_2^-$ oxidation was derived from $^{18}O_2$. When the cells were incubated with $^{15}NO_2^-$ and $H_2^{18}O$ 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 $^{15}NO_2^-$ oxidation in the presence of ^{18}O phosphate (all 4 'O' 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 'O' in $^{15}NO_3^-$ are derived from $P^{18}O_4^{2-}$ during nitrite oxidation by *Nitrobacter agilis* In another experiment in which cells were incubated for 18h with $P^{18}O_4^{2-}$

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 $^{15}N^{16}O_3^-$ + $^{15}N^{16}O_2^{18}O^-$ + $^{15}N^{16}O^{18}O_2^-$ produced by cells in presence of $H_2^{18}O$.

(D) 50mM, $^{15}N^{16}O_3^-$ produced by cells in presence of $P^{18}O_4^{2-}$.

Experimental details are described in Section 2.2.15.2.

FIG. 62

(A)

(B)

(C)

(D)

4   0   -4   -8

Hz
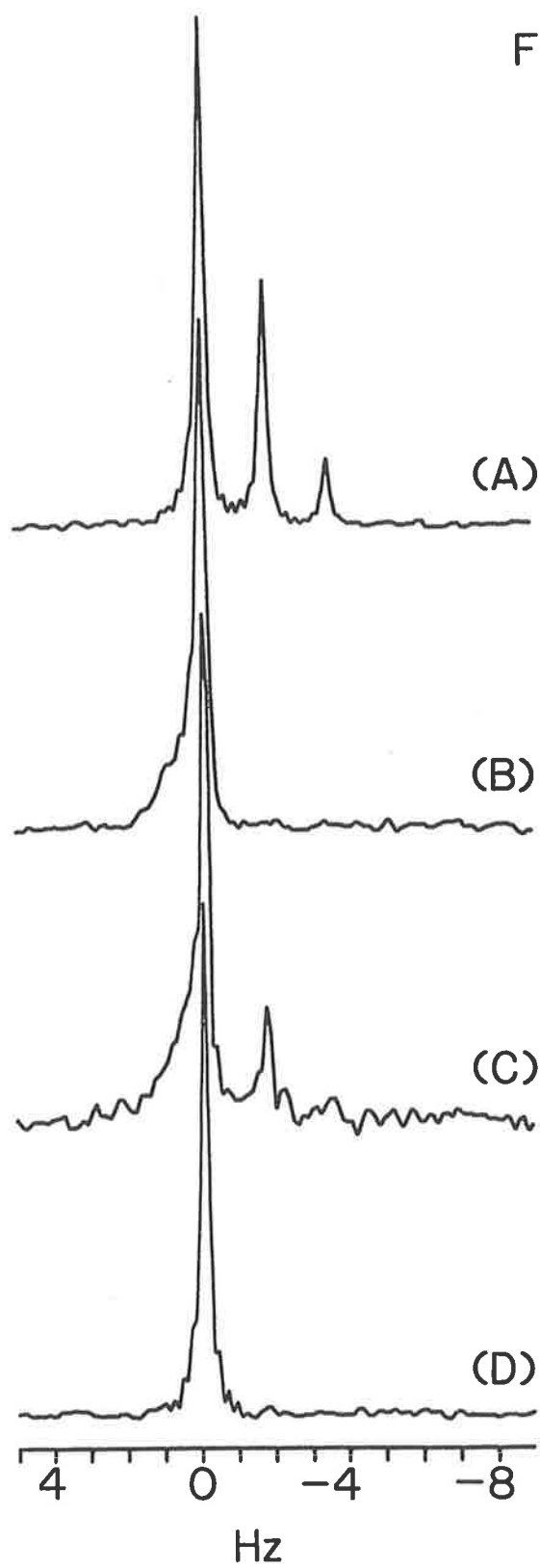
and $^{15}NO_2^-$, the NMR spectrum was similar to that observed in Fig.62D. Thus there appeared to be no measurable biological or chemical exchange of ^{18}O between either $P^{18}O_4^{2-}$ and H_2O, $P^{18}O_4^{2-}$ and $^{15}NO_3^-$ or $P^{18}O_4^{2-}$ and $^{15}NO_2^-$.

