ENERGY SUPPLY FOR DINITROGEN FIXATION BY *AZOTOBACTER VINELANDII* AND BY BACTEROIDS OF *RHIZOBIUM LEGUMINOSARUM*

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Proefschrift

ter verkrijging van de graad
van doctor in de Landbouwwetenschappen,
op gezag van de Rector Magnificus,
dr. H.C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op vrijdag 29 februari 1980
des namiddags te vier uur in de Aula
van de Landbouwhogeschool te Wageningen

STELLINGEN

1

Zonder het te beseffen laten Thorneley *et al.* zien, dat de door hen gebruikte nitrogenase preparaten uit *Azotobacter* en *Klebsiella* een relatief hoge myokinase activiteit bevatten.

-Thorneley, R.N.F., Lowe, D.J., Eady, R.R. and Miller, R.W. (1979) Biochem.Soc.Trans. *7*, 633-636.

2

De metingen van de hydrogenase-gekatalyseerde H_2-productie activiteit met behulp van dithioniet-gereduceerd methyl viologeen bij lage pH worden doorgaans niet correct uitgevoerd, omdat geen rekening gehouden wordt met de verhoging van de potentiaal van het (bi)sulfiet-dithioniet redoxkoppel bij verlaging van de pH.

-Adams, M.W.W. and Hall, D.O. (1979) Arch.Biochem.Biophys. *195*, 288-299.
-Adams, M.W.W. and Hall, D.O. (1979) Biochem.J. *183*, 11-22.
-Arp, D.J. and Burris, R.H. (1979) Biochim.Biophys.Acta, *570*, 221-230.
-Mayhew, S.G. (1978) Eur.J.Biochem. *85*, 535-547.

3

Bij het gebruik van methylammonium om de pH gradiënt over een membraan te meten, dient terdege rekening gehouden te worden met de mogelijke aanwezigheid van een ammonium transport systeem.

-Rottenberg, H. (1975) Bioenergetics, *7*, 61-74.
-Michels, P.A.M. (1978) Proefschrift, Universiteit van Groningen.

4

Het uitbrengen van een symposium-boek ruim anderhalf jaar na dato is gelijk mosterd na de maaltijd.

-Proc.Third Int.Symp. on Nitrogen Fixation (juli 1978) Madison, Wisconsin, in the press.
-Proc.Sixth Int.Symp. on Flavins and Flavoproteins (maart 1978) Kobe, Japan, in the press.

5

In limnologisch en oceanografisch onderzoek wordt veel te weinig aandacht besteed aan de rol van organisch detritus in het voedselweb.

6

Moleculaire clonering van recombinant DNA lijkt op het ogenblik eerder doel dan middel.

7

Het is onbegrijpelijk dat men in leerboeken voor organische chemie allerlei reactietypes beschrijft, zonder dat men oog heeft voor enzym-katalytische principes.

8

Het wordt helaas te weinig onderkend dat de resultaten, die worden verkregen met de EXAFS (extended X-ray absorption fine structure) techniek pas zinvolle informatie oplevert, indien de structuur van het te onderzoeken chromofoor reeds goed bekend is.

-Teo, B-K. and Averill, B.A. (1979) Biochem.Biophys.Res.Commun. *88*, 1454-1461.

9

Hoe groter de politieke instabiliteit van een land, des te groter en fraaier de postzegels.

Aan Anneke

Aan mijn ouders

VOORWOORD

Het is de gewoonte om op de omslag van een proefschrift alleen de naam van de betrokken promovendus te vermelden. Eigenlijk een hoogst merkwaardige gewoonte, want die ene naam wekt de indruk dat de inhoud van het proefschrift slechts het resultaat is van één persoon. Dat is natuurlijk grote ONZIN: dit proefschrift is het resultaat van intensieve samenwerking. Vandaar dat ik zou willen voorstellen om in de toekomst achter de naam van de promovendus "en andere" toe te voegen. Een prettige bijkomstigheid van deze extra toevoeging zal ongetwijfeld zijn, dat een potentiële lezer zich zal afvragen wie deze "en andere" dan wel zijn. Om hierop een antwoord te krijgen moet de lezer het proefschrift openen. De eerste drempel is nu overschreden. Een ware onthulling volgt dan in het voorwoord. De hoop lijkt nu gerechtvaardigd dat de lezer zo geboeid raakt, dat hij of zij de tweede drempel overschrijdt en werkelijk het proefschrift gaat lezen.

Wie zijn deze "en andere", die ik hierbij gezamenlijk wil bedanken voor de bijdragen die ze hebben geleverd tot het gereed komen van dit proefschrift?

Allereerst mijn promotor Cees Veeger voor de enthousiaste en kundige begeleiding van het onderzoek. In het bijzonder wil ik mijn co-referent Huub Haaker bedanken voor de inspirerende en kritische manier waarop hij optimaal participeerde in het onderzoek. De samenwerking met hem heb ik als ideaal ervaren. Dit geldt evenzeer voor de samenwerking met mijn labgenoten Gerard Scherings en Louise van Zeeland-Wolbers. Verder dank ik Arie Bruinink, Eylon Shlomi, Willy Krone en Reint Harkink, die in het kader van hun ingenieurs studie aan dit onderzoekproject hebben meegewerkt. Zeer erkentelijk ben ik mijn kamergenoot Cees van Dijk voor het bepraten van vele zaken en het ongewild meeroken in de afgelopen drie jaar.

Bijzondere dank ben ik verschuldigd aan de heren Houwers, Van Velsen en Van Geffen van de afdeling Microbiologie voor het met zorg kweken van de ontelbare erwtenplantjes. Zij hebben voor een belangrijk deel de basis van dit proefschrift gelegd.

Een woord van dank gaat tevens uit naar Wil Konings van de afdeling Microbiologie in Haren. Zijn hulp in de "flow dialyse" experimenten kende geen grenzen en bleek onmisbaar. Verder Sander Ottema, die verreweg de vervelenste taak van de

"flow dialyse" experimenten op zich heeft genomen; het schoonmaken van de vele telpotjes.

Voorts dank ik Rob Goldbach van de vakgroep Moleculaire Biologie voor de verleende hulp in het isotopen laboratorium en alle leden van de "Knollenclub".

Een speciale vermelding verdienen Bery Sachteleben, Jenny Toppenberg-Fang, alsmede Jill Zeilstra-Ryalls. Bery voor zijn praatjes en zijn tekenwerk van grote klasse, Jenny voor haar niet te overtreffen type capaciteiten en Jill voor de correctie van de engelse tekst.

CONTENTS

LIST OF ABBREVIATIONS

ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
ATPase	ATP phosphohydrolase (EG 3.6.1.3)
BSA	bovine serum albumin
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
Cl_4CF_3Bza	see TTFB
CoA	coenzym A
DMO	5,5-dimethyloxazolidine-2,4-dione
DNA	deoxyribonucleic acid
DNP	2,4-dinitrophenol
EDTA	ethylene diaminetetra acetic acid
NAD^+	oxidized nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
$NADP^+$	oxidized nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
P_i	inorganic phosphate
PCB^-	phenyldicarbaun decarborane
Ph_3MeP^+	triphenylmethylphosphonium
Ph_4P^+	tetraphenylphosphonium
pK_a	negative logarithm of the acid-dissociation constant
SCN^-	thiocyanate
Tes	*N*-tris (hydroxymethyl)methyl-2-aminoethanesulfonate
Tris	Tris (hydroxymethyl)-aminomethane
TTFB	4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazol
ΔpH	transmembrane proton concentration difference
$\Delta\Psi$	electrical membrane potential
$\Delta\tilde{\mu}_{H^+}$	protonmotive force

1. GENERAL INTRODUCTION

Nitrogen (N) is one of the most vital elements in nature, since all forms of life absolutely require nitrogen for their growth. Although the atmospheric reserve of nitrogen in the form of dinitrogen (N_2) is virtually inexhaustible, most plants and animals are limited in their growth by an inadequate supply of nitrogen. This paradox becomes self-evident when one considers the fact that atmospheric dinitrogen is so inert that most organisms cannot utilize this form of nitrogen. Dinitrogen can enter biological systems only when it has been "fixed"; thus, combined with other elements such as hydrogen or oxygen. In nature, atmospheric dinitrogen can be fixed by only a relatively small number of microorganisms. No higher organisms have developed the cellular equipment required to fix dinitrogen, although several participate indirectly by forming symbiotic associations with *dinitrogen-fixing* bacteria [1].

The fixation can also be accomplished industrially through the manufacture of ammonia from hydrogen gas and atmospheric dinitrogen; the so-called Haber-Bosch process. However, the bulk of all fixed nitrogen on earth is of biological origin [2]. The energy necessary for industrial fixation is provided by fossil fuels. Since the cost of nitrogen fertilizers is closely correlated with the cost of fossil fuels and most food-producing plants require fertilizer, it is clear that nowadays the process of biological dinitrogen fixation is under current investigation.

1.1. BIOLOGICAL DINITROGEN FIXATION

Although the process of dinitrogen fixation is a faculty reserved to a relatively few genera of microorganisms, the list of dinitrogen-fixing organisms is expanding and covers a wide range of microbial types. Among these are free-living bacteria, cyanobacteria, those that thrive only in a symbiotic association with plants or animals and some that can adapt either mode of life. A few of these bacteria are photosynthetic, some require oxygen, while others can fix dinitrogen only when oxygen is excluded from their environment (Table 1, [3]). In spite of the large diversity among these organisms, they all share the enzyme

system nitrogenase, which catalyzes the reduction of dinitrogen to ammonia. The nitrogenase system in all bacteria studied, consists of at least two different

	Genus or type	Species (examples only)
Strict anaerobes	*Clostridium* *Desulfovibrio* Methane-producing bacteria	*C.pasteurianum* *D.vulgaris*
facultative (aerobic when not fixing dinitrogen)	*Klebsiella* *Bacillus*	*K.pneumoniae* *B.polymyxa*
microaerophiles (normal aerobes when not fixing dinitrogen)	*Mycobacterium* *Spirillum* some *Rhizobia*	*M.flavum* *S.lipoferum*
aerobes	*Azotobacter* *Azotococcus*	*A.vinelandii* *A.agilis*
phototrophs (anaerobes)	*Chromatium* *Chlorobium*	*C.vinosum* *C.limicola*
phototrophs (facultative)	*Rhodospirillum* *Rhodopseudomonas*	*R.rubrum* *Rps.capsulata*
phototrophs (microaerophiles)	*Plectonema*	*P.boryanum*
phototrophs (aerobic)	*Anabaena* *Nostoc* *Gloeocapsa*	*A.cylindrica* *N.muscorum* *G.alpicola*

Table 1. Some dinitrogen-fixing microorganisms. After Postgate [3].

components. The larger component (molecular weight 220,000) contains molybdenum and iron and is commonly called Mo-Fe protein or component I. The smaller component (molecular weight 55,000) contains only iron and is named the Fe-protein or component II. Because the actual reaction is taking place at component I, Burris recently suggested to call component I dinitrogenase, and component II dinitrogenase reductase [4]. Detailed information concerning the properties of this complex enzyme system is given by Orme-Johnson *et al.* [5].

The nitrogenase complex requires energy for its activity. The equation given below shows that the overall reaction is exergonic.

$$N_2 + 2H^+ + 3H_2 \rightleftharpoons 2\ NH_4^+ \qquad \Delta G^{o'} = -80\ \text{kJ/reaction}$$

This implies that, in the presence of a suitable catalyst and enough reducing power (H_2), the reduction of dinitrogen will proceed spontaneously. In accordance

with this view, it would seem sufficient for dinitrogen-fixing organisms to produce a strong reductant for the process to occur. However, in addition to energy in the form of reducing equivalents, the nitrogenase system requires another source of chemical energy, that obtained from the hydrolysis of ATP (Fig. 1). During electron transfer from dinitrogenase reductase to dinitrogenase, ATP is converted into ADP. Since ADP is a strong inhibitor of the actual nitrogenase reaction, the ATP/ADP-ratio has long been recognized as an important regulator of nitrogenase activity. In laboratory experiments the reduction of 1 molecule dinitrogen into 2 molecules of ammonium required 12 to 15 molecules of MgATP [6]. However, the number of ATP molecules required by the nitrogenase reaction strongly depends on factors such as temperature [7], the pH [8], the amount of dinitrogenase reductase relative to the amount of dinitrogenase [9], the source of the two components and the rate of electron transfer between the two components [4].

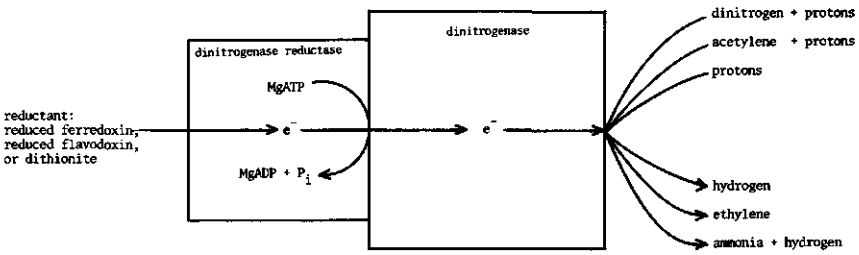

Figure 1. Reactions of the nitrogenase complex.

Presumably, ATP is necessary to overcome the activation energy of the reduction of dinitrogen by nitrogenase [10,11]. In addition to dinitrogen a wide range of other substrates including protons and acetylene, function in the nitrogenase reaction (Fig. 1). Reduction of acetylene (C_2H_2) to another gas ethylene (C_2H_4) is particularly important, due to its relative ease of detection by means of gas chromatography. Earlier methods used to estimate dinitrogen fixation involved either tracing heavy isotopes of nitrogen, or measuring the increase in the total nitrogen content of an organism. Both procedures are time-consuming and not very sensitive. The acetylene-reduction assay is commonly employed in studying the nitrogenase activity of both enzyme preparations, as well as intact organisms. However, the acetylene-reduction method also has disadvantages. For example, acety-

lene cannot be used to measure the nitrogenase activity of methane-oxidizing bacteria, since acetylene inhibits their metabolism [12].

As was mentioned previously, the nitrogenase complex breaks the tight triple bond of molecular nitrogen only when it is protected against damage by oxygen and when it is supplied with sufficient energy. Aerobic dinitrogen-fixing organisms are thus faced with the problem of maintaining their intracellular oxygen concentration at a level that is too low to inhibit nitrogenase activity, yet high enough to produce the required amount of energy needed to fix dinitrogen. Several protective mechanisms against inactivation by oxygen can be distinguished. All of these are fine examples of the way cells can adapt themselves to their natural habitat.

1. The free-living dinitrogen fixer *Azotobacter vinelandii* keeps the environment of nitrogenase free of oxygen by adapting its respiration rate to the oxygen input during growth (respiration protection) [13,14]. Furthermore, the nitrogenase components can be protected from oxygen inactivation by the formation of a complex with a third, ferredoxin-type protein (conformational protection) [15,16].
2. In the symbiotic association between *Rhizobia* species and legumes this problem is overcome by the presence of leghaemoglobin in the root nodules. This oxygen-binding protein is probably located in the space between the symbiotic bacteria (bacteroids) and the membrane which enclose them within the plant host cell [17]. Leghaemoglobin facilitates a high flux of oxygen at an extremely low free-oxygen concentration to each bacteroid [17,18]. In this way, the bacteroid produces enough energy for dinitrogen fixation; and at the same time, the dinitrogen-fixing system is protected against damage by oxygen through the oxygen-buffering capacity of leghaemoglobin.
3. In cyanobacteria, formerly called blue-green algae, the process of dinitrogen fixation is reserved only to specialized cells, called heterocysts. This type of cell is thought to protect nitrogenase against oxygen inactivation by slime production, a thick wall (physical protection) [19], the absence of an oxygen evolving photosystem II [20], and a high rate of respiration [21].

Some obligate aerobic organisms such as *Rhizobia* strains, which are capable of fixing dinitrogen in liquid culture, and *Mycobacterium flavum*, do not exhibit a protective mechanism. These aerobes fix dinitrogen only under micro-aerophilic conditions (see [3]).

1.2 ENERGY GENERATING SYSTEMS IN BACTERIA

In vitro, nitrogenase activity is usually measured merely by adding MgATP (or an ATP-generating system), along with a strong reducing agent, such as sodium dithionite. However, intact cells must derive their energy from their metabolism. In this paragraph, general aspects of energy-generating systems in bacteria will be considered.

Metabolic energy, required for maintenance and growth of bacteria, can be generated by several fundamentally different biochemical mechanisms. In most bacteria, energy for energy-requiring processes such as ATP synthesis, active transport or cellular mobility is made available by the following mechanisms:

1. By scalar reactions. In a sequence of reactions, energy is converted at the substrate level into energy rich compounds, such as ATP and acetyl-CoA (substrate level phosphorylation, see 1.2.1).
2. By chemiosmotic reactions. In reactions directed vectorially, chemical energy is converted into osmotic energy. The most frequently occuring vectorial reactions are redox reactions *via* electron transfer chains. General aspects of electron transfer chains are described in 1.2.2. Another way of generating chemiosmotic energy is exhibited by certain extreme halophilic bacteria; *via* a purple pigment, called bacteriorhodopsin [22]. Recently, a third vectorial process that yields metabolic energy has been recognized [23]. On theoretical grounds it was deduced that fermenting bacteria can derive some additional metabolic energy by the excretion of fermentation products. In the next paragraph (1.3) the molecular basis of energy conversion by vectorial reactions will be discussed.

Scalar reactions play an important role in bacteria which rely entirely upon fermentation. In contrast, in bacteria which obtain their energy from electron transfer reactions, scalar reactions play a minor role in the total energy production of the cell.

1.2.1. substrate level phosphorylation

Several catabolic pathways are known to yield energy in the form of ATP and/or reducing equivalents. It is beyond the scope of this thesis to deal extensively with all the reactions which provide energy at the substrate level. For detailed information, the reader is referred to a review article of Thauer *et al.* [24], which covers this field. However, in fermenting organisms, such as *Clostridium*

pasteurianum, *Bacillus polymyxa* and *Klebsiella pneumoniae*, energy for energy-requiring processes, among which falls dinitrogen fixation, is generated by the central pathway of metabolism, the so-called phosphoroclastic reaction (Fig. 2A) [25-27]. The breakdown of pyruvate results in the formation of ATP, and the reduction of the electron carrier protein ferredoxin (or flavodoxin).