The observations of GC/MS and NMR studies taken together indicate that one and only one oxygen in NO_3^- produced as a result of NO_2^- oxidation by *Nitrobacter agilis* originates from H_2O and not from O_2 or PO_4^{2-}, 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 sphero-plasts 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), O-phenanthroline, α-α'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 NO_2^- oxidation in membrane vesicles substantiates the idea that NO_2^- 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 NO_2^- 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 Mg^{2+} dependent ATPase has been detected in membrane vesicles of *Nitrobacter agilis* (Section 3.1.7). ATPase activity requiring either Mg^{2+} or Ca^{2+} 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 (egs. CCCP, DNP) but was strongly inhibited by classical ATPase inhibitors (eg. DCCD). One important observation in the present study is that the ATPase inhibitors (DCCD, vanadate, diethylstilbestrol and NBD chloride) also restrict NO_2^- oxidation to approximately the same extent as for ATPase itself. The ATPase however was not affected by the inhibitors of NO_2^- oxidation (eg. uncouplers). This indicates a possible functional relationship between NO_2^- oxidase and ATPase in the bacterium. The delipidation of membranes by phospholipase A_2 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 NO_2^- oxidation by *Nitrobacter agilis*, the further assimilation of NO_2^- (or NO_3^-) 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 $^{15}NO_2^-$, $^{15}NO_3^-$, $^{15}NH_2OH$ and $^{15}NH_4^+$ respectively into cell nitrogen; of these $^{15}NH_4^+$ was most readily utilized. These results are in agreement with those of Wallace and Nicholas (1968). This also

confirms the finding that NH_4^+ enhances the growth of this bacterium in pure cultures (Section 3.2.1). Thus in its natural habitat, NH_4^+ would be an important source of nitrogen for *Nitrobacter agilis*.

Cobley (1976a) reported that NO_2^- oxidation by electron transport particles of *Nitrobacter winogradskyi* was stimulated by NH_4Cl (maximum stimulation 35% at 2mM). In the present study with washed cells by *Nitrobacter agilis*, no such stimulation was observed. Although 2mM NH_4Cl stimulated the growth of the bacterium, higher concentrations inhibited NO_2^- oxidation. This effect was reversed by increasing the concentration of 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 NH_4^+ would inhibit NO_2^- oxidation until such time as enough NO_2^- was produced (eg. from the oxidation of NH_4^+ by *Nitrosomonas*) to overcome the inhibition. Since NH_4^+ even at relatively high concentrations does not inhibit NO_2^- oxidation completely (Fig. 12), its accumulation would only retard and not completely inhibit the rate of NO_2^- conversion to 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 *Nitrosomonas 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 $^{15}NO_2^-$ or $^{15}NH_4^+$ 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 NO_2^- and NO_3^- *via* NH_4^+ when there is no exogenous 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*.