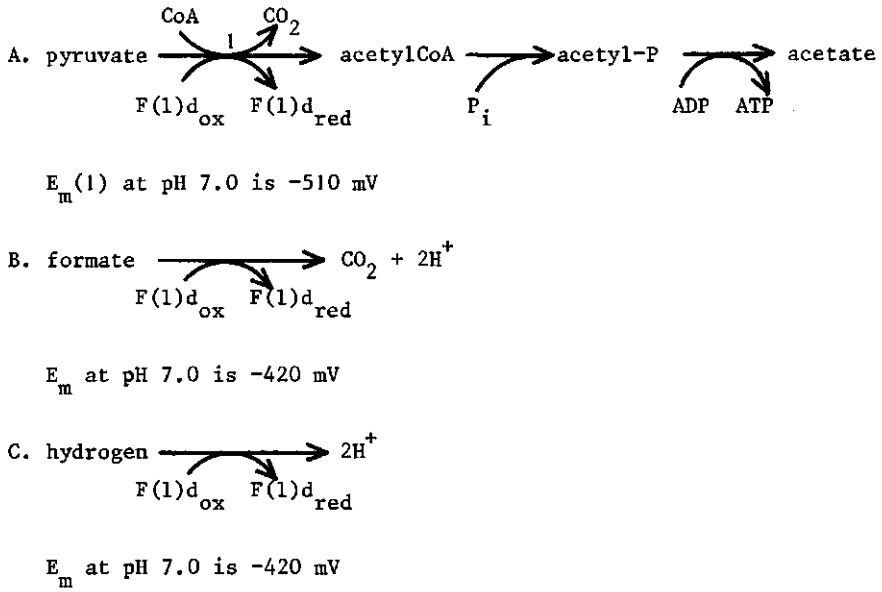

Figure 2. Energy-providing reactions for nitrogenase in fermenting bacteria. CoA, coenzym A; Fd, ferredoxin; Fld, flavodoxin; ox, oxidized; red, reduced.

Reduced ferredoxin (or flavodoxin) serves as the natural electron donor for nitrogenase. The acetyl-phosphate synthesized in this reaction transfers its "high-energy" phosphate to ADP, thus forming ATP. Under appropriate conditions, reducing equivalents with a sufficiently low redox potential can also be generated by decarboxylation of formate (Fig. 2B), or by the oxidation of H_2 (Fig. 2C) [26,27]. However, under physiological conditions, these reactions do not contribute significantly to the generation of reducing power for dinitrogen fixation, since they are supposed to function in precisely the opposite direction. In general, the formation of ATP is the rate-limiting step in fermenting organisms. Reducing power is available in excess (see [6]). Along these lines, one can see that it would be of interest to investigate the possibility that the efflux of fermentation products in whole cells provides additional ATP for the nitrogenase reaction.

Because reducing power is in excess in these organisms, immediate regulation of the nitrogenase activity by the intracellular ATP/ADP-ratio can be of physiological importance (see [5]).

1.2.2. electron transfer systems

In most other bacteria, energy is generated by oxidation-reduction reactions *via* an electron transfer chain. This chain is located in the cytoplasmic membrane of bacteria, and is either arranged in a linear or cyclic fashion. Cyclic electron transfer chains are only involved in bacteria which derive their energy directly from light.

1.2.2.1 linear electron transfer chains

This type of chain consists of a series of electron carriers of successively higher redox potential. The energy contained within an oxidizable substrate is liberated during electron transfer along the chain and subsequently converted into metabolically useful energy, some of which is in the form of ATP. Despite the fact that there is a large diversity among individual components of the chain, the overall features of electron transfer chains are similar in both bacteria, as well as in higher organisms. Fig. 3 visualizes a general scheme of bacterial electron transfer systems.

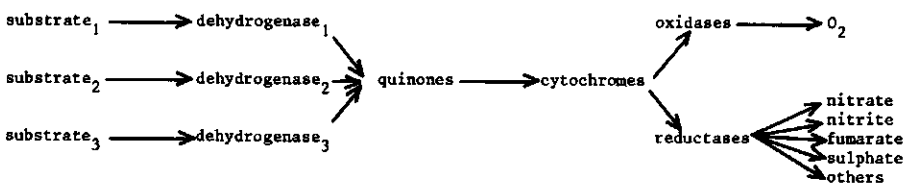

Figure 3. Generalized scheme of electron transfer systems in bacteria.

Several classes of proteins are known to participate in electron transfer. The first major group is that of the dehydrogenases. At the level of the dehydrogenases, the substrate releases its reducing equivalents into the chain. The second important group is that of the quinones. These relatively small molecules shuttle

between the dehydrogenases and the cytochromes. Finally, these haem-containing proteins transfer electrons to complex cytochromes, called oxidases, or to reductases. In addition, there are iron-sulphur proteins present which are involved in electron transfer at several stages along the chain. For detailed information, the reader is referred to review articles, which cover this field extensively [24,28].

In higher organisms, oxygen serves as a terminal electron acceptor. In contrast, bacteria can use several other terminal electron acceptors as well (Fig. 3). In accordance with the nature of the terminal acceptor one makes a distinction between "aerobic" or "anaerobic" respiratory chains. Of all the bacteria which use a respiratory chain, only a few are capable of fixing dinitrogen. Moreover, among these organisms, most thrive on aerobic respiration. To our knowledge only *Desulfovibrio* species are capable of fixing dinitrogen by employing an anaerobic respiratory chain [29]. The best-known group of obligate aerobic dinitrogen fixers is the family *Azotobacteraceae*. Another important group is the family *Rhizobiaceae*. In contrast to the *Azotobacter* species, the latter organisms normally fix dinitrogen in symbiosis with legumes.

How obligate aerobes derive their energy from metabolism for dinitrogen fixation is only partly known. ATP is generated by a process called oxidative phosphorylation (see 1.3.4.1). How reducing power is generated is still a topic of debate (see 1.3.4.4). In this thesis the relation between energy metabolism and dinitrogen fixation is studied in two aerobes; *Azotobacter vinelandii* and bacteroids of *Rhizobium leguminosarum*.

1.2.2.2. cyclic electron transfer systems

In most photosynthetic bacteria, solar energy is captured by antenna pigments (carotenoids and bacteriochlorophyll), and subsequently converted into metabolic energy *via* a cyclic electron transfer chain (Fig. 4).

The ability to fix dinitrogen among photosynthetic bacteria, is a common feature. Examples are the purple non-sulphur bacteria, such as several *Rhodopseudomonas* [30] and *Rhodospirillum* species [31]; the purple sulphur bacterium *Chromatium*, and the green sulphur bacterium *Chlorobium* [32]. Initial studies on dinitrogen fixation by photosynthetic bacteria indicated that the reduction of dinitrogen occurs only under anaerobic conditions in light. However, recent evidence indicates that in the purple bacterium *Rps.capsulata* the reduction of dinitrogen is not obligatorily coupled to the activity of the photosynthetic apparatus [33]. It

can be demonstrated that this organism can fix dinitrogen in darkness with alternative energy conversion systems; namely, aerobic sugar fermentation and aerobic respiration at low oxygen tension. Whether the strict anaerobes *Chlorobium* and *Chromatium*, are able to fix dinitrogen under fermentative conditions is not yet known. In bacteria of the genus *Chlorobia*, the possibility exists that the primary electron acceptor in the cyclic electron transfer chain (X, -550 mV), donates electrons directly to strong reductants, such as ferredoxin [34]. However, in most photosynthetic bacteria, the midpoint potential of the primary electron acceptor is too high (-50 to -150 mV) for a direct reduction of ferredoxin. At present, it is not known how reducing equivalents for nitrogenase are generated in photosynthetic bacteria. A possible mechanism is discussed in Chapter 6.

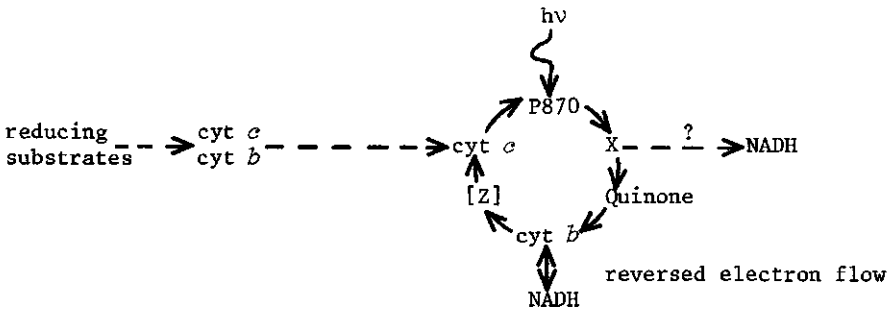

Figure 4. A generalized scheme for cyclic electron flow in photosynthetic bacteria. P870, photoreaction center bacteriochlorophyll; X is likely to be an iron protein or iron-quinone complex; Z is an intermediate suggested from kinetic evidence. Reducing substrates; in sulphur bacteria these are hydrogen sulphide or thiosulphate, in non-sulphur bacteria these are organic molecules such as malate or succinate. After Jones [35].

A very special group of dinitrogen-fixing organisms, which also use light as an energy source, is the cyanobacteria. Well-known genera are *Anabaena* and *Nostoc*. In light, cyclic phosphorylation was shown to supply the ATP for nitrogenase [36]. Again, little is known about the way in which reducing equivalents are generated in cyanobacteria. A possible mechanism for the electron transfer to nitrogenase is given in Chapter 6. For an extensive discussion see [37].

1.3. MOLECULAR MECHANISM OF ENERGY CONVERSION

1.3.1. introduction to protonmotive force

The search for the mechanism that explains the coupling between electron transfer and energy-requiring processes has extended for over 25 years and has produced numerous hypotheses [38]. The most useful and successful of these hypotheses is that known as Mitchell's chemiosmotic theory, of which an early account can be found in 1961 [39-44]. This theory recognizes specifically the role of the cytoplasmic membrane and its supramolecular structure. In its original version, Mitchell postulated that the flow of reducing equivalents along a membrane-bound electron transfer chain results in the vectorial translocation of protons across the membrane. To accomplish proton translocation from one site of the membrane to the other, the chain was considered to be an alternating sequence of hydrogen and electron carriers, arranged across the membrane in loops (Fig. 5A). The localisation of carriers in the membrane is still a topic of debate, as an alternating arrangement implies that very defined proton/electron ratios should be found. In several membrane systems, ratios have been found that differ from those predicted by the chemiosmotic theory. Based on these discrepancies, Williams [45,46] and Papa [47] developed alternative models which, although they explain different proton/electron stoichiometries, have no convincing evidence to date for their existence.

According to the chemiosmotic theory, membranes are poorly permeable to ions; especially, protons and hydroxyl ions. Because of this, an electrochemical proton difference ($\Delta\tilde{\mu}_{H^+}$) can be built up across the membrane during electron transfer along the respiratory chain. The translocation of protons across the membrane intrinsically involves a net movement of charge across the membrane. Therefore, the electrochemical gradient or "protonmotive force" consists of an electrical and a concentration component, according to the relation:

$$\Delta\tilde{\mu}_{H^+} = \Delta\Psi - Z\Delta pH,$$

where $\Delta\Psi$ is the potential difference between the inner and outer surface of the cytoplasmic membrane (membrane potential) and ΔpH is the transmembrane difference in proton concentration. The gradients are usually expressed in millivolts. $Z = 2.3\frac{RT}{F}$ and is a factor converting ΔpH into millivolts.

1.3.2 protonmotive force-generating systems

Initially, only electron transfer systems were considered to generate a $\Delta\tilde{\mu}_{H^+}$. Now, it has been firmly established that the hydrolysis of ATP via a membrane-bound ATPase also generates a $\Delta\tilde{\mu}_{H^+}$ (Fig. 5B, see [38]). Furthermore, a unique protein called bacteriorhodopsin, was isolated from certain extremely halophilic bacteria which generates a $\Delta\tilde{\mu}_{H^+}$ directly from light, without the involvement of electron carriers (Fig. 5C) [22]. As mentioned above, Konings and coworkers [23] proposed a very simple way for fermenting bacteria to generate a $\Delta\tilde{\mu}_{H^+}$ (Fig. 5D). Although they have no experimental evidence, it seems to be worthwhile to consider their hypothesis as an additional energy-generating mechanism.

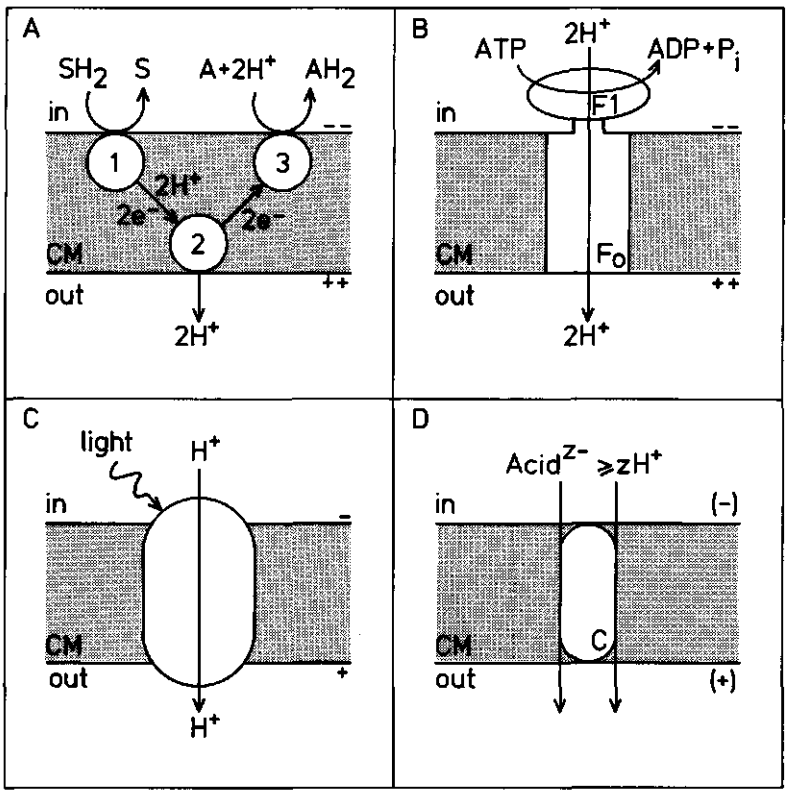

Figure 5. $\Delta\tilde{\mu}_{H^+}$ generating mechanisms in bacteria. (A), electron transfer chain. SH_2 and A represent the electron donor and acceptor, respectively. 1, 2 and 3 are components of the chain arranged in a loop. After Boonstra [48]; (B), ATPase; F_0 and F_1 are components of the Mg^{2+}-ATPase; (C), bacteriorhodopsin; (D), excretion of fermentation products; C, carrier protein. CM, cytoplasmic membrane.

1.3.3. relation between ATP and the protonmotive force

It is now becoming apparent that chemiosmotic phenomena are involved in various systems that perform physiologically useful work (Fig. 6). Traditionally, only ATP and other "high-energy" compounds have been assigned this unique position in cellular economics. Energy-consuming reactions were considered to be driven by ATP, or by thermodynamically equivalent compounds, such as GTP and NADH. According to this view, ATP itself was considered to be the universal energy currency. One of the major contributions of the chemiosmotic theory to the understanding of energy fluxes in bacteria has been the recognition that this description is seriously oversimplified. Numerous studies left no doubt that several membrane systems can do work without the intermediacy of ATP (see review [49]).

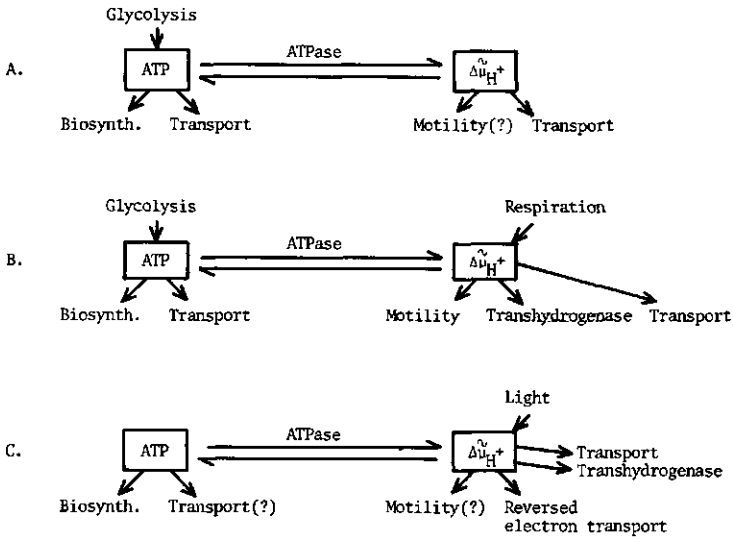

Figure 6. Generalized scheme of energy fluxes in bacteria. (A), fermentative metabolism; (B), metabolism of aerobes and facultative anaerobes; (C), metabolism of photosynthetic bacteria. After Harold [49].

Today, ATP and $\Delta\tilde{\mu}_{H^+}$ are both considered to be energy currencies, which are interconvertible through the Mg^{2+}-dependent ATPase complex. Fig. 6 deals with energy fluxes in bacteria, but it should be noted that the capacity to generate a $\Delta\tilde{\mu}_{H^+}$ is also a common feature among mitochondria and chloroplasts.

In fermentative bacteria, metabolically useful energy is generated mainly during glycolysis and other catabolic pathways by scalar reactions that generate reducing power, ATP and other "energy-rich" compounds (Fig. 6A). In some energy-

requiring processes, among which dinitrogen fixation, ATP and reducing equivalents are used directly. In others, the chemical energy of ATP is transformed first into chemiosmotic energy ($\Delta\tilde{\mu}_{H^+}$) *via* the proton-translocating Mg^{2+}-ATPase, and subsequently used to perform work such as transport and possibly motility. In bacteria which use an electron transfer chain (cyclic or linear) to obtain their metabolic energy, the energy conversion is the other way around (Fig. 6B and C). First, a $\Delta\tilde{\mu}_{H^+}$ is built up by electron transport coupled to proton translocation. Subsequently, the generated $\Delta\tilde{\mu}_{H^+}$ is used by several enzyme systems to do work, such as ATP synthesis, reversed electron transport, motility and transport.

1.3.4. protonmotive force-utilizing systems

In Fig. 7, models are given that visualize how enzyme systems utilize the $\Delta\tilde{\mu}_{H^+}$ to perform physiologically useful work. The list of processes which require a $\Delta\tilde{\mu}_{H^+}$ is still expanding. Recently, evidence was presented, which suggests that the synthesis of methane by *Methanobacterium ruminantium* is also dependent on $\Delta\tilde{\mu}_{H^+}$ [50]. Furthermore, an important step in the infection process of bacteriophages seems to be related to $\Delta\tilde{\mu}_{H^+}$ [51]. Evidence along quite another line may be contained in the report of Mevel-Ninio *et al.* [52], which suggests that the synthesis of DNA in *E.coli* requires the energized state of the membrane.

1.3.4.1. ATP synthesis

Bacterial membranes, like those of mitochondria and chloroplasts, often appear in photomicrographs to contain knobs that project into the cytoplasm. These knobs have been identified as the Mg^{2+}-dependent ATPase complex [53]. Several laboratories have described proton translocation by purified ATPases from different sources incorporated into artificial membranes [54-56], leaving no doubt that proton translocation is an intrinsic function of this enzyme complex. This enzyme complex is comprised of two distinct regions; the water soluble headpiece, F_1, and the membrane portion, or basepiece, F_o. Although the precise molecular mechanism of action is still a topic of debate [38], the hydrolysis of ATP in the F_1 part of the complex brings about the electrogenic extrusion of protons through a proton channel in the F_o-fragment of the ATPase complex; and, conversely, a $\Delta\tilde{\mu}_{H^+}$ drives the synthesis of ATP by a reverse flow of protons down the gradient (Fig. 7A).

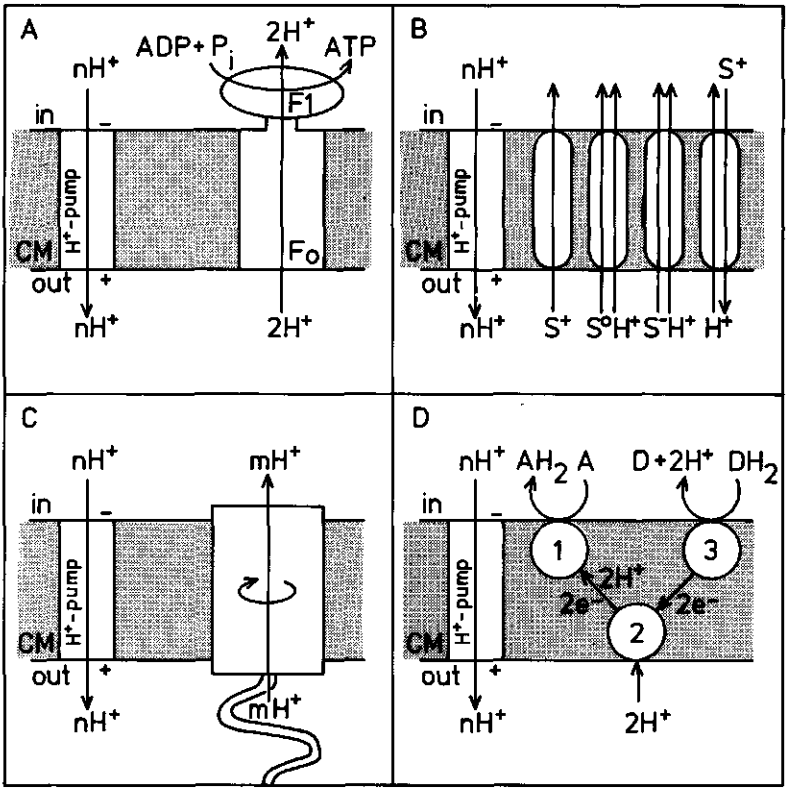

Figure 7. Models for the coupling between $\Delta\tilde{\mu}_{H^+}$ and energy-requiring processes. (A), ATP synthesis; (B), active transport; (C), motility; (D), reversed electron flow. CM, cytoplasmic membrane. n, number of protons extruded by the $\Delta\tilde{\mu}_{H^+}$-generating proton pump; m, number of protons consumed by the energy-requiring process considered. S, solute species; S^o, uncharged; S^+, cationic; S^-, anionic; 1,2 and 3 are components of the respiratory chain with successively higher redox potential; D, electron donor; A, electron acceptor.

1.3.4.2. active transport

In bacteria, the cytoplasmic membrane also functions as a selective barrier in the transfer of specific solutes from one side of the membrane to the other. According to the chemiosmotic theory, several transport systems are driven by $\Delta\tilde{\mu}_{H^+}$, or one of its components. The molecular mechanism of the enzyme system determines the coupling with $\Delta\tilde{\mu}_{H^+}$. As shown in Fig. 7B, uptake of cationic metabolites (potassium, lysine) respond directly to the $\Delta\Psi$, interior negative, while anionic metabolites (phosphate, glutamate) would be transported in symport with protons; thus, according to the ΔpH, interior alkaline. Uncharged solutes (galactosides, proline) would travel by symport with protons, accumulating in response

to the $\Delta\tilde{\mu}_{H^+}$. Furthermore, antiport carriers link extrusion of Na^+ or Ca^{2+} to the entry of protons. After a period of confusion and controversy [57-59], a general consensus with these principles has been reached. Today more refined models have appeared in the literature that also take into consideration changes in the proton/solute stoichiometry with variation of the external pH [60].

1.3.4.3. motility

Many bacteria swim actively in response to environmental stimuli, such as attractants or repellents, changes in pH, or light intensity. The organs for locomotion are the flagella, long whiplike filaments inserted into the cellular envelope [61]. It has long been a puzzling question as to how metabolism can generate motion and how information to guide movement is sensed, processed, and transmitted to the flagella. Today, it is clear that the rotation of the flagella is powered by the influx of protons (Fig. 7C). No ATP seems to be necessary for cellular motility [62]. In the bacterium, *Escherichia coli*, the root of the flagellum is a ring of 16 proteins, apposed to a similar ring in the cell wall. If a proton must pass through each protein to rotate the flagellum one sixteenth of a turn, 256 protons would be consumed in each revolution [63].

1.3.4.4. reversed electron flow

With the exception of motility, all of the activities mentioned above are reversible. As was stated earlier, the synthesis of ATP is driven by $\Delta\tilde{\mu}_{H^+}$; and, conversely, the hydrolysis of ATP generates a $\Delta\tilde{\mu}_{H^+}$. Also it has been demonstrated that a carrier-mediated reversed substrate transport can generate a $\Delta\tilde{\mu}_{H^+}$ [64,65]. The same applies for the flow of electrons along the respiratory chain. The electron flow along a respiratory chain is determined by the electronmotive force, and the energy made available in this way is partially converted into a protonmotive force. However, under appropriate conditions, a $\Delta\tilde{\mu}_{H^+}$ can push electrons against the electronmotive force. This process is commonly termed reversed electron flow. Reversed electron flow is thought to be involved in NADH generation by several photosynthetic bacteria, certain (non-photosynthetic) chemoautotrophs, and occurs in other bacteria as well [66]. The mechanism must be envisaged as a reversal of that by which a $\Delta\tilde{\mu}_{H^+}$ is generated between NADH and quinone.

Haaker *et al.* found that electron transport to nitrogenase in *A.vinelandii* was sensitive to de-energization of the cytoplasmic membrane [67]. In their ex-

periments, proton-conducting uncouplers were used to de-energize the membrane. Since no scalar reactions are known to occur in *Azotobacter* which generate enough reducing power for nitrogenase, their results suggest that electron transport to nitrogenase is driven by the $\Delta\tilde{\mu}_H^+$ and therefore behaves like a reversed electron flow. For instance, from NAD(P)H *via* ferredoxin or flavodoxin to the nitrogenase complex.

1.4. SPECIAL TOPICS

1.4.1. mode of action of ionophores

Much of the work presented above has been elucidated by means of ionophores. These compounds have been found to be very useful tools in the study of chemiosmotic phenomena. Ionophores function by specifically altering the permeability of the membrane for certain ions.

The best known ionophores are the so-called proton ionophores or proton-conducting uncouplers, examples of which are 2,4-dinitrophenol (DNP), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 4,5,6,7-tetrachloro-2-trifluoromethyl-benzimidazol (TTFB). They are all weak acids ($pK_a \simeq 5$), and are soluble within the membrane both in their protonated and unprotonated forms (Fig. 8A). Proton-conducting uncouplers are known to induce a collapse of both the $\Delta\Psi$ and ΔpH [68]. These uncouplers are therefore potent inhibitors of processes that require a $\Delta\tilde{\mu}_H^+$, or one of its components.

Another commonly used ionophore is valinomycin. This cyclic, hydrophobic molecule binds potassium very strongly and is soluble within the membrane with or without bound potassium. It facilitates the electrogenic movement of potassium across the membrane, causing dissipation of the $\Delta\Psi$ and, in some instances, reciprocal enhancement of the ΔpH (Fig. 8B) [69-71].

Nigericin is also a frequently used ionophore. It is a cyclic molecule which can bind either a proton or a potassium ion. In either state, it is soluble within the membrane. Nigericin mainly transports potassium across the membrane in exchange for protons. Consequently, nigericin dissipates the ΔpH and, in some cases, enhances the $\Delta\Psi$ (Fig. 8C) [69-71]. Whether these ionophores inhibit, stimulate or have no effect at all depends on the nature of the coupling between $\Delta\tilde{\mu}_H^+$ and the energy-requiring process concerned. Detailed information about the properties of ionophores and their biological applications has been reviewed by Pressman [72].

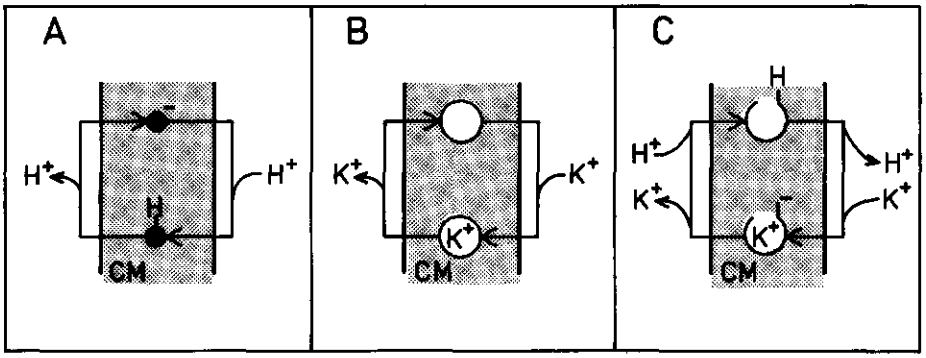

Figure 8. Action of ionophores. (A), proton ionophores; (B), potassium ionophores; (C), potassium-proton ionophore. CM, cytoplasmic membrane.

1.4.2. determination of $\Delta\tilde{\mu}_{H^+}$

A major step towards the understanding of chemiosmotic phenomena has been the development of accurate methods for measuring $\Delta\Psi$ and ΔpH. Although description of some of these methods is included herein, it is beyond the scope of this thesis to deal extensively with all the methods described in the literature. For detailed information the reader is referred to an article on this field [73].

One of the methods for determining $\Delta\Psi$ is based on the property of lipophylic ions to become distributed across the membrane in response to the $\Delta\Psi$ [74,75]. In whole cells, or membrane systems with the same orientation as the cell, inside negative, positively charged ions such as triphenylmethylphosphonium (Ph_3MeP^+) or tetraphenylphosphonium (Ph_4P^+) are frequently used ([69,70,75], Chapter 4 and 5). On the other hand, permeant anions are employed as a $\Delta\Psi$-probe, when the inside is positive; thus, in membrane systems oriented inside-out. Examples of permeant anions are, thiocyanate (SCN^-) and phenyldicarbaun decarborane (PCB^-) [76]. Several methods are used to measure the distribution of ions across the membrane. At present, the flow dialysis technique is the most succesful. With this technique, the concentration of the ion at both sides of the membrane can easily and continuously be determined, using radioactively labeled compounds [69]. From the steady-state concentration gradient of the ion across the membrane the $\Delta\Psi$ can be calculated by means of the Nernst equation [69,70]. In quite a similar way, the ΔpH can be estimated from the transmembrane distribution of acids or bases, which are membrane permeable in the neutral form [73]. Weak acids, such as acetate and 5,5-dimethyloxazolidine-2,4-dione (DMO), are concentrated in membrane systems

with the same orientation as cells, interior alkaline. In contrast, weak bases, such as methylammonium, are accumulated in membrane systems which are oriented inside-out; thus, interior acidic.

The magnitude of $\Delta\Psi$ and ΔpH have been determined in various bacterial cells and cell membrane vesicles [68,70,77-83]. Although the findings differ considerably, depending on the species, on the type of vesicle and on the method of determination, several general conclusions can be drawn. In all the bacterial cells that were tested, the proton pumps are directed outward. In all cases, the operation of the pump is associated with the formation of a $\Delta\Psi$. The magnitude of the ΔpH strongly depends on the external pH [68,77], on the ion composition of the medium [81] and on metabolic activities [82]. In most bacteria, the internal pH is maintained at a constant value of about 7.5. Therefore, the ΔpH is large at a low external pH, and decreases to very low values at a neutral pH. In some systems, such as *E.coli* [68] and alkalophilic bacteria [83], at pH's above 7.5, the ΔpH is even inverted, and the cell becomes more acidic than the medium. In contrast, the $\Delta\Psi$ is essentially constant in the pH range 5-9. In general, the $\Delta\tilde{\mu}_{H^+}$ amounts to 100-240 mV and is high at low external pH's and low at high external pH's, where only the $\Delta\Psi$ is significant.

1.5. OUTLINE OF THIS THESIS

This thesis deals with the energy supply for dinitrogen fixation in two obligate aerobic organisms; the free-living bacterium *Azotobacter vinelandii* and the symbiotic dinitrogen-fixer *Rhizobium leguminosarum*. As mentioned in the introduction, the nitrogenase complex functions only when cells generate enough energy in the form of ATP and in the form of reducing equivalents. In general, it is well understood how aerobes generate ATP. In contrast, little is known about how these cells derive reducing equivalents from their metabolism at a sufficiently low potential for nitrogenase. The results of Haaker *et al.* with *A.vinelandii* suggest that the $\Delta\tilde{\mu}_{H^+}$ is involved in the generation of reducing equivalents for nitrogenase. In this thesis, the involvement of $\Delta\tilde{\mu}_{H^+}$ in electron transport to nitrogenase is studied in more detail for *A.vinelandii*, and extended to bacteroids of *R.leguminosarum*. The different Chapters of this thesis describe the following aspects:

2. Characterization of some factors controlling the nitrogenase activity in bacteroids of *R.leguminosarum*.

3. Studies on the efficiency of ATP formation in membrane preparations of *A.vinelandii* and bacteroids of *R.leguminosarum*.
4. Studies on the mechanism of electron transport to nitrogenase in bacteroids of *R.leguminosarum*.
5. The influence of ammonium on dinitrogen fixation by *A.vinelandii* and by bacteroids of *R.leguminosarum*.
6. Discussion.

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2. Involvement of the Cytoplasmic Membrane in Nitrogen Fixation by *Rhizobium leguminosarum* Bacteroids

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1. The nitrogen-fixing efficiency of freshly prepared suspensions of *Rhizobium leguminosarum* bacteroids from pea root nodules was considerably enhanced by addition of bovine serum albumin. Evidence was found that during preparation of bacteroids the cell membrane is exposed to the uncoupling effect of free fatty acids and to plant phospholipase D activity. Both effects could be counteracted by bovine serum albumin.

2. A technique was developed by which concentrations of free O_2 and nitrogenase activity could be measured simultaneously under conditions of steady-state respiration.

By means of this system it could be shown that in contrast to previous claims, high ATP/ADP ratios can be achieved in bacteroids even with a high concentration of O_2 in the medium.

3. Nitrogen fixation was found to be controlled by the ATP/ADP ratio, the generation of reducing equivalents and the switch-off phenomenon. It was demonstrated that the generation of reducing equivalents for nitrogenase is regulated by the energized state and the integrity of the bacteroid cell membrane.

The data indicate that the process of aerobic nitrogen fixation in *R. leguminosarum* bacteroids resembles that of *Azotobacter vinelandii*.

The involvement of oxyleghaemoglobin in the nitrogen fixation process of symbiotic bacteria has been well established [1–4]. Addition of purified oxyleghaemoglobin to respiring bacteroids, isolated from soybean root nodules, greatly enhances their nitrogen-fixing efficiency. The principal function of oxyleghaemoglobin was shown to be the delivery of O_2 at a high flux but a low partial pressure to the bacteroid surface [2, 5–7]. Other O_2-binding proteins were also shown to facilitate O_2 diffusion across the 'unstirred layer' around each bacteroid [2].

Current evidence suggests that soybean bacteroids contain at least two terminal oxidase systems with different affinities for O_2 and phosphorylating capacities [2–4, 7, 8]. It is believed that one of these systems accepts O_2 only from O_2-binding proteins and is more efficient in providing high cellular ATP concentrations than the other oxidase which operates at higher O_2 concentrations [4, 7].

Little is known about the supply of reducing equivalents to nitrogenase in bacteroids. Appleby et al. [4] noted that changes in the nitrogenase activity of soybean bacteroids could not be explained just by differences in ATP/ADP ratios induced by the addition of carbonyl cyanide *m*-chlorophenylhydrazone as an uncoupling agent. Although these authors favour a different interpretation, in our opinion their results suggest that in addition to the ATP/ADP ratio, the way of generating reducing equivalents is equally important in supporting nitrogenase activity *in vivo*.

In the present work we devised a technique by which concentrations of free O_2 could be measured simultaneously with the rate of acetylene reduction under conditions of steady-state respiration. Evidence is presented that, as in *Azotobacter vinelandii* [9], the energized state and the integrity of the cytoplasmic membrane of pea nodule bacteroids controls the supply of reducing equivalents to nitrogenase. Furthermore, we demonstrate that high ATP/ADP ratios are not limited to a specific low O_2 concentration range as previously thought [7], but that they also occur at high free O_2 concentrations.

MATERIALS AND METHODS

Growth Conditions and Protein Preparations

Root nodules were produced under controlled conditions on peas (*Pisum sativum* L. cv. Rondo) by inoculation with *Rhizobium leguminosarum* (strain

Abbreviation. Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate.

PRE). The plants were grown in gravel and the nodules were harvested with a wire-stripper 20–25 days after inoculation.