NH₂OH ──────────→ NH₂OH   proteins

NO₂⁻

NADH NAD   NADH NAD   NADPH NADP

NO₃⁻ 2 NO₂⁻ 3 NH₄⁺ 5 Glu

NO₃⁻

MEMBRANE   CYTOPLASM   Gln ──────→ other compounds

1) *nitrite oxidase*
2) *nitrate reductase*
3) *nitrite reductase*
4) *hydroxylamine reductase*
5) *glutamate dehydrogenase*
6) *glutamine synthetase*
7) *glutamate synthase*
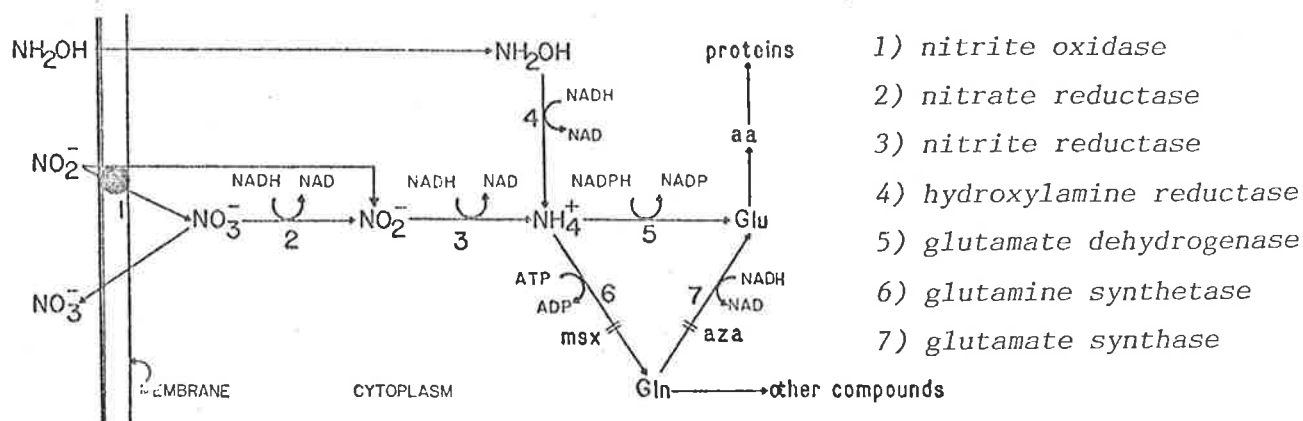
4.2.2 *Purification, properties and regulation of glutamine synthetase and glutamate dehydrogenase from Nitrobacter agilis and Nitrosomonas europaea*

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 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* ($\simeq$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_4Cl 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 in-
activation of the NH_4^+ binding site by glutamine would preclude the binding of
NH_2OH 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 adenylylation/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 indi-
cates 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 adenylylation
/deadenylylation (Ginsburg and Stadtman, 1973). The adenylylated and de-
adenylylated forms of GS differ in their regulatory properties (Bender *et al.*,
1977). It is well known that the adenylylation 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 adenylylated 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 adenylylated even when the cells were grown with NO_2^-, 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 adenylylation of GS. Relatively low concentrations of CTAB ($2.5\mu g\ ml^{-1}$) 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 adenylylated form of GS from *Nitrobacter agilis* could be deadenylylated by treatment with snake venom phospho-diesterase. This confirms that the *Nitrobacter* enzyme is indeed regulated by an adenylylation/deadenylylation mechanism. Another line of evidence to support this conclusion is that differentially adenylylated 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 *Klebsialla 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 ($\simeq 120mM$) of NH_4^+ would be expected to have a highly adenylylated form of GS. The results pre-sented 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 adenylylated: (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 adenylylated 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) adenylyla-tion 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 adenylylation 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}C] ATP followed by an ammonia shock, the [^{14}C] label was associated with GS, but phospho-diesterase treatment of this enzyme did not remove the [^{14}C] adenine moiety. In a similar experiment with toluene treated cells of *Nitrosomonas europaea* [^{14}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 NH_4^+ and NADPH (substrate stimulation) so that two distinct K_m values were obtained for either substrate. Cells of *Nitrobacter agilis* contained a high concentration of NH_4^+ (approximately 30mM) and this explains why (a) GS is highly adenylylated (Section 3.3.3) and (b) $NADP^+$-GDH (K_m NH_4^+, 6.3 to 33mM) operates in the direction of glutamate production even when no exogenous NH_4^+ is available to the bacterium. *Nitrosomonas europaea* has only biosynthetic GDH activity ($NADP^+$-GDH) which can function in either direction ie. 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 NH_4Cl (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 ie. 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 NH_4^+ concentration observed under laboratory growth conditions support the view that GDH is the main pathway for NH_4^+ assimilation in *Nitrobacter agilis*. Accumulation of NH_4^+ *via* the assimilatory NO_2^- reductase (Wallace and Nicholas, 1968) appears to be under minimal regulatory control. Under natural growth conditions in the soil, the availability of N source (NO_2^- and/or NH_4^+) for *Nitrobacter agilis* can vary greatly and this may account for the fact that the bacterium has two NH_4^+ assimilation pathways ie. GDH and GS/GOGAT. The following schemes are suggested assuming that NO_2^- reductase is responsible for the production of NH_4^+ in *Nitrobacter agilis* when exogenous NH_4^+