The bacteroids were isolated anaerobically from these nodules in 25 mM Tes/KOH pH 7.4, 2 mM $MgSO_4$, 0.3 M sucrose, 4% soluble polyvinylpyrrolidone and 20 mM dithionite, using an anaerobic screw press according to Bergersen et al. [10]. Bacteroids were washed once or twice (see text) by centrifuging in 25 mM Tes/KOH, pH 7.4 containing 2 mM $MgSO_4$ and 0.3 M sucrose. They were finally suspended in the same medium in a concentration of 10–15 mg protein/ml. During experiments the bacteroid suspensions were stored under anaerobic conditions at 0 °C in 6.5-ml bottles fitted with Subaseal caps. The protein content of the bacteroid suspension is 70% of its dry weight.

Leghaemoglobin was prepared as described by Appleby [8] with the following modifications. All purification procedures were performed in 25 mM Tes/KOH pH 7.2. After dialysis, the preparation was fractionated twice on a column of Sephadex G-75, concentrated to 1–2 mM in a Amicon Ultrafiltrator (UM 10), and stored at −20 °C. The leghaemoglobin fraction was found to be at least 96% pure by sodium dodecylsulphate gel electrophoresis. Leghaemoglobin and myoglobin were oxygenated as described by others [2].

Concentrations of the haemoproteins were determined by pyridine haemochromogen assay (cf. [2]) and haem was extracted from myoglobin with acid (1%)/acetone [11]. Native apomyoglobin was obtained after haem extraction by dissolving the dried protein precipitate in water and dialysis against 25 mM Tes/KOH pH 7.4 for 2 h.

Analytical Methods

All experiments were carried out in a magnetically-stirred vessel with an O_2 electrode located at the bottom (Rank Brothers, Bottisham, Cambridge) so that the free O_2 concentration and the nitrogenase activity could be determined at the same time. This system was made gas-tight with a rubber stopper, through which reactants could be added by syringe. An amplifier was connected between the output of the O_2 electrode and the recorder in order to detect low O_2 concentrations [6]. The electrode was calibrated by adding small amounts of air-saturated water to the anaerobic reaction solution.

The standard incubation mixture contained at 25 °C: 25 mM Tes/KOH, 2 mM $MgSO_4$, 0.3 M sucrose and 50 mM sodium succinate as substrate, final pH 7.4. This mixture and the reaction vessel were made anaerobic by flushing with argon. After addition of the bacteroids and various proteins (see text) at time zero (final incubation volume 2 ml), variable amounts of oxygen followed by acetylene (final concentration 10%) were added to the gas phase (6 ml). After five minutes stirring when the desired level of free O_2 had been reached in the reaction mixture, aliquots (0.1 ml) of the gas phase were removed at intervals during the following 8 min and analysed for ethylene by gas chromatography [9]. The rate of acetylene reduction was linear with time. At 13–15 min the cells were fixed by rapidly adding $HClO_4$ to the incubation mixture up to a final concentration of 4% (w/v). The levels of ATP, ADP and AMP were determined as earlier described [9].

The steady-state concentration of free O_2 in the solution is determined by the partial pressure of O_2 in the gas phase, the rate of stirring and the rate at which O_2 was consumed by the bacteroids. In the present experiments the stirring speed was usually held constant and the desired concentrations of free O_2 was therefore obtained by varying the partial pressure of O_2 in the gas phase. It was sometimes necessary to make slight adjustments of the stirring speed during the course of an experiment when the free O_2 concentration was seen to vary. The O_2 input into the solution ($nmol \times min^{-1} \times ml^{-1}$) at the standard stirring speed was calculated by adding known amounts of O_2 to the gas phase and measuring the initial rate of increase of the O_2 tension in the anaerobic liquid phase.

Toluene/EDTA-treated cells were prepared according to Van Straten [12].

Bacteroid protein was determined by the biuret method and phospholipase activity according to Yang [13].

Biochemicals and Gasses

All gasses were purchased from Hoekloos (Amsterdam); bovine serum albumin and myoglobin (whale skeletal muscles) were from Sigma, and all enzymes from Boehringer.

RESULTS AND DISCUSSION

Experimental System

Several techniques have been developed to follow respiration and nitrogenase activity in bacteroid suspensions. A widely used method is the 'shaking assay' system [1, 2, 14]. The usefulness of this method was questioned by Stokes [5], who deduced mathematically that with haemoprotein present other effects might occur in addition to that of facilitated O_2 diffusion. Further, the free O_2 concentration cannot be measured and therefore it is not certain whether equilibrium conditions exist. Bergersen and Turner [6] devised a 'no-gas-phase' system, in which the free O_2 concentration, the deoxygenation of O_2-binding

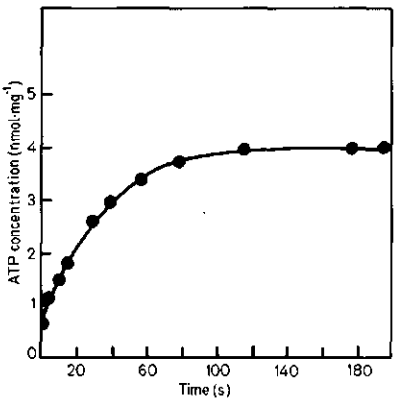

Fig. 1. *Time course of ATP synthesis during oxidation at constant free O_2 concentration.* Bacteroids were isolated and washed twice as described in Materials and Methods. In order to eliminate possible temperature effects the temperature of the stored bacteroid suspension was raised from 0 to 25 °C 5 min before addition of 2.5 mg bacteroid protein to the incubation mixture which contained 100 μM myoglobin. The free O_2 concentration was maintained at 5 ± 0.5 μM during the assay. At suitable time intervals the total incubation was fixed with $HClO_4$. After neutralization the ATP content was determined

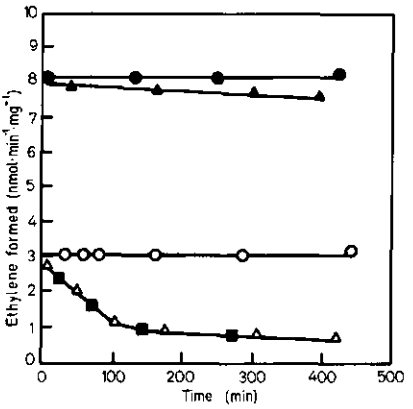

Fig. 2. *The effect of bovine serum albumin and the washing procedure on the succinate-driven nitrogenase activity of bacteroids.* Two bacteroid suspensions were prepared and stored as described in Materials and Methods. One was washed once in 2 ml, the other twice in 20 ml fresh medium. Before injecting 0.66 mg bacteroid protein, fatty-acid-free bovine serum albumin (final concentration 3%) was added to the incubation mixture. Polyvinylpyrrolidone was added just after the isolation procedure was complete to a part of the suspension which was washed once (final concentration 2%). The reactions were started after different storage times of the bacteroid suspension and nitrogenase activity was measured at a fixed optimal free O_2 concentration of 1.5 ± 0.2 μM. (Δ——Δ) Once washed, without bovine serum albumin; (○——○) twice washed, without bovine serum albumin; (▲——▲) once washed, with fatty-acid-free bovine serum albumin; (●——●) twice washed, with fatty-acid-free bovine serum albumin; (■——■) once washed, with polyvinylpyrrolidone

protein and the acetylene reduction could be recorded. It is clear that with this system it is not possible to achieve the steady-state conditions which are likely to occur *in vivo*. Our system is a compromise between these two methods. Both steady-state free O_2 concentration and nitrogenase activity can be assayed simultaneously. Fig. 1 shows that when pea nodule bacteroids were added to the medium, which was held at a constant O_2 concentration of 5 μM, the intracellular level of ATP increased during about 2 min before it reached its steady-state level. Bergersen and Turner [7] terminated the reaction in their system at shorter times than 2 min and consequently they found low, but rising, ATP levels at O_2 concentrations between 10.0 and 0.5 μM in soybean bacteroids. Their measurements were made under non-equilibrium conditions and their conclusion that high ATP levels only occur when the concentration of free O_2 is low (0.01 – 0.1 μM) is therefore incorrect. Further evidence against the conclusion of Bergersen and Turner [7] comes from experiments described below.

Effect of Bovine Serum Albumin on Bacteroid Nitrogenase Activity and Oxidative Phosphorylation

It appeared necessary with our system that the nitrogen fixing capacity of a freshly prepared bacteroid suspension is preserved during several hours. Fig. 2 demonstrates that the succinate-driven nitrogenase activity remained constant during storage for at least 400 min when the bacteroids had been washed twice in the final isolation step from nodules. However the nitrogenase activity of bacteroids that had been washed only once, and in a smaller volume, declined rapidly. In both cases, addition of bovine serum albumin to the incubation mixture greatly enhanced the acetylene reduction rates, even after a 7-h storage period. Furthermore, it appeared that fatty-acid-free bovine serum albumin was more effective than non-fatty-acid-free bovine serum albumin.

Bovine serum albumin is often used in the isolation of functional chloroplasts [15] and mitochondria [16] from plant tissue. Its beneficial effect is reported to be that it eliminates the uncoupling by binding fatty acids present endogenously or formed by the action of lipolytic acylhydrolases [17]. These considerations plus the results presented in Fig. 2 strongly suggest that freshly prepared pea nodule bacteroids are partially uncoupled by free fatty acids. The presence of polyvinylpyrrolidone during storage (Fig. 2) did not affect the stability and we therefore conclude that the observed decrease in nitrogenase activity was not due to phenolic substances that had possibly

Table 1. *Effect linoleic acid on oxygen input, nitrogenase activity and oxidative phosphorylation of bacteroids*
The usual isolation procedure for bacteroids was performed, except that 2.5% bovine serum albumin (34 mg/mg bacteroid protein) was added to the first washing medium, to remove free fatty acids. Linoleic acid, dissolved in 96% ethanol, was added just after 2.1 mg bacteroids were injected to the reaction mixture. The total incubation was fixed with $HClO_4$ after 8 min when constant rates of acetylene reduction and constant optimal free O_2 concentration had been reached. After neutralization the cell extract was analyzed for ATP and ADP. Fatty-acid-free bovine serum albumin (final concentration 3%) was added 5 min after the bacteroids were incubated with linoleic acid and the same procedure followed

Linoleic acid	Addition	Oxygen input	C_2H_4 evolution	ATP/ADP
μg		nmol $\times min^{-1}$ $\times ml^{-1}$	nmol $\times min^{-1}$ $\times mg^{-1}$	
–	–	60	12	3.5
–	Ethanol (30 μl)	61.5	12	3.5
18	–	57.5	11.3	3.2
36	–	54	7.7	1.6
54	–	45.5	3.5	1.0
36	3% Bovine serum albumin	60	11.6	3.4

been left behind after the isolation procedure. Like soybean nodules [18], pea nodules might contain large amounts of endogeneous fatty acids. Table 1 shows that linoleic acid, a fatty acid that is present in large amounts in soybean nodules, was an uncoupler for bacteroids and that bovine serum albumin counteracted its effect. Linoleic acid slightly inhibited oxygen consumption by bacteroids (Table 1), an effect similar to that observed with mitochondria [17]. Furthermore, it was deduced from the data of Fig. 2 that the bacteroid membrane is attacked by plant enzymes during the isolation procedure. Conclusive evidence was obtained from measurements of the lipolytic enzyme activity in the supernatant of nodule homogenates (not illustrated). From these results we concluded that nodules probably contain only phospholipase D. This enzyme catalyzes the hydrolysis of phospholipids to phosphatidic acids and is reported to occur widely in the plant kingdom and is particularly active in storage tissues, such as pea seeds [19,20].

The decrease in nitrogenase activity as observed in Fig. 2, could be completely simulated by adding 45 ng pure phospholipase D to cells that had been washed with bovine serum albumin. The effects of the pure enzyme could also be counteracted by bovine serum albumin. A final albumin concentration of 30–40 mg/mg bacteroid protein in the incubation vessel or during the first washing procedure was sufficient to counteract the effect of phospholipase D and endogeneous free fatty acid on the bacteroid membrane. Bacteroids, free fatty acids and plant phospholipase D are probably spatially separated in the nodule. For this reason, freshly prepared bacteroids do not represent the situation *in vivo*. This consideration suggests that bovine serum albumin treatment during the isolation procedure of bacteroids is necessary.

Bergersen et al. [1] used bovine serum albumin in the 'shaking assay' to maintain the concentration of soluble protein when the concentration of leghaemoglobin was decreased. Their data do not indicate any stimulation of the reaction by added serum albumin to respiring soybean bacteroids. Their failure to observe a stimulation of nitrogenase activity may have been due to the fact that the albumin that they used was not fatty acid free, or because the amount of albumin per mg bacteroid was rather low.

Role of Bovine Serum Albumin, Oxymyoglobin and Oxyleghaemoglobin in Supporting Nitrogenase Activity

Several reports have appeared in the literature on attempts to assess the effect of O_2 on the nitrogenase activity in bacteroids [2,4,6]. In these experiments both the 'shaking assay' system and the 'no-gas-phase' system were used. As indicated previously, we doubted whether the use of these methods led to results of relevance to the situation *in vivo*. We therefore studied the effect of oxygen on the nitrogenase activity under controlled steady-state conditions (Fig. 3).

The data presented in Fig. 3A, B support the conclusion that freshly prepared bacteroids of *R. leguminosarum* are partially uncoupled. Bovine serum albumin stimulated both steady-state nitrogenase activity, O_2 consumption and oxidative phosphorylation, without affecting the free O_2 concentration at which maximal acetylene reduction occurs. Furthermore, as the oxygen supply was increased, the ATP/ADP ratio rose to a fairly constant level that was maintained at higher O_2 concentrations even when the nitrogenase activity declined. Similar phenomena were observed when excess myoglobin or leghaemoglobin were present in the incubation vessel (Fig. 3C, D).

The observed decline in nitrogen fixation is therefore not due to a decreased ATP supply, as proposed by Bergersen and Turner [7] for soybean bacteroids, but rather to inhibition by excess O_2, the so-called switch off mechanism [21,22]. These data show a striking resemblance with those obtained with *A. vinelandii* [9], and it seems likely therefore that bacteroids, like *A. vinelandii* [23], might possess a branched electron transport chain [7] of which one branch generates ATP at high O_2 concentrations, and the other branch synthesises ATP at much lower O_2 concentrations in the medium.

It is clearly demonstrated in Fig. 3A, C, D, that with haemoprotein in the assay mixture, the whole peak of nitrogenase activity is shifted to lower free O_2 concentrations. Similar results were obtained by Bergersen and Turner [6] with soybean bacteroids, although slightly different 'O_2-buffering' zones were reported for myoglobin and leghaemoglobin. As a consequence of facilitated oxygen diffusion via the oxygen carrier, bacteroids will 'see' more oxygen, at a given free (unbound) O_2 concentration than would be the case in the absence of the O_2-carrier. For this reason the nitrogenase will be switched off at a lower free O_2 concentration.

The similar effects of bovine serum albumin, myoglobin and leghaemoglobin on the nitrogen-fixing efficiency, might suggest that the same mechanism underlies the mode of action of these proteins. However, apomyoglobin had no effect on respiring bacteroids and exactly the same curves as presented in Fig. 3A were obtained. Therefore, the idea that the two O_2-binding proteins could reverse the uncoupling effect on the bacteroid membranes can be disregarded. Our results confirm the general concept, that the O_2-carriers exert their effect only by facilitated O_2-diffusion.

Fig. 3E shows the effect of bovine serum albumin plus oxymyoglobin on the nitrogen-fixing efficiency. Both the rates of O_2 consumption and acetylene reduction were considerably enhanced and the effects of the two proteins were roughly additive. As was expected, the overall nitrogenase activity shifted to a lower free O_2 concentration. The nitrogenase activity was doubled, but the steady-state ATP/ADP ratio was hardly influenced. It appears that under these circumstances nitrogenase was controlled by the supply of electrons rather than by the ATP/ADP ratio. Furthermore, the maximum nitrogenase activity observed in Fig. 3E was more than 90% of the activity obtained by toluene/EDTA-treatment of the cells. The latter activity can be used as a quantitative measure of the amount of potentially active enzyme present in the bacteroid [14], and we conclude therefore that the physiological nitrogen-fixing system in bacteroids is highly effective.

These data indicate that in pea nodule bacteroids, as in *A. vinelandii* [9], the generation of reducing equivalents to nitrogenase is controlled by the energized state and the integrity of the cell membrane.

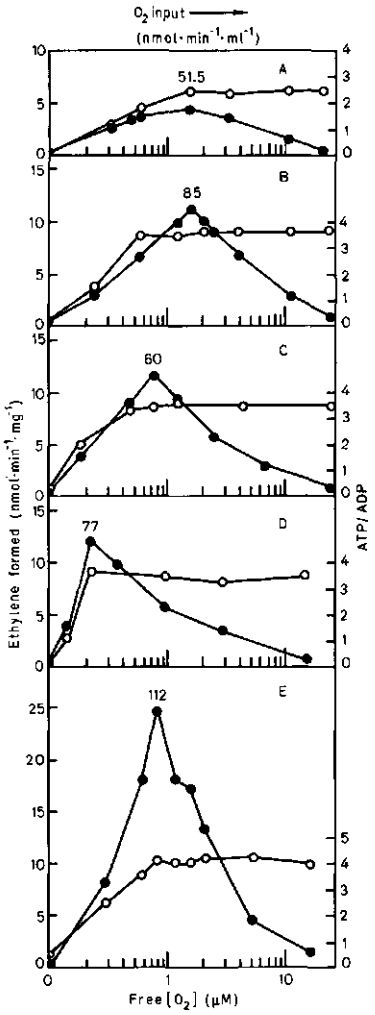

Fig. 3. *The relationship between the free dissolved O_2 concentration, O_2 consumption, nitrogenase activity and the ATP/ADP ratio of pea nodule bacteroids as influenced by different effectors.* The experimental conditions are described in Materials and Methods. The figures are composed of the results obtained from three separate experiments. The oxygen input at maximum nitrogenase activity is given. A logarithmic scale indicates the free O_2 concentration. (●——●) Nitrogenase activity; (○——○) ATP/ADP. (A) Without addition; (B) 3.1% fatty-acid-free bovine serum albumin; (C) 130 μM myoglobin; (D) 110 μM leghaemoglobin; (E) 130 μM myoglobin plus 3.1% fatty-acid-free bovine serum albumin

Influence of Uncoupler on Nitrogenase Activity and Oxidative Phosphorylation

To investigate the involvement of the energized state of the cytoplasmic membrane in the nitrogen fixing process of pea nodule bacteroids, the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone was used. As in soybean bacteroids [4] it proved a suitable uncoupler. In order to eliminate the uncoupling by free fatty acids, the bacteroids were washed with bovine serum albumin. Furthermore, excess amounts of myoglobin was added to obtain optimally functioning cells. In the experiments the uncoupler was added at maximum nitrogenase activity. The results of Fig. 4A

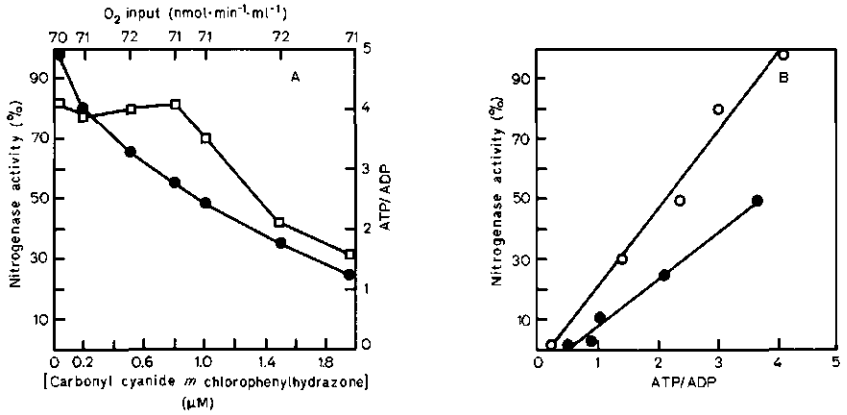

Fig. 4. *Influence of carbonyl cyanide* m-*chlorophenylhydrazone on O_2 consumption, nitrogenase activity and ATP/ADP ratio.* Experiments were performed as described in Materials and Methods in the presence of 121 μM myoglobin and at a fixed free dissolved O_2 concentration of 0.65 ± 0.5 μM. (A) (●——●) Nitrogenase activity; (□——□) ATP/ADP. (B) Relationship between bacteroid nitrogenase activity and ATP/ADP ratio in the presence (●——●) and absence (○——○) of carbonyl cyanide *m*-chlorophenylhydrazone

show that at low carbonyl cyanide *m*-chlorophenylhydrazone concentrations the nitrogenase activity declines, but there was little or no effect on the ATP/ADP ratio. At concentrations higher than 1 μM an approximately linear relationship was observed between the ATP/ADP ratio and the nitrogenase activity (Fig. 4B). The data without uncoupler, obtained from experiments carried out at O_2 concentrations lower than that necessary for maximum nitrogenase activity, thus the increasing part of the curve (comparable with Fig. 3E), were also linear but had a different slope. Experiments using the fluorescent probe 9-amino-6-chloro-2-methoxyacridine have shown that all carbonyl cyanide *m*-chlorophenylhydrazone concentrations used lower the energized state of the membrane (cf. [9]). Since the only known effect of carbonyl cyanide *m*-chlorophenylhydrazone is to lower the energized state of membranes, these results show that as well as being controlled by the ATP/ADP ratio, the nitrogenase activity is influenced by supply of electrons via the energized state of the bacteroids membrane. Furthermore, recent evidence with *A. vinelandii* indicates that oligomycin, which slows down the rate of ATP synthesis does not inhibit nitrogenase activity [28]. Appleby et al. [4] also noted that the correlation between the ATP/ADP ratio and the nitrogenase activity was poor when they used carbonyl cyanide *m*-chlorophenylhydrazone as an uncoupler. Their explanation that there are separate domains of ATP formation and accumulation within the bacteroid, and that one of these domains is more sensitive to uncoupling seems very unlikely to us. Our results show that their observation can also be explained in terms of a limitation in electron supply to nitrogenase. The energized state of the membrane is mainly determined by oxygen input at sub-optimum free O_2-concentrations. At higher O_2-concentrations the nitrogenase activity is switched off at maximum ATP/ADP ratio and membrane energization. Bacteroids, like *A. vinelandii*, contain flavodoxin [25,26, 27]. In the latter organism it has been shown that flavodoxin is a good electron donor for nitrogenase and that the switch-off state may be caused by autooxidation of flavodoxin [22,24]. We suggest that the same mechanism applies for *R. leguminosarum* bacteroids. Work is in progress to characterize the interaction between the energized state of the membrane and the generation of reducing equivalents for nitrogenase.

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Eur. J. Biochem. 97, 369–377 (1979)

3. On the Efficiency of Oxidative Phosphorylation in Membrane Vesicles of *Azotobacter vinelandii* and of *Rhizobium leguminosarum* Bacteroids

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1. The effect of O_2 on the rate of ATP synthesis and the P/O ratio has been studied in membrane vesicles of *Azotobacter vinelandii* and *Rhizobium leguminosarum* bacteroids (strain PRE). It was observed that the P/O ratio rises to a maximum value when the O_2 input is increased, but declines when O_2 becomes detectable in the medium.

2. Evidence is presented, that in *A. vinelandii* H_2 donates its electrons at a fourth proton-translocating site. The presence and the efficiency of this site is independent of the growth conditions and it appeared that no flavoproteins are directly involved in the oxidation of H_2. The unidirectional hydrogenase in membrane vesicles of *A. vinelandii* is not inhibited by acetylene. The function of hydrogenase in the process of nitrogen fixation by *A. vinelandii* is discussed.

3. Two types of membrane vesicles of *R. leguminosarum* bacteroids were prepared. Membranes isolated in a low salt medium lose the ability to couple succinate oxidation to oxidative phosphorylation; upon isolation in a high salt medium this ability is preserved. In contrast the efficiency of oxidative phosphorylation with NADH is not affected by the isolation procedure. The efficiency of energy coupling in membranes vesicles of *R. leguminosarum* bacteroids can be enhanced by the addition of fatty-acid-free bovine serum albumin. No active membrane-bound hydrogenase could be detected in membrane vesicles of *R. leguminosarum* bacteroids.

Several reports have appeared in the literature on attempts to assess the efficiency of oxidative phosphorylation in intact cells and isolated respiratory membranes of *Azotobacter vinelandii* [1–5]. The reported P/O ratios of respiratory membrane vesicles are usually low (< 0.5 with NADH) [1,5–7], although Ackrell and Jones [3] obtained values as high as 1.1. Much higher P/O ratios were calculated during studies on whole cells of this organism and the authors suggest that there are at least two and more likely three coupling sites [2,4]. One site at the level of NADH dehydrogenase, one in the central ubiquinone–cytochrome *b* region and a third coupling site at the minor branch of the terminal cytochrome system; the major cytochrome branch appears to be uncoupled.

No data are present in the literature about the efficiency of energy coupling in membrane vesicles of *Rhizobium leguminosarum* bacteroids. In general P/O ratios are obtained from measurements at air-saturated O_2 concentrations, which are non-physiological for aerobic N_2-fixing organisms. We, therefore devised a technique which enabled us to maintain different rates of respiration at non-saturating O_2 concentrations and to study the effect of O_2 on the P/O ratio in membrane vesicles of both *A. vinelandii* and *R. leguminosarum* bacteroids.

It has been known for several years that a number of nitrogen fixing organisms contain a hydrogenase, which enables these organisms to recover some of the energy lost during the wasteful reduction of protons by nitrogenase [8–13]. The membrane energization and oxidative phosphorylation connected with H_2 oxidation in membrane vesicles of both *A. vinelandii* and *R. leguminosarum* bacteroids was studied in addition.

MATERIALS AND METHODS

Growth Conditions and Vesicle Preparations

A. vinelandii ATCC strain OP was cultured on a Burk's nitrogen-free basic salt medium [14] in a New Brunswick chemostat (type C30) equipped with an

Abbreviation. Tes, 2-{[tris(hydroxymethyl)methyl]amino}-ethanesulfonic acid.

O_2 electrode. Two types of cells were used: cells grown O_2-limited or N_2-limited; dilution rate 0.1 h^{-1} [15]. Cells were harvested, washed with water and resuspended to a final concentration of 30–40 mg protein/ml in a medium containing 50 mM Tes/KOH, 5 mM EDTA, 1 mg lysozyme/ml and 0.2% fatty-acid-free bovine serum albumin, final pH 8.0. After 30 min stirring at 24 °C, $MgCl_2$ was added to 10 mM, followed by sonication for 4 × 30 s in a MSE sonifier. Cellular debris and unbroken cells were removed by centrifugation at 28000 × *g* for 15 min. Membrane vesicles were sedimented by further centrifugation of the cell extract at 100000 × *g* for 30 min and were resuspended in 50 mM Tes/KOH, 2.5 mM $MgCl_2$, and 1.2% fatty-acid-free bovine serum albumin, pH 7.0 to a final concentration of 30–40 mg protein/ml. All procedures were performed anaerobically at 4 °C.

R. leguminosarum bacteroids (strain PRE) were produced on peas and isolated from the root nodules as described earlier [16]. Membrane vesicles were prepared as for *A. vinelandii*, except that 0.3 M sucrose or 0.33 M NaCl (see text) was present during the procedure. The membrane vesicles were freshly prepared and stored at 0 °C during the course of experiments.

Analytical Methods

P/O ratios were determined with the assay system previously described [16, 17]. With this system it is possible to control the respiration rate of a membrane suspension from zero to the maximum respiration rate and to measure the free O_2 concentration continously. For *A. vinelandii* the standard incubation mixture contained: 50 mM Tes/KOH, 2.5 mM $MgCl_2$, 10 mM glucose and 5 mM potassium phosphate, final pH 7.0. The incubation mixture (total volume 2.5 ml) and the reaction vessel were made anaerobic by flushing with argon. The vesicles, substrate and 15 U of a dialyzed hexokinase solution were added and incubated for 3 min before a determined amount of O_2 was added to the gas phase. After a further 3 min stirring at a standard speed to allow the system to reach equilibrium (see text), an anaerobic solution of ADP (final concentration 0.5 mM) was added to the incubation mixture. This mixture was incubated for 2.5 min and the reaction was stopped by adding $HClO_4$ to a final concentration of 4% (w/v). The amount of glucose 6-phosphate was determined [18]. The rate of O_2 uptake was calculated from the O_2 input data as described earlier [16]. Experiments with vesicles of *R. leguminosarum* bacteroids were performed as for *A. vinelandii*, except that the assay temperature was 25 °C instead of 30 °C and the 5 ml incubation mixture contained 0.3 M sucrose or 0.33 M NaCl (see text). Anomalies introduced by the O_2 uptake at the gas-liquid interface were minimized if necessary by increasing the amount of liquid so that the surface-to-volume ratio decreased.

In order to establish whether or not constant rates of respiration were obtained in the assay system described above, oxygenated myoglobin [19] was added to a final concentration of 200 µM and its degree of oxygenation [20] was followed spectrophotometrically during the course of the experiment. Absorption spectra were recorded between 500 and 600 nm at intervals of about 1 min by placing the experimental system in the light beam of an Aminco-Chance dual-wavelength spectrophotometer.

Acridine fluorescence was measured by placing the experimental system in the light beam of an Aminco SPF-500 fluorimeter and the fluorescence of 9-amino-6-chloro-2-methoxyacridine (2 µM) was followed during the course of a P/O ratio experiment. The excitation wavelength was 420 nm and the emission wavelength 500 nm.

The reduced-minus-oxidized difference spectra of membrane vesicles of *A. vinelandii* were determined in 50 mM Tes/KOH and 2.5 mM $MgCl_2$, final pH 7.0, using an Aminco-Chance dual-wavelength spectrophotometer. Vesicles were added to a concentration of 3.4 mg protein/ml. The test system was reduced either by NADH (final concentration 0.6 mM) or by H_2. H_2 was added to the gas phase of a closed cuvet, which was shaken prior to recording the spectrum. The reference system was kept in the oxidized state by shaking in air immediately before scanning. The time course of the reduction or oxidation of flavoproteins was recorded by placing the experimental system in the light beam of an Aminco-Chance dual-wavelength spectrophotometer and follow the absorbance at 455 nm with regard to a reference wavelength of 700 nm. Vesicles were added to the anaerobic reaction mixture to a concentration of 1.6 mg protein/ml and NADH, malate, H_2 and H_2O_2 were supplied at the times indicated to a final concentration of respectectively 0.6 mM, 5 mM, 100% in the gas phase and 0.005%.

Protein concentrations were determined by the biuret method.

Biochemicals and Gases

All gases were purchased from Hoekloos (Amsterdam); fatty-acid-free bovine serum albumin and myoglobin (whale skeletal muscles) were from Sigma, and all enzymes from Boehringer.

RESULTS

Effect of O_2 on the Coupling between Oxidation and Phosphorylation in Membrane Vesicles of A. vinelandii

When membrane vesicles of *A. vinelandii* are subjected to a constant input of O_2 from the gas phase

into the liquid phase in the system described in Materials and Methods, the rate of respiration becomes constant within 2 min as measured by the rapid oxygenation of myoglobin towards an equilibrium state. The degree of oxygenation of myoglobin proved to be a very sensitive test for changes in the respiration rate, provided that the O_2 input is kept constant (not shown). This implies that the 3-min time interval between the addition of O_2 to the assay system and the addition of ADP are sufficient for the vesicles to reach steady-state respiration rates. At high rates of O_2 input, when the O_2 input exceeds the rate of respiration, O_2 becomes detectable in the medium. When under these conditions ADP is supplied to the incubation mixture the free O_2 concentration declines (about 20%) which indicates that the rate of respiration increases, due to the so-called respiratory control [1, 21]. This phenomenon has been taken into consideration in calculating the P/O ratios.

Fig. 1 shows the effect of O_2 on the rate of ATP synthesis and the energy coupling as measured by P/O ratios on membrane vesicles prepared from *A. vinelandii* cells that had been grown O_2-limited. When the O_2 supply into the vesicle suspension is increased the P/O ratio with NADH as substrate rises to a maximum value, but declines when O_2 becomes detectable in the medium. At the same time the rate of ATP synthesis rises to a fairly constant level. The drop in the P/O ratio at high rates of O_2 input is also observed when malate, H_2 or H_2 plus NADH are supplied to the incubation mixture, except that with H_2 as substrate the rate of ATP synthesis declines rapidly at high rates of O_2 input. This decline does not appear to be due to a limited amount of H_2, but is caused presumably by inhibition of the hydrogenase by excess O_2. The decrease in the P/O ratio observed at high rates of O_2 input is caused by an enhanced flow of electrons through the non-phosphorylating pathway from cytochrome *b* to cytochrome *d*, since cytochrome *d* has a lower affinity for O_2 than the oxidase of the phosphorylating pathway. The P/O ratios reported in the literature for membrane vesicles of *A. vinelandii* [1, 3, 5–7] were obtained from experiments in air-saturated solutions and are therefore comparable with the values obtained here at high rate of O_2 input. Hyndman et al. [22] demonstrated the esterification of inorganic phosphate in conjunction with the oxidation of H_2 in cell-free particles of *A. vinelandii*, but no P/O values were reported. Fig. 1A and C show that the maximum P/O ratio obtained with H_2 as a donor is comparable with the one obtained with NADH and that the coupling between oxidation of H_2 and phosphorylation is equally efficient. Conform earlier work [3, 5] our results show that in cells grown O_2-limited the P/O ratio with malate is lower than that with NADH. Fig. 1D shows the effect of oxidation of NADH plus H_2 on the rate of

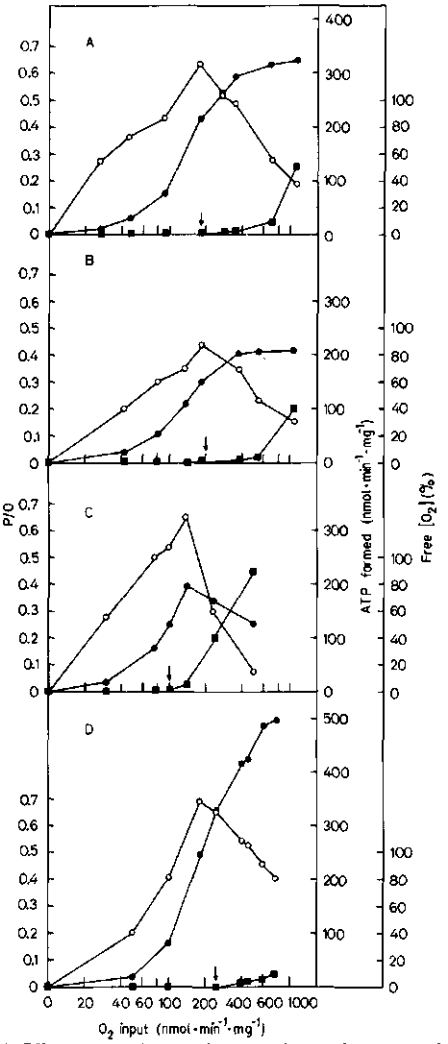

Fig. 1. *Effect of O_2 on the rate of ATP synthesis and energy coupling in membrane vesicles of* A. vinelandii, *which were grown O_2-limited.* Vesicles were isolated and the experiments were performed as described in Materials and Methods. A logarithmic scale indicates the O_2 input and arrows indicate the rate of O_2 input at which O_2 becomes detectable in the medium. Although the arrows do not represent the maximum oxidation capacity, they are an indication of the respiration rate. This figure is composed of the results obtained from several experiments. For each assay 0.22 mg vesicle protein was added. (○——○) P/O ratio; (●——●) rate of ATP synthesis; (■——■) O_2 concentration expressed as percentage saturation. (A) NADH, final concentration 0.6 mM; (B) malate, final concentration 5 mM; (C) H_2, 50% in the gas phase; (D) NADH plus H_2

ATP synthesis and energy coupling. The rate of ATP production as well as the maximum respiration rate were increased as compared with the values obtained

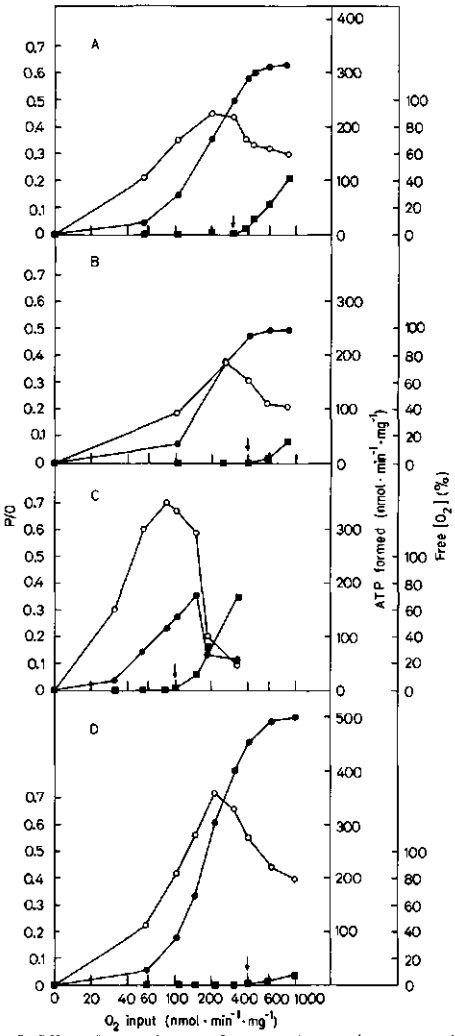

Fig. 2. *Effect of O_2 on the rate of ATP synthesis and energy coupling in membrane vesicles of* A. vinelandii, *which were grown N_2-limited.* Conditions as in Fig. 1

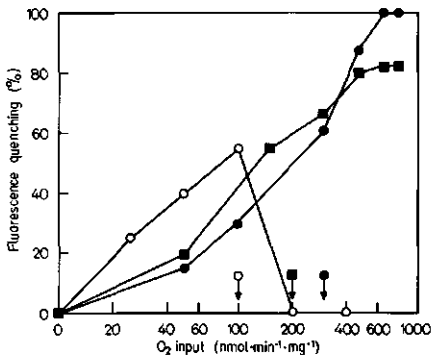

Fig. 3. *Quenching of 9-amino-6-chloro-2-methoxyacridine fluorescence by membrane vesicles of* A. vinelandii *upon energization by oxidation of different substrates.* Vesicles were isolated from cells grown O_2-limited and the experiments were performed as described in Materials and Methods. For each experiment 0.31 mg vesicle protein was added. Arrows indicate for the different substrates the rate of O_2 input at which O_2 becomes detectable in the medium. (■——■) NADH, final concentration 0.6 mM; (●——●) malate, final concentration 5 mM; (○——○) H_2, 50% in the gas phase

for NADH or H_2 alone. The same phenomenon was observed when malate and NADH were added together as substrates (not shown). This suggests that the rate-limiting step in the production of ATP is at the level of the primary dehydrogenases. No inhibition of the hydrogenase was observed in Fig. 1 D at high rates of O_2 input, since the O_2 concentration in the medium is rather low, due to the high rate of respiration.