is unavailable to bacterium. 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_mNH_4 = 0.2mM).
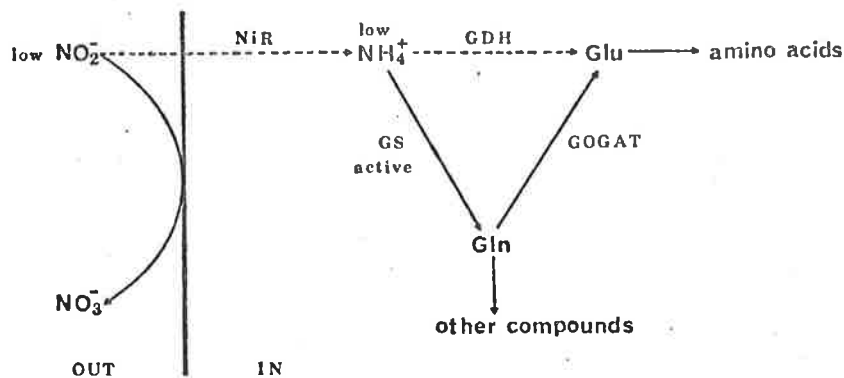
When an ample supply of NO_2^- is available (eg. culture growth conditions), NO_2^- reductase would produce sufficient amounts of NH_4^+ that would result in adenylylation of GS. The NH_4^+ thus produced would then be predominately assimilated by the GDH pathway.
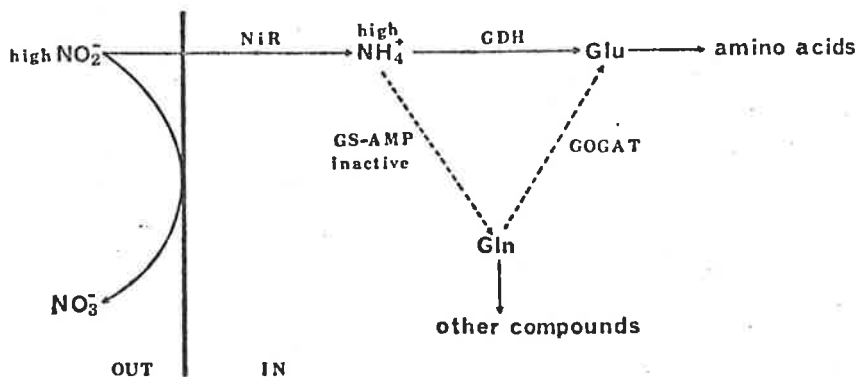
4.3   ENERGY CONSERVATION IN *NITROSOMONAS EUROPAEA* AND *NITROBACTER AGILIS*

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 (ΔpH, inside alkaline). This results in a proton-motive force (Δp) 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 $\rightarrow H^+/O$ 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 $\rightarrow H^+/O$ 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 ($\rightarrow H^+/O \simeq 4$) 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 $\rightarrow H^+/O$ 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 $\rightarrow H^+/O$ ratios recorded for *Nitrosomonas europaea* are quantitatively in accord with the finding that the first step in NH_4^+ oxidation ($NH_4^+ + O \rightarrow NH_2OH + H^+$) 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 $\rightarrow H^+/O$ ratio for NH_4^+ is 3.4. Substracting 0.66 stoichiometric protons per O atom gives a ratio of 2.74. Inasmuch as the first of the three O atoms is utilized in an O insertion reaction, the effective $\rightarrow H^+/O$ ratio becomes $3/2 \times 2.74 = 4.1$ for the two O 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 $\rightarrow H^+/O$ 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 electroneutral 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 $NH_2OH + 2O \rightarrow NO_2^- + H_2O + H^+$ ($\Delta G^{\circ\prime}$ = -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; Sone *et al.*, 1977; Hinkle and Yu, 1979).