Fig. 2 shows the effect of O_2 on the rate of ATP synthesis and the energy coupling on membrane vesicles isolated from *A. vinelandii* cells, that had been grown N_2-limited. Compared with the membranes isolated from cells grown O_2-limited the capacity of the non-phosphorylating pathway is enhanced due to a higher content of cytochrome *b* and *d* [5]. The data are in general similar to those presented in Fig. 1, except that the P/O ratios with NADH are almost equal to those obtained with malate. Thus confirming the observation [3, 5, 23–25] that membranes isolated from cells that have been grown N_2-limited have a low phosphorylation efficiency at site I. The maximum rate of ATP synthesis as coupled to NADH-oxidation was not affected by the different growth conditions, although due to the higher NADH-respiration rate, site I phosphorylation becomes 'uncoupled'. In this respect the efficiency of oxidative phosphorylation at the level of site II and III is hardly influenced. It could be argued that the decrease in the phosphorylation efficiency at site I is due to the appearance of a KCN-insensitive NADH oxidase activity when cells are adapted to high rates of respiration. However, we could not observe any significant KCN-insensitive NADH oxidase activity in both types of vesicles. Fig. 2 C and D demonstrate that the vesicles isolated from the two types of cells respond similarly to H_2 oxidation and to oxidation of NADH plus H_2.

Smith et al. [26] reported that the activity of the unidirectional hydrogenase in *A. chroococcum* is inhibited by acetylene. However, we could not observe any inhibition of H_2-respiration when acetylene (final concentration 20%) was added to the gas phase of the experimental system. Exactly the same curves were obtained as presented in the Fig. 1 C and 2 C.

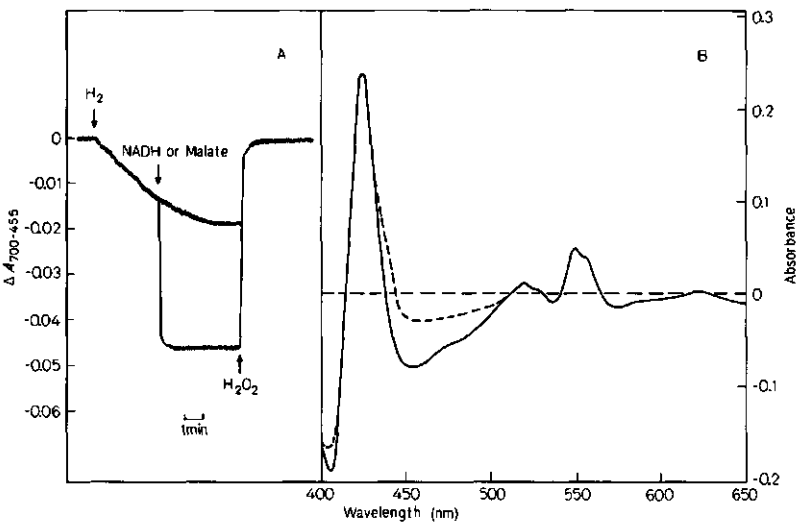

Fig. 4. *Effect of H_2 oxidation on the components of the respiratory system of* A. vinelandii. Vesicles were isolated from cells that had been grown O_2-limited and experiments were performed as described in Materials and Methods. (A) Time course of the reduction or oxidation of flavoproteins; (B) reduced-minus-oxidized difference spectra. (----) H_2; (——) NADH

Fig. 1 and 2 clearly show that the rate of ATP synthesis is not directly proportional with the respiration rate. The sigmoidal shape of the curve demonstrates that the membrane must be energized to a certain degree before full ATP synthesis occurs. We used 9-amino-6-chloro-2-methoxyacridine as a fluorescent probe in order to get a rough estimation of the energized state of the membrane at the threshold to high rates of ATP synthesis. This probe is often used to detect changes in the energy levels of cytoplasmic membranes [16,27]. Fig. 3 shows the quenching of the fluorescence as coupled to respiration of different substrates. In combination with Fig. 1 it can be derived, that for all substrates used, the rate of ATP synthesis starts to increase rapidly when 20–30% of the fluorescence is quenched. Optimal P/O ratios and maximal rates of ATP synthesis are observed when respectively 50–60% or about 80% of the fluorescence is quenched. Clearly demonstrated in this figure is that at low O_2 input H_2-oxidation results in a higher energized state of the membrane than that observed during oxidation of malate or NADH. This is in agreement with the observation described above that the coupling between H_2-oxidation and phosphorylation is rather efficient. Furthermore at high rates of O_2 input the quenching of the fluorescence as coupled to H_2-oxidation decreases. This is conform the results presented in the Fig. 1C and 2C, where excess O_2 is shown to inhibit oxidative phosphorylation as coupled to the oxidation of H_2. However, it should be noted that this inhibition of hydrogenase is dependent on the amount of O_2 and the exposure time to O_2. It takes about 10 min before the quenching of the fluorescence is diminished at high rates of O_2 input.

Fig. 4 shows that the bulk of the flavoproteins involved in the respiratory chain of *A. vinelandii* is only partially and very slowly reduced by H_2. This is in contrast to the results obtained with NADH or malate where a rapid and large decrease of the absorbance at 455 nm could be observed. This slow decrease in absorbance upon H_2 oxidation was not caused by a limited supply of H_2 from the gas phase into the liquid phase. Thus we conclude that flavoproteins are not directly involved in the respiratory chain from H_2 to the terminal cytochrome systems. The slow decrease in absorbance at 455 nm upon reduction with H_2 is probably due to the partial reduction of flavoproteins in the absence of their substrates. As far as we know this is the only example of an aerobic respiratory chain in which flavoproteins are not involved.

Effect of O_2 on the Coupling between Oxidation and Phosphorylation in Membrane Vesicles of R. leguminosarum *Bacteroids*

The method of studying the effect of O_2 on the rate of ATP synthesis and the P/O ratio of membrane vesicles of bacteroids is in principle the same as that for vesicles of *A. vinelandii*, except that a few modifications appeared to be necessary. Firstly, the res-

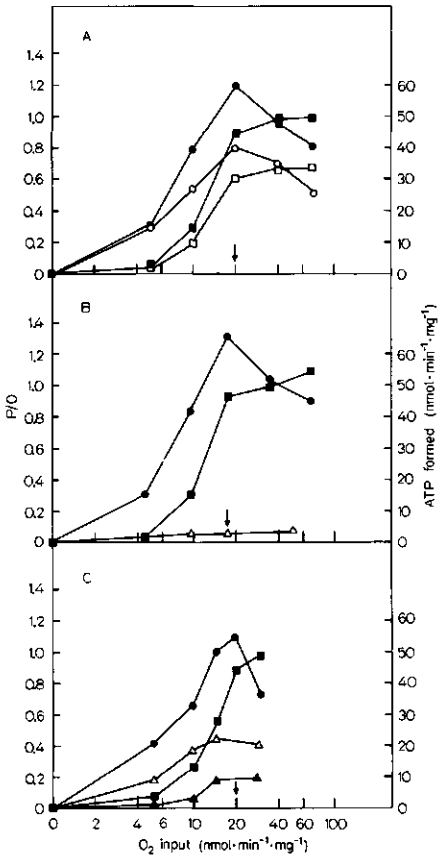

Fig. 5. *Effect of O_2 on the rate of the ATP synthesis and energy coupling in membranes vesicles of* R. leguminosarum *bacteroids.* $NaCl^+$ and $NaCl^-$ vesicles were isolated and the experiments were performed as described in Materials and Methods. A logarithmic scale indicates the O_2 input and arrows indicate the rate of O_2 input at which O_2 becomes detectable in the medium. For each assay 2.2 mg vesicle protein was used. Succinate and NADH were added to a final concentration of respectively 10 mM and 0.6 mM. (●——●) P/O ratio with NADH in bovine serum albumin treated vesicles; (○——○) P/O ratio with NADH in vesicles which are not treated with bovine serum albumin; (△——△) P/O ratio with succinate in bovine serum albumin vesicles; (■——■) rate of ATP synthesis with NADH in bovine-serum-albumin-treated vesicles; (□——□) rate of ATP synthesis with NADH in vesicles which are not treated with bovine serum albumin; (▲——▲) rate of ATP synthesis with succinate in bovine serum albumin treated vesicles. (A) and (B) $NaCl^-$ vesicles; (C) $NaCl^+$ vesicles

piration rate of the bacteroidal vesicles is about 20-times lower than that of *A. vinelandii* vesicles, making it necessary to add more vesicles to the incubation mixture. Secondly, in order to avoid gas-liquid phase interactions the volume of the assay mixture was set at 5 ml. The 3-min time interval between the addition of O_2 to the gas phase and the addition of ADP to the incubation mixture proved to be sufficient to reach constant rates of respiration with the bacteroidal membrane.

In Fig. 5A the effect of fatty-acid-free bovine serum albumin on the efficiency of oxidative phosphorylation is reported. The results support the conclusion previously reported [16] that the cytoplasmic membrane is partially uncoupled by free fatty acids. Furthermore it is demonstrated that the coupling between NADH oxidation and phosphorylation is very efficient in bacteroidal vesicles and that, like in *A.vinelandii*, the P/O ratio drops at high rates of O_2 input. Bacteroids, like *A. vinelandii*, contain a branched electron transport system [19, 28, 29]. The decline in the P/O ratio observed at high rates of O_2 input thus can also be explained by an enhanced flow of electrons through the non-phosphorylating pathway(s).

We reported recently [17] that a difference can be observed between vesicles prepared from bacteroids in the presence of high salt concentrations ($NaCl^+$) or in the presence of sucrose ($NaCl^-$). The rate of succinate oxidation is the same in both vesicles preparations, but $NaCl^+$ vesicles can be energized by succinate oxidation, while $NaCl^-$ vesicles cannot or only to a small extent. Oxidation of NADH leads to the same level of membrane energization with both types of vesicles. Fig. 5 B and C give the effect of O_2 on the rate of ATP synthesis and on the P/O ratio with both types of vesicles. The vesicles were washed with fatty-acid-free bovine serum albumin in order to get highly coupled membranes. The results obtained this way are in complete agreement with the results to be expected from the fluorescence data. In $NaCl^-$ vesicles very low P/O ratios with succinate are observed while in $NaCl^+$ vesicles the ability to couple succinate oxidation to phosphorylation is maintained. The reason for this difference is not known yet, but is possibly due to the dissociation of an essential component in a medium with a low salt concentration, since the ability to couple succinate oxidation to phosphorylation can be restored by the addition of concentrated supernatant to the incubation mixture. The coupling between NADH oxidation and phosphorylation is, in accordance with the expectation, effective with both types of vesicles. H_2 was not oxidized at all (O_2 appeared immediately in the medium) by $NaCl^-$ and $NaCl^+$ vesicles. We therefore conclude that there is no active hydrogenase present in this strain of *R. leguminosarum*.

DISCUSSION

The data presented in this paper extend previous studies [1, 3, 5–7, 30] on the process of oxidative phosphorylation in membrane vesicles of *A. vinelandii* and *R. leguminosarum* bacteroids. The efficiency of oxidative phosphorylation is always found to be

maximal at an O_2 input, optimal for the process of N_2 fixation in whole cells. Thus at an O_2 input at which the O_2 concentration in the medium is too low to inhibit the nitrogenase activity, yet high enough to energize the cytoplasmic membrane to an extent which allows both optimum rates of ATP synthesis as well as the generation of functional amounts of reducing equivalents. The only difference we could observe between the phosphorylation efficiency of membrane vesicles isolated from *A. vinelandii* cells which had been grown O_2-limited or N_2-limited is the low efficiency at site I in the latter case. However, the total amount of phosphate-energy generated by the process of oxidative phosphorylation appears to be the same in cells which had been grown at low or high rates of aeration. In our opinion, the uncoupling at site I can be regarded as a regulatory mechanism for an efficient supply of ATP under the different growth conditions.

Theoretically there are several possibilities for the entry of the electrons generated upon H_2 oxidation by hydrogenase into the respiratory chain of *A. vinelandii*. Firstly, at the level of site III of the terminal cytochrome branch. This possibility can be excluded on basis of the observation that low concentrations of KCN (25 μM) immediately inhibit the oxidation of 2,6-dichloroindophenol [3], but not the oxidation of H_2. Secondly, at the level of site II in the central ubiquinone−cytochrome *b* region. This possibility seems very unlikely to us, since the phosphorylation efficiency with H_2 is the same as with NADH and higher than with malate which suggests the involvement of three rather than two coupling sites. Thirdly, at the level of NADH dehydrogenase (site I). Our data indicate that the bulk of the flavoproteins is not reduced during H_2 oxidation. Therefore it is unlikely that H_2 donates its electrons at the level of NADH dehydrogenase which is a flavoprotein. Furthermore the efficiency of oxidative phosphorylation as coupled to H_2 oxidation is not affected by the different growth conditions. We therefore propose that membrane vesicles of *A. vinelandii* contain four instead of the three generally accepted coupling or proton-translocating sites [31] (Fig. 6). This fourth site is to be located at the level of hydrogenase. The Fe-S containing enzyme hydrogenase splits protons from electrons without the involvement of a flavin group and thus can in the proper orientation within the membrane act as a proton pump. Attempts to inhibit site I phosphorylation in order to maintain the phosphorylation capacity at the proposed site IV were unsuccessful, due to the rather insensitivity of site I towards respiratory chain inhibitors such as rotenone and amytal [32, 33]. Additional evidence which favours the existence of a fourth proton-translocating site at the level of hydrogenase comes from kinetical interpretation of the data presented in the Fig. 1 and 2. It is clear that according to the theory of Mitchell [34−36] the rate of ATP synthesis in respiring membrane vesicles is dependent on the rate at which protons are translocated across the membrane,

Table 1. *Hill-coefficients of the rate of ATP synthesis versus the respiration rate in membrane vesicles of* A. vinelandii, *grown O_2-limited or N_2-limited*

Vesicles were isolated as described in Materials and Methods. The data were obtained from the results presented in Fig. 1 and 2. Additional experiments were performed in order to obtain the values for NADH plus malate. Mean values are given. Due to the inhibition of hydrogenase by excess of O_2, no values can be given for the oxidation of H_2

Electron donor	Hill coefficient	
	O_2-limited	N_2-limited
Malate	1.9	1.9
NADH	2.4	1.9
NADH plus malate	2.4	2.4
NADH plus H_2	2.7	2.7

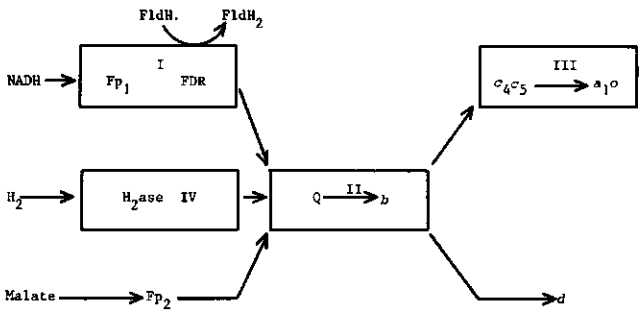

Fig. 6. *A proposal for the localization of hydrogenase in the respiratory system of* A. vinelandii. The composition of the respiratory system as described by Downs and Jones [26] is used as a basis for this scheme, Fp, flavoprotein; FDR, flavodoxinreductase; FldH., flavodoxin semiquinone; $FldH_2$, flavodoxin hydroquinone; H_2ase, hydrogenase; Q, ubiquinone; a_1, *b*, c_4, c_5, *d*, *o*, cytochromes; I, II, III, IV represent the four proton-translocating sites

thus dependent on the respiration rate and the amount of functioning proton-translocating sites. In our opinion it is therefore possible to estimate the amount of proton-translocating sites involved during respiration of different substrates by calculating the Hill-coefficient from the rate of ATP synthesis versus the respiration rate (Table 1). However, it should be noted that this is only correct when the respiration rate is directly proportional with the energized state of the membrane. Our data indicate that this is only true for all substrates when O_2 is absent in the medium. As soon as O_2 becomes detectable in the medium this relationship is disturbed by the enhanced influence of the non-phosphorylating cytochrome *d* pathway in the respiration rate. This phenomenon has only a slight effect on the Hill coefficient.