In *Nitrobacter* the free energy available in the aerobic oxidation of a high potential reductant NO_2^- ($NO_2^- + O \rightarrow NO_3^-$; $\Delta G^{\circ\prime}$ = -18 kcal or -75.4 kJ mol^{-1}) is used to drive the reduction of a low potential oxidant NAD^+ and to produce ATP (Aleem, 1968, 1977).

$$NO_2^- + NAD^+ + H_2O \rightarrow NO_3^- + NADH + H^+ \qquad \Delta G^{\circ\prime} = 38 \text{ kcal}$$

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; Cobley, 1976b). The coupling of NO_2^- respiration to phosphorylation and NAD^+ reduction is generally assumed to be by way of the proton-motive force (Cobley, 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 *Nitrobacter* is that of Cobley (1976b) who demonstrated with electron transport particles of *Nitrobacter winogradskyi* that the aerobic oxidation of NO_2^- resulted in a small but reversible alkalinization of the medium. Although the effect was abolished by agents known to collapse Δp, 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 $\leq$-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 $\rightarrow H^+/O$ 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 concen-
trations (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 *Nitrosomonas 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 *Nitrosomonas
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 *Nitrosomonas 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 *Nitrosomonas europaea* and a variety
of other bacteria there is no evidence that permeant ions inhibit the re-
spiratory 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 ($\tilde{\mu}_H+$) gives rise to a proton-motive force
(Δp). This proton gradient consists of an electrical potential ($\Delta\psi$) and a pH
gradient (ΔpH). The two components of Δp have the following relationship:
$\Delta p = \Delta\psi - 2.3RT/F \Delta pH$. Whereas Δp 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 hungaetei* (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* pHi remained 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 $\Delta\psi$ (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 $\Delta\psi$, 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 concentratins (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 eg. *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 $\Delta \psi$ also inhibited NO_2^- oxidation by membrane particles of *Nitrobacter winogradskyi*, Cobley (1976a,b) predicted that NO_2^- oxidation requires $\Delta \psi$. The way in which NO_2^- oxidation is mediated by $\Delta \psi$ 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 $\Delta \psi$. 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 $\Delta\psi$ 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 ΔpH and $\Delta\psi$ in nitrifying bacteria is quite similar to that reported for other bacteria eg. *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 $\Delta\psi$. 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 $\Delta\psi$. 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 $\Delta\Psi$ 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 $\Delta\psi$ 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.
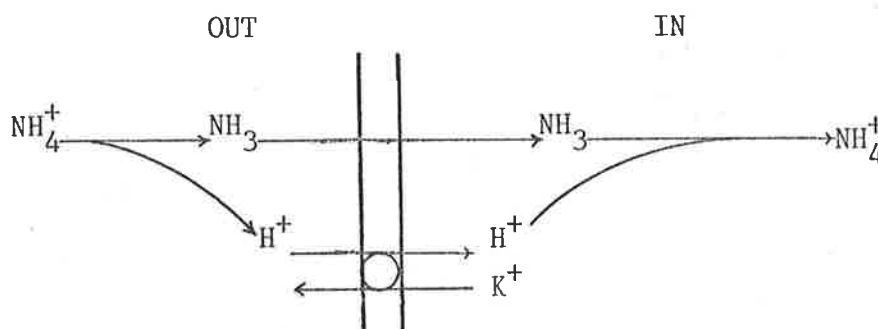
Since the internal pH is decreased by the extrusion of K^+ and the antiporter is relatively inactive at lower pH (Nakamura *et al.*, 1982), a high concentration of amine is required for the bulk release of cellular K^+. It appears that the cells of *Nitrosomonas europaea* grown with high NH_4^+ have relatively low and variable intracellular K^+ (Table 41) which could well be associated with K^+/H^+ antiporter system and NH_4^+ transport into the cell.

The depolarization of $\Delta\psi$ 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 $\Delta\psi$ by about 60mV but this depolarization of $\Delta\psi$ was compensated by an approximately 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 $\Delta\psi$ 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 ΔpH (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 ΔpH. Such interconversions are well known in energy transducing membranes eg. lipid soluble cation $TPMP^+$ causes an extensive conversion of $\Delta\psi$ into ΔpH 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 ΔpH 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 $^{22}Na^+$ loaded cells it is shown in the present work that both *Nitrosomonas europaea* and *Nitrobacter agilis* lack the respiration dependent Na^+ pump. Extrusion of $^{22}Na^+$ from $^{22}Na^+$ loaded cells required K^+ as a counter-ion in both *Nitrosomonas europaea* and *Nitrobacter agilis* but the extent of $^{22}Na^+$ 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_4Cl also resulted in the extrusion of $^{22}Na^+$, 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^+/Na^+ and Na^+/H^+. At least one of these antiporters (K^+/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:

OUT   IN
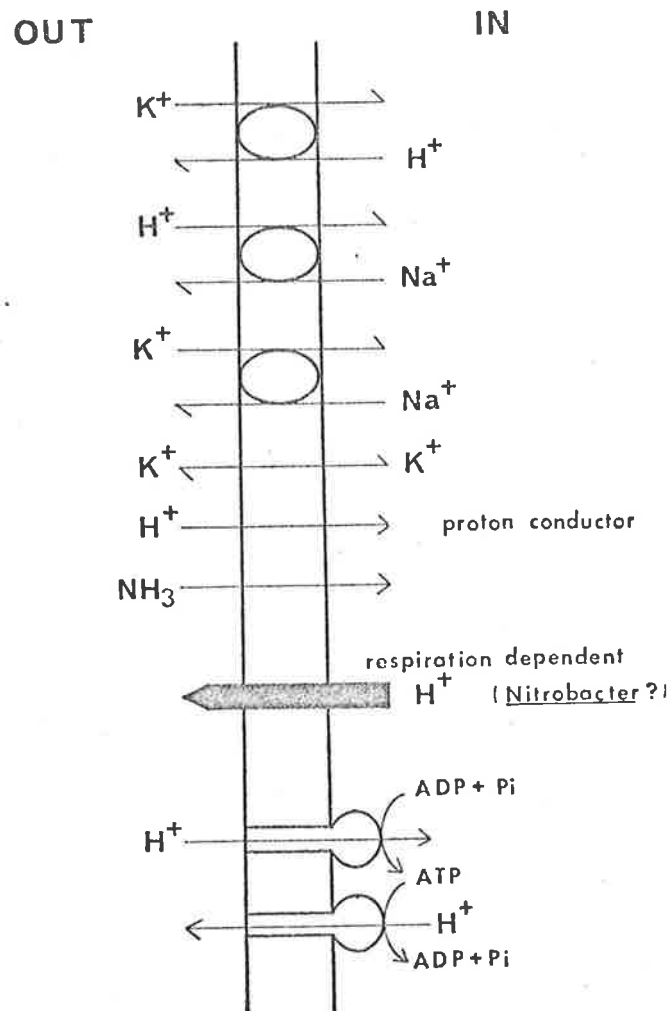
4.3.4 $^{15}N, ^{18}O$ isotope studies of NO_2^- oxidation by Nitrobacter agilis