The data presented in Table 1 amplify the conclusion that the proton-translocating capacity at site I is low or even diminished when cells are adapted to high rates of O_2 input (N_2-limited). The results with NADH plus H_2 as electron donors confirm our statement that four instead of three proton translocating sites are present in membrane vesicles of *A. vinelandii*. Furthermore, the uncoupling of site I can be abolished by the addition of an extra electron donor. We therefore propose that the degree of reduction of ubiquinone determines the proton-translocating efficiency of site I.

We can only speculate about the function of hydrogenase in the process of N_2 fixation by *A. vinelandii*. Recent evidence indicates that the generation of reducing equivalents for N_2 fixation in *A. vinelandii* is coupled to NADH respiration via an energized cytoplasmic membrane [37]. The fact that H_2 oxidation does not follow exactly the same respiratory chain as the NADH oxidation may explain the observation of Walker and Yates [38] that the nitrogenase activity in whole cells of *A. chroococcum* is relatively low during H_2 respiration. In other words, the oxidation of H_2 only recovers the ATP lost during wasteful production of H_2 by nitrogenase and is at best loosely connected with the machinery which produces reducing equivalents for nitrogenase, the rate-limiting step in the process of aerobic N_2 fixation [16,18] (see Fig. 6).

Our results with membrane vesicles of *R. leguminosarum* bacteroids show that the coupling between NADH oxidation and phosphorylation is independent of the way the membranes are isolated and better than in membrane vesicles of *A. vinelandii*. It is surprising that the coupling between succinate oxidation and phosphorylation behaves differently. One possible explanation could be that there are different pathways for the oxidation of succinate and NADH, and that only the oxidation of succinate is effectively coupled to the process of N_2 fixation. This idea is supported by the observation [42] that bacteroids preferentially use succinate in the process of N_2 fixation. Although in membranes of *Rhizobium* bacteroids several cytochromes have been detected [39,40], the problem of its composition has yet to be solved. Furthermore, the nature of the couplings factor described above and its possible role in the high-affinity system, which functions during oxyleghaemoglobin-facilitated respiration [29,41] needs further investigation.

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4. THE INVOLVEMENT OF THE MEMBRANE POTENTIAL IN NITROGEN FIXATION BY BACTEROIDS OF *RHIZOBIUM LEGUMINOSARUM*

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1. Introduction

One of the major problems concerning nitrogen fixation in obligate aerobes is the generation of reducing equivalents for nitrogenase. Current evidence with *Azotobacter vinelandii* [1–5] and with pea nodule bacteroids [6–8] indicates that the generation of reducing equivalents rather than the ATP production is the rate limiting factor in the process of aerobic nitrogen fixation. It was shown that the energized state and the integrity of the cytoplasmic membrane controls the supply of reducing equivalents to nitrogenase. Recently, a proposal has been made for the electron transport to nitrogenase in *A. vinelandii* [2]. According to this hypothesis the protonmotive force across the cytoplasmic membrane, generated by respiration, drives the thermodynamically unfavourable reduction of flavodoxin to its hydroquinone form by NADH via a NADH flavodoxin oxidoreductase. This driving force, the so-called electrochemical proton gradient ($\Delta\tilde{\mu}_{H^+}$), is composed of an electrical ($\Delta\Psi$) and a concentration gradient (ΔpH) across the membrane [9–11] and specific ionophores are known to dissipate either one of these gradients [12].

Here we investigated the effect of ionophores on nitrogen fixation by respiring bacteroids of *Rhizobium leguminosarum*. Evidence is presented that the membrane potential ($\Delta\Psi$) across the cytoplasmic membrane of bacteroids regulates the generation of reducing equivalents for nitrogenase.

Abbreviations: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate; BSA, bovine serum albumin; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazol; Ph_4P^+, tetraphenylphosphoniumbromide

2. Materials and methods

2.1. *Bacteroid suspensions and myoglobin preparation*

Bacteroids of *R. leguminosarum* (strain PRE) were produced under controlled conditions on peas (*Pisum sativum* L. cv. Rondo) and isolated from the root nodules as in [6]. Bacteroids were washed twice by centrifugation in a medium containing 25 mM Tes/NaOH, 2.0 mM $MgCl_2$, 1.0 mM KCl, 0.3 M sucrose and 2.5% fatty acid-free BSA, final pH 7.4. They were finally resuspended and stored at 0°C at ~60 mg protein/ml in the same medium except that BSA was omitted.

Myoglobin was oxygenated as described [13].

2.2. *Analytical methods*

The nitrogenase activity (acetylene reduction) of bacteroids was measured in the assay system of [6,8]. In all experiments the oxygen supply was sufficient to allow maximal rates of acetylene reduction (see [6], fig.3). The standard incubation buffer contained: 25 mM Tes/NaOH, 2.0 mM $MgCl_2$; 1.0 mM KCl, 0.3 M sucrose and 20 mM sodium succinate, final pH 7.4 or 6.7. When the rate of acetylene reduction was constant ionophores were added and incubation was continued for $\geqslant$8 min.

Flow dialysis experiments were conducted aerobically at 25°C as in [14]. The upper and lower chambers were separated by dialysis tubing (Vishking; pore

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diam. 2.4 nm). The upper chamber (0.85 ml) contained the standard buffer at a given pH and 230 μM oxygenated myoglobin. Reactions were started by the addition of cells (0.05 ml), since the endogeneous rate of respiration was rather high. Further additions were made as indicated. The same buffer without myoglobin was pumped through the lower chamber at 3 ml/min. Fractions (1.0 ml) were collected and assayed for radioactivity. The experiments were performed under optimal conditions for nitrogen fixation. This was done by flushing pure oxygen over the medium in the upper chamber and by adjusting the stirring speed or the amount of cells. ΔpH and the $\Delta\Psi$ were calculated from the steady-state concentration gradients of acetate and tetraphenylphosphoniumbromide (Ph_4P^+) [14,15], respectively. The internal volume of the bacteroid was determined as in [16] and was calculated to be 5.2 μl/mg cell protein. Flow dialysis figures are presented as in [17].

The intracellular levels of ATP, ADP and AMP were determined as in [1].

Protein concentrations were determined by the biuret method.

[U-^{14}C]Acetic acid (60 Ci/mol) was obtained from the Radiochemical Centre (Amersham, Buckinghamshire). [U-^{3}H]Tetraphenylphosphoniumbromide (113.5 Ci/mol) was a generous gift of Dr H. R. Kaback.

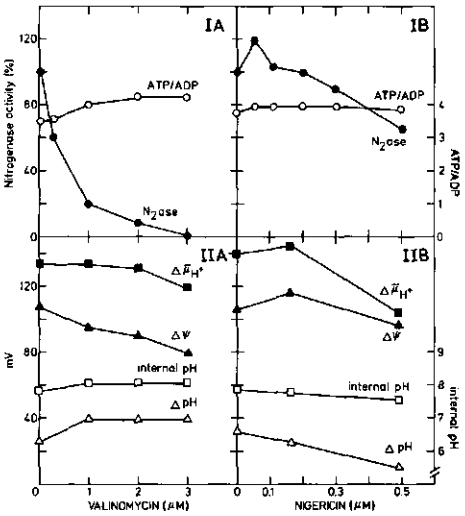

Fig.1. Effect of valinomycin (panels A) and nigericin (panels B) on nitrogenase activity (N_2ase), ATP/ADP ratio (I), $\Delta\tilde{\mu}_{H^+}$, $\Delta\Psi$, ΔpH and internal pH (II) in bacteroids of *R. leguminosarum*. Bacteroids were isolated and experiments were performed at an external pH 7.4 as described in section 2. To the standard incubation buffer bacteroids and myoglobin were added to final conc. 4.0 mg protein/ml and 230 μM, respectively. Data in panels II were obtained from flow dialysis as presented and described in fig.2.

3. Results and discussion

To investigate the role of $\Delta\tilde{\mu}_{H^+}$ or its components on the process of nitrogen fixation valinomycin and nigericin were used. Valinomycin, facilitates the electrogenic movement of K^+ across the membrane, causing dissipation of the membrane potential ($\Delta\Psi$) and, in some instances, reciprocal enhancement of the transmembrane pH difference (ΔpH). Nigericin, on the other hand, facilitates the electroneutral exchange of H^+ mainly for K^+, causing dissipation of the ΔpH and, in some cases, reciprocal enhancement of the $\Delta\Psi$ [12,14,15,18].

Figure 1 illustrates the effect of valinomycin (panels A) and nigericin (panels B) on the nitrogenase activity and the ATP/ADP ratio (I) as well as on $\Delta\tilde{\mu}_{H^+}$, $\Delta\Psi$, ΔpH and the intracellular pH (II) in bacteroids of *R. leguminosarum*. The panels IIA and B compile the data obtained from flow dialysis experiments as presented in fig.2. Addition of increasing amounts of valinomycin resulted in a decrease of the nitrogenase activity and an increase of the ATP/ADP ratio, while the rate of succinate oxidation was hardly influenced. At low concentration ($\leqslant 2$ μM) valinomycin hardly affected the $\Delta\tilde{\mu}_{H^+}$, since the decrease in $\Delta\Psi$ was compensated by an increase in ΔpH. This phenomenon is quite similar to that observed in membrane vesicles of *Escherichia coli* [14]. Under optimal conditions for nitrogen fixation the ΔpH was calculated to be 0.45 pH units. The intracellular pH is therefore ~7.9. As the concentration of valinomycin was increased, the internal pH rose to a fairly constant level of ~8.1.

Nigericin at <0.2 μM stimulated the nitrogenase activity significantly, while at higher concentrations

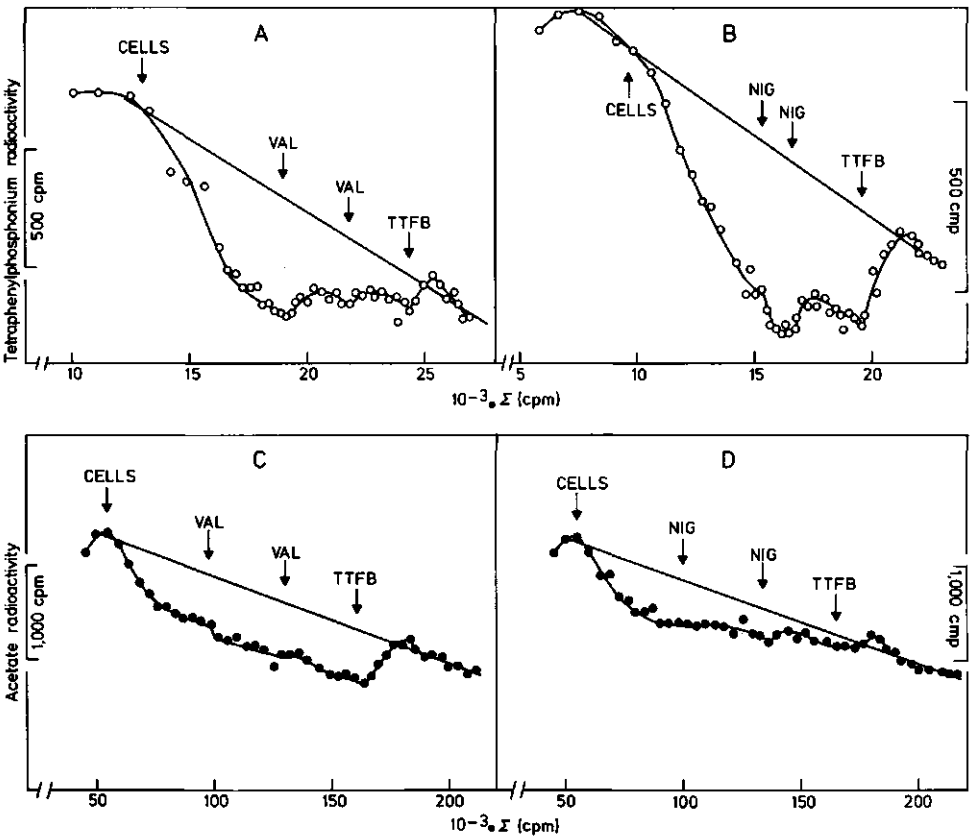

Fig.2. Effect of valinomycin and nigericin on uptake of tetraphenylphosphonium (*A,B*) and acetate (*C,D*) by bacteroids of *R. leguminosarum*, as determined by flow dialysis. Bacteroids were isolated and experiments were performed at an external pH 7.4 as described in section 2. Oxygenated myoglobin, [^{3}H]tetraphenylphosphonium and [^{14}C]acetate were added to the upper chamber at final conc. 230 μM, 24 μM and 30 μM, respectively. Reactions were started by the addition of 0.05 ml cells, final conc. 5.3 mg/ml. Valinomycin (VAL), nigericin (NIG) and uncoupler (TTFB) were added at the times indicated by arrows up to final conc. 1 μM (first addition, 2 μM second addition), 0.14 μM (first addition, 0.35 μM second addition) and 10 μM, respectively. Figures are presented as described [17].

the rate of acetylene reduction was inhibited (panel IB). The ATP/ADP ratio and the rate or respiration remained unchanged within the concentration range used. The increase in nitrogenase activity was accompanied by an increase in $\Delta\Psi$, while the transmembrane pH difference as well as the internal pH decreased.

Nigericin at >0.2 μM had an uncoupling effect, as can be seen by the decline in $\Delta\tilde{\mu}_{H^+}$.

In vivo nitrogenase functions only when cells generate enough energy in the form of ATP and reducing equivalents with a sufficiently low potential. Since the intracellular level of ATP (expressed in the ATP/ADP

ratio) is hardly influenced by the addition of valinomycin or nigericin, it is clear that the decline or stimulation of the nitrogenase activity by the addition of these ionophores is not due to a decreased supply of ATP to nitrogenase. Furthermore, our results indicate that the decline in nitrogenase activity is not caused by a decrease in $\Delta\tilde{\mu}_{H^+}$. In our opinion, two possible explanations remain which can account for the observed effects of these ionophores on nitrogen fixation by bacteroids:

1. By an effect of the internal pH on nitrogenase itself or on the electron transport system to nitrogenase;
2. By a direct effect of $\Delta\Psi$ on the electron transport system to nitrogenase.

Figure 3 shows the relationship between the nitrogenase activity and $\Delta\tilde{\mu}_{H^+}$ (A), ΔpH (B) and $\Delta\Psi$ (C). In addition the internal pH values are given. It should be noted that the nitrogenase activity resembles the flow of reducing equivalents to nitrogenase, since the ATP/ADP ratios under the applied experimental conditions are hardly influenced. This figure also contains the results obtained from experiments performed at an external pH 6.7 instead of 7.4 (open symbols). At an external pH 6.7 the nitrogenase activity, at a fixed oxygen supply, was still 90% of the activity at 7.4. At pH <6.7 the nitrogenase activity declined dramatically. Like in several bacteria [19–21] the ΔpH varied markedly with external pH. Despite an increase in ΔpH, at lower external pH, the internal pH and the $\Delta\Psi$ remained fairly constant. Intracellular pH values at extracellular pH 6.7–7.4 were almost constant at 7.65–7.85. Consequently, $\Delta\tilde{\mu}_{H^+}$ rose from about −130 mV at pH 7.4 to −160 mV at pH 6.7, while the nitrogenase activity was 10% lower and the ATP/ADP ratio hardly changed (not shown). The effect of valinomycin on the nitrogenase activity appeared to be independent of the external pH. At 1 μM the nitrogenase activity dropped in both cases to 20%. However, nigericin was far more effective at pH 6.7 than at pH 7.4. This observation is consistent with the finding that at a higher external pH the ΔpH is only small. At an external pH 6.7 nigericin at 0.014 μM stimulated the nitrogenase activity maximally by 40%, while at pH 7.4 more nigericin (0.06 μM) was necessary for maximal stimulation of the nitrogenase activity (20%).

Figure 3 clearly demonstrates that there is no correlation between the flow of reducing equivalents to nitrogenase and $\Delta\tilde{\mu}_{H^+}$ or ΔpH in bacteroids of *R. leguminosarum*. The nitrogenase activity changes markedly, while $\Delta\tilde{\mu}_{H^+}$ or ΔpH are not influenced. On the other hand, there is a clear relationship between the nitrogenase activity and $\Delta\Psi$. All data, whether they are obtained from different experiments performed at different external pH or in the presence of ionophore fit into this relationship. Since the internal pH does not change significantly under these conditions (fig.3C), it is clear that electron transport to nitrogenase is regulated by the $\Delta\Psi$ and extremely sensitive

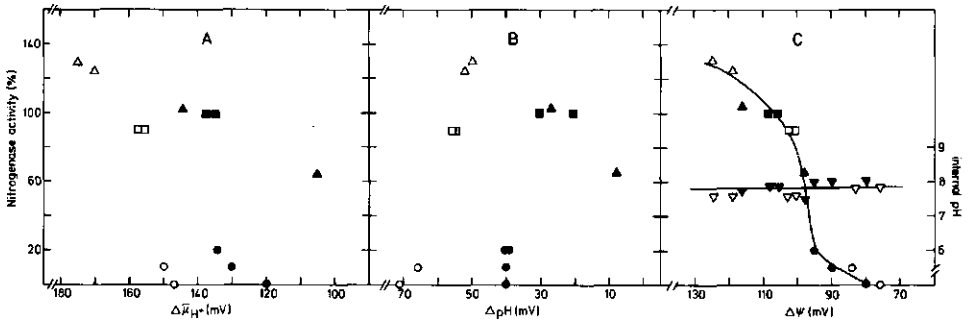

Fig.3. Relationship between nitrogenase activity and $\Delta\tilde{\mu}_{H^+}$ (A), ΔpH (B), $\Delta\Psi$ (C) in bacteroids of *R. leguminosarum*. Data at an external pH 7.4 (dark symbols) are obtained from experiments presented in fig.1. Open symbols represent the results obtained at an external pH 6.7 (see text). 100% nitrogenase activity ≃20 nmol ethylene produced .min^{-1}.mg bacteroid protein^{-1} at pH 7.4. (□,■) Without addition; (△,▲) with nigericin; (○,●) with valinomycin; (▽,▼) internal pH.

towards changes in $\Delta\Psi$. At $\Delta\Psi < 80$ mV no functional reducing equivalents are generated, while at > 120 mV the machinery which produces reducing equivalents seems to function maximally. Especially small changes in $\Delta\Psi$ centered around -100 mV are accompanied by marked changes in the flow of reducing equivalents to nitrogenase.