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 ΔpH) 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 NO_3^- and PO_4^{2-} or 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}N-NMR and GC/MS studies using stable isotopes of ^{15}N and ^{18}O (Section 3.5.5). The results of both ^{15}N-NMR and GC/MS studies clearly indicate that the third 'O' in NO_3^- produced from NO_2^- originated from H_2O and not from either O_2 or PO_4^{2-}. These findings are in agreement with those of Aleem *et al.* (1965). The GC/MS studies of NO_2^- oxidation reported in Section 3.5.5.1 indicate that there was no apparent exchange of ^{18}O between H_2O and $P^{18}O_4^{2-}$ so that it is unlikely that ^{18}O would have been lost from $P^{18}O_4^{2-}$ 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}N-NMR study of NO_2^- oxidation by *Nitrobacter agilis* (Section 3.5.5.2) indicate that when the cells were incubated with $^{15}NO_2^-$ and H_2O, the three resonances in the NMR spectrum represented $^{15}N^{16}O_3^-$ (100%), $^{15}N^{16}O_2^{18}O^-$ (19%) and $^{15}N^{16}O^{18}O_2^-$ (2.3%). Since the $^{15}NO_2^-$ stock contained about 30% $^{15}NO_3^-$, the ratio of $^{15}N^{16}O_3^-$: ($^{15}N^{16}O_2^{18}O^-$ + $^{15}N^{16}O^{18}O_2^-$) is close to the theoretical value (100 : 32). This result proves that all the 'O' in nitrate was derived from water. The oxidation of NO_2^- to 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 $^{15}N^{16}O^{18}O_2^-$ resonance in the ^{15}N-NMR spectrum (Fig.62) is thus unusual. A chemical exchange of ^{18}O between $H_2^{18}O$ and $^{15}N^{16}O_2^{18}O^-$ at alkaline pH is unlikely. A possible explanation of the appearance of $^{15}N^{16}O^{18}O_2^-$ could be associated with the recycling of $^{15}NO_2^-$ by nitrite oxidase (I) and dissimilatory type nitrate reductase (II) (Sewell and Aleem, 1979) in thick cell suspensions which tend to become anaerobic.

$$^{15}N^{16}O_2^- \xrightarrow[\quad(I)\quad]{H_2^{18}O} {}^{15}N^{16}O_2^{18}O^-$$

$$^{15}N^{16}O_2^- + H_2^{18}O \xrightarrow{(I)} {}^{15}N^{16}O_2^{18}O^- \xrightarrow{(II)}$$

$$^{15}N^{16}O^{18}O^- \xrightarrow[H_2^{18}O]{(I)} {}^{15}N^{16}O^{18}O_2^-$$
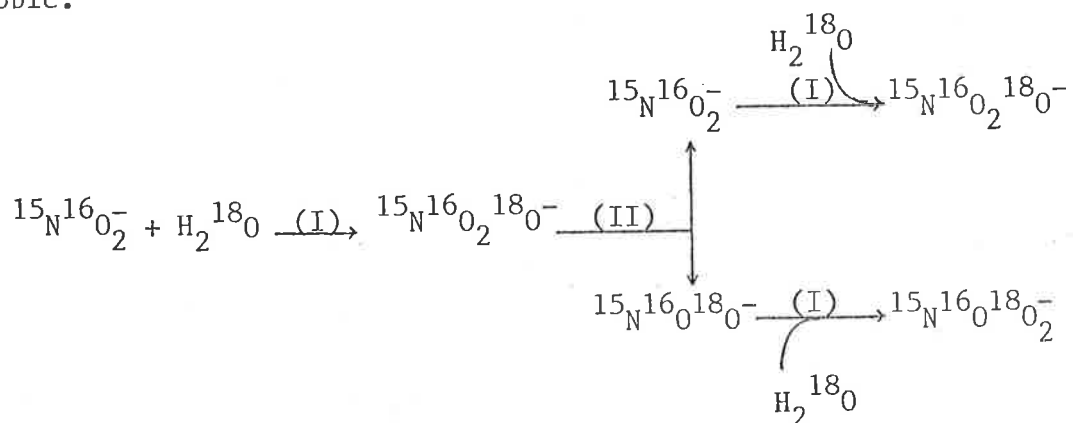
In conclusion, it is possible that *Nitrobacter agilis* conforms to the classical chemiosmotic coupling mechanism for ATP biosynthesis but the non-detection of a respiratory proton pump in this bacterium is unusual and further work is awaited to resolve this interesting anomaly.

5. BIBLIOGRAPHY

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