We also studied the effect of valinomycin and nigericin on the ATP/ADP ratio and on the nitrogenase activity of the free-living nitrogen fixer *A. vinelandii* (not shown). Valinomycin and nigericin were found to be very active in *A. vinelandii* provided cells were pretreated with EDTA [22]. However, both the rate of acetylene reduction as well as the ATP/ADP ratio dropped considerably when low concentrations of valinomycin or nigericin were added. *A. vinelandii* accumulates K^+ actively during respiration and K^+ is required for an optimal functioning metabolism [23]. For example, the rate of respiration decreased ~2-fold when K^+ was omitted from the medium. Furthermore, without K^+ little nitrogenase activity could be observed. Since valinomycin and nigericin disturb the K^+ gradient in vivo, it seems reasonable to suggest that they also influence essential processes for nitrogen fixation. We observed no influence of K^+ on the metabolism of *R. leguminosarum* bacteroids. In our opinion, bacteroids do not need a K^+-accumulating system, since bacteroids are localized within the plant host cell which already contains a relatively high concentration of K^+.

At this stage of investigation the mechanism of a $\Delta\Psi$-dependent formation of reducing equivalents in bacteroids of *R. leguminosarum* is not understood. Work is in progress to characterize the function of the $\Delta\Psi$ in the generation of reducing equivalents with a sufficiently low potential for nitrogenase in isolated membrane vesicles of *R. leguminosarum* bacteroids.

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We wish to thank Mr A. Houwers and his collegues of the Department of Microbiology, Wageningen, for growing the plant material, Drs S. Ottema for his help in the flow dialysis assays and Mr B. J. Sachteleben for drawing the figures.

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. SHORT-TERM EFFECT OF AMMONIUM CHLORIDE ON NITROGEN FIXATION BY *AZOTOBACTER VINELANDII AND* AND BY BACTEROIDS OF *RHIZOBIUM LEGUMINOSARUM*

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.1. SUMMARY

. Evidence is presented suggesting, that the direct depressing effect of ammonium chloride on dinitrogen fixation by *Azotobacter vinelandii* is due to inhibition of the electron transport system to nitrogenase. Furthermore, we were able to confirm the observation [Houwaard, F. (1979) Appl.Environ.Microbiol. in press] that ammonium chloride has no short-term effect on dinitrogen fixation by isolated bacteroids of *Rhizobium leguminosarum*.

. By means of the flow dialysis technique, it could be demonstrated that in *A.vinelandii* ammonium is taken up as a cation in response to the $\Delta\Psi$, and that uptake of ammonium specifically inhibits the flow of reducing equivalents to nitrogenase by lowering the $\Delta\Psi$ across the cytoplasmic membrane. In *A.vinelandii*, as in bacteroids, the generation of reducing equivalents at a potential low enough to reduce nitrogenase, was found to be extremely sensitive towards changes in $\Delta\Psi$. At $\Delta\Psi$-values less than 80 mV, interior negative, no such reducing equivalents are generated, while at a $\Delta\Psi$-value of 110 mV, nitrogenase is optimally supplied with reducing equivalents. The nature of the ammonium transport system in *A.vinelandii* and its significance as a regulator for the rapid "switch off/switch on" of nitrogenase activity is discussed.

. Bacteroids of *R.leguminosarum* did not accumulate ammonium and no effect on $\Delta\Psi$ was observed for ammonium. On the contrary, it could be demonstrated that bacteroids excrete ammonium in response to the ΔpH.

5.2. INTRODUCTION

It has been firmly established that the process of dinitrogen fixation by free-living bacteria [1-4], as well as by symbiotic associations [5,6], is inhibited in the presence of fixed nitrogen; especially ammonium. In the free-living dinitrogen-fixer *Azotobacter* two effects of ammonium can be distinguished. Firstly, the energetically expensive nitrogenase reaction is rapidly switched off (short-term effect) [1]. Secondly, the synthesis of nitrogenase is repressed, resulting in a decline of total activity (long-term effect) [2,7]. On the other hand, fixed nitrogen does not affect the nitrogenase activity of isolated bacteroids of *Rhizobium leguminosarum* [8]. However, when these bacteroids are in symbiosis with the plant host cell, fixed nitrogen affects the nitrogenase activity [5,6].

Little is known about the short-term effect of ammonium on the process of dinitrogen fixation. *In vivo*, nitrogenase functions only when cells generate enough energy in the form of ATP and reducing equivalents. Since cell-free nitrogenases are insensitive to ammonium concentrations that completely inhibit the nitrogenase activity in whole cells [9,10], it has been suggested that ammonium in *Azotobacter* depresses the flow of reducing equivalents and/or the supply of ATP to nitrogenase [1,11].

In the present paper, we investigated the short-term effect of ammonium chloride on dinitrogen fixation by the free-living organism *Azotobacter vinelandii*, and the symbiotic dinitrogen-fixer *Rhizobium leguminosarum*. Evidence is presented suggesting, that electron transfer to nitrogenase in *A.vinelandii*, as in *R.leguminosarum* bacteroids [12], is regulated exclusively by the electrical component ($\Delta\Psi$) of the protonmotive force, and that uptake of ammonium by *A.vinelandii* specifically switches off the flow of reducing equivalents to nitrogenase by lowering the $\Delta\Psi$. In contrast, bacteroids excrete newly-fixed ammonium, while the $\Delta\Psi$ as well as the ATP/ADP ratio are not affected by externally added ammonium.

5.3. MATERIALS AND METHODS

5.3.1. Growth Conditions of the Bacteria

A.vinelandii ATCC strain OP was cultured in batches of 0.5 liter on a Burk's nitrogen-free basic salt medium [13], supplied with sucrose or sodium succinate as sole carbon and energy source. Cells were harvested by centrifugation at the

end of the logarithmic growth, washed once with distilled water and finally suspended in 25 mM Tris/HCl, 2.5 mM $MgCl_2$ and 1 mM KCl, final pH 7.6, up to a concentration of 40-60 mg protein/ml. Cells were prepared freshly and stored at 0°C during the course of the experiments.

Bacteroids of *R. leguminosarum* (strain PRE) were produced under controlled conditions on peas (*Pisum sativum* L. cv. Rondo) and isolated from the root nodules as described previously [12,14]. Bacteroids were suspended in 25 mM Tes/NaOH, 2.0 mM $MgCl_2$, 1.0 mM KCl and 0.3 M sucrose, final pH 7.4 to a concentration of about 60 mg protein/ml and stored at 0°C.

5.3.2. Analytical Methods

The nitrogenase activity (acetylene reduction) of *A. vinelandii* and of *R. leguminosarum* bacteroids was measured in the assay system described previously [14]. Incubation mixtures for *A. vinelandii* and bacteroids were used as described above. Glucose or sodium succinate were added as substrates, final concentration 20 mM (see text). In all experiments, the oxygen input was sufficient to allow maximum rates of acetylene reduction. When the rate of acetylene reduction was constant, ammonium chloride was added and the incubation was continued for at least 6 minutes.

The intracellular levels of ATP, ADP and AMP were determined as described in [15].

Uptake studies were performed at 25°C with the flow dialysis technique as described by Ramos *et al.* [16,17]. The upper and lower chambers were separated by dialysis tubing (Visking; pore diameter 2.4 nm). The upper chamber contained the same mixture as was pumped through the lower chamber at a rate of 3 or 4 ml per minute (see text). For *A. vinelandii*, the standard incubation mixture consisted of 25 mM Tris/HCl, 2.5 mM $MgCl_2$, 20 mM substrate and, where indicated, KCl, final pH 7.6. For bacteroids, the standard dialysis mixture contained: 25 mM Tes/NaOH, 2.0 mM $MgCl_2$, 20 mM sodium succinate, 1.0 mM KCl and 0.3 M sucrose, final pH 7.4. Prior to use, the incubation mixtures were flushed with pure oxygen. Since the endogeneous rate of respiration was rather high, reactions were not started by adding substrate, but by adding cells (0.05 ml) to the upper chamber, final volume 0.85 ml. Further additions were made as indicated. In the case of bacteroids, the upper chamber also contained oxygenated myoglobin, final concentration 230 µM. Fractions of 1.5 ml were collected and assayed for radioactivity. During the experiments care was taken that the oxygen input into the cell suspension was optimal for dinitrogen fixation. This was done by flushing pure oxygen over the upper

chamber, and by adjusting the stirring speed or the amount of cells. ΔpH and ΔΨ were calculated from the steady-state concentration gradients of acetate or 5,5-dimethyloxazolidine-2,4-dione and tetraphenylphosphonium bromide (Ph_4P^+) respectively [16-18]. The internal volume of bacteroids was calculated to be 5.2 µl per mg of cell protein [12] and a value of 5.0 µl per mg of cell protein was used for *A.vinelandii* [19].

Efflux studies were also performed with the flow dialysis technique, using bacteroids preloaded with methylammonium. This was done by incubating a suspension of bacteroids (55 mg protein/ml) with 25 mM methylammonium, of which 20 µM was radioactively labelled, for 2 hours at $0^{o}C$ under anaerobic conditions. Experiments were started by adding 0.9 ml of this suspension to the gas-tight upper chamber, which was made anaerobic by flushing with argon. The anaerobic standard dialysis mixture for bacteroids was pumped through the lower chamber at a rate of 3.5 ml/min. Pure oxygen was added to the gas phase at the time indicated.

Myoglobin was oxygenated as described [20].

Protein concentrations were determined by the biuret method.

5.3.3. Chemicals

[^{14}C] Methylaminehydrochloride (61.9 Ci/mol) and [^{14}C] Acetic acid (60 Ci/mol) were obtained from the Radiochemical Centre (Amersham, Buckinghamshire, England). [^{14}C] 5,5-dimethyloxazolidine-2,4-dione (11 Ci/mol) was obtained from New England Nuclear (NEN) and [U-^{3}H] Tetraphenylphosphonium bromide (113.5 Ci/mol) was a generous gift of Dr. H.R. Kaback. All other (bio-)chemicals were reagent grade, and were obtained from commercial sources.

5.4. RESULTS

5.4.1. Effect of Ammonium Chloride on Nitrogenase Activity and Oxidative Phosphorylation

Fig. 1 shows the short-term effect of increasing ammonium chloride concentrations on the nitrogenase activity and the ATP/ADP ratio in *A.vinelandii* and in bacteroids of *R.leguminosarum*. In succinate-grown cells of *A.vinelandii*, 0.2 mM ammonium chloride rapidly (within 1 min) inhibits the nitrogenase activity (Fig. 1A), while the rate of respiration is hardly influenced (7% enhancement). The depressing effect of ammonium on dinitrogen fixation by sucrose-grown cells was

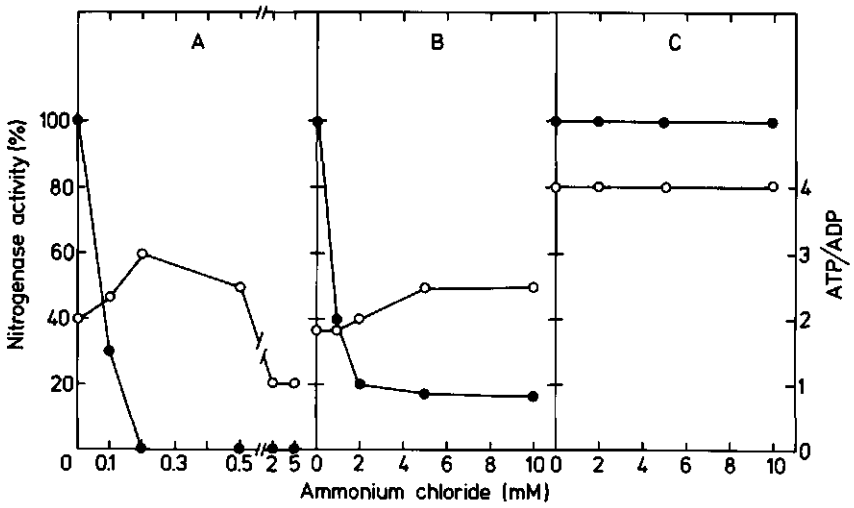

Fig. 1. Effect of ammonium chloride on nitrogenase activity and ATP/ADP ratio in *A.vinelandii* and bacteroids of *R.leguminosarum*.
Experiments were carried out as described in Materials and Methods, using *A.vinelandii* grown on succinate (A) or on sucrose (B), and using isolated bacteroids of *R.leguminosarum* (C). For sucrose-grown cells, glucose was used as substrate. In the case of succinate-grown cells, or bacteroids succinate served as substrate. *A.vinelandii* cells and bacteroids were added to final concentrations of 1.6 mg protein/ml and 3.8 mg protein/ml, respectively. (●-●), Nitrogenase activity; (○-○), ATP/ADP ratio.

found to be less pronounced (Fig. 1B). In sucrose-grown cells, it appeared not to be possible to inhibit the nitrogenase activity completely with ammonium. On the contrary, no effect of ammonium chloride, even at concentrations of 10 mM, could be observed on the nitrogenase activity of isolated bacteroids (Fig. 1C). The observation that ammonium inhibits the nitrogenase activity of *A.vinelandii*, but not that of bacteroids, has also been reported by other investigators [1,8,11].

It has been suggested that the inhibitory effect of ammonium on dinitrogen fixation by *A.vinelandii* is due to a decreased supply of ATP (lower ATP/ADP ratio) for nitrogenase, since the conversion of ammonium into glutamine via glutamine synthetase requires ATP [11]. However, Fig. 1A and B clearly show that the decline of the nitrogenase activity is accompanied by an increase in the ATP/ADP ratio.

The ATP supply for nitrogenase was lowered only when succinate-grown cells were exposed to ammonium concentrations higher than 0.5 mM. Under these circumstances, ammonium seems to act like an uncoupler. In bacteroids, neither the ATP/ADP ratio nor the nitrogenase activity is affected by the addition of ammonium chloride (Fig. 1C).

In principle ammonium can influence dinitrogen fixation directly in several ways. Firstly, the influence can be exerted by means of product inhibition of nitrogenase. This possibility can be excluded, since ammonium up to a concentration of 5 mM has no effect on the rate of acetylene reduction of isolated nitrogenase [9], nor on the activity in cell-free extracts [1,10]. A second way in which ammonium can influence dinitrogen fixation is by means of a decrease in the supply of ATP to nitrogenase. Our results clearly indicate that this is not the case. Thirdly, ammonium can directly affect dinitrogen fixation by inhibiting the transfer of electrons to nitrogenase.

In previous studies [15,22], evidence has been presented suggesting, that in *A.vinelandii*, the flow of reducing equivalents to nitrogenase is regulated by the energized state of the cytoplasmic membrane. It has been proposed that the protonmotive force across the membrane, generated by respiration, drives the generation of reducing equivalents for nitrogenase [22]. According to the chemiosmotic theory, as postulated by Mitchell [23-25], this driving force ($\Delta\tilde{\mu}_{H^+}$) consists of an electrical gradient ($\Delta\Psi$) and a concentration gradient (ΔpH) across the membrane. An explanation for the depressing effect of ammonium on dinitrogen fixation by *A.vinelandii* might therefore be found in a decrease of the protonmotive force, or one of its components.

5.4.2. Uptake of Ammonium

In whole cells, ammonium can affect the energized state of the membrane only when it is taken up. Information concerning the uptake of ammonium was obtained by studies with the radioactive analogue of ammonium, methylammonium. Methylammonium is often used in order to demonstrate the existence of ammonium transport systems in bacteria [26,27], as well as in eukaryotic microbes [28-30]. It appeared to be a useful analogue, since methylammonium, although less pronounced than ammonium, affects dinitrogen fixation by *A.vinelandii*. Weak bases, like methylammonium, permeate rapidly across membranes in their electroneutral form. The method to study methylammonium uptake is, therefore, by means of flow dialysis. A treatise on this technique is discussed in [18].

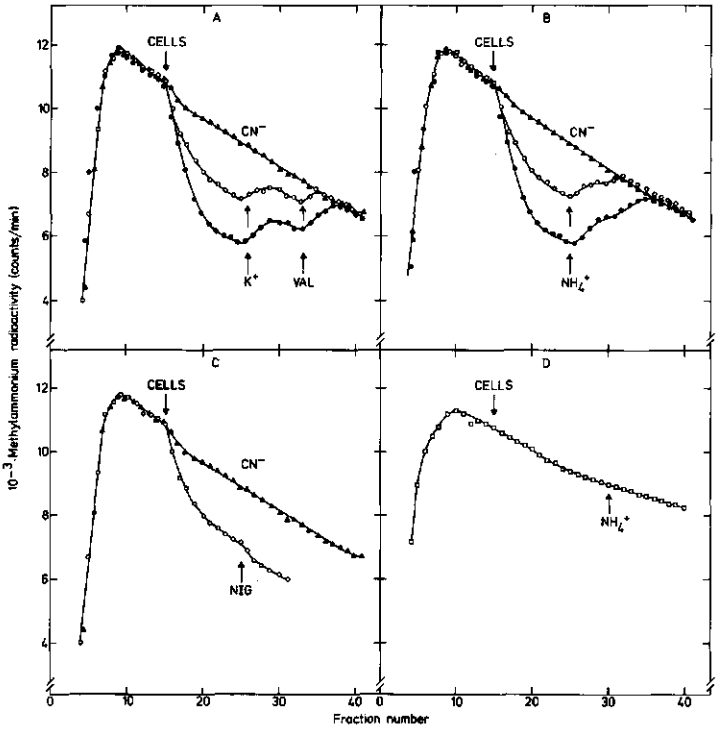

Figure 2. Uptake of methylammonium by *A.vinelandii* and bacteroids of *R.leguminosarum*, as determined by flow dialysis.
Assays were performed at 25°C, as described in Materials and Methods. The reaction volume was 0.85 ml. Substrates (glucose or succinate, see Fig. 1) were added to the standard incubation mixture to final concentrations of 20 mM. [^{14}C] Methylammonium, *A.vinelandii* cells and bacteroids were supplied to the upper chamber to final concentrations of 230 µM (8.8 Ci/mol), 5.3 mg protein/ml and 3.6 mg protein/ml, respectively. Prior to the addition of ionophore, EDTA (final concentration 3.1 mM) was added to the incubation mixture. In the case of bacteroids the upper chamber also contained 230 µM oxygenated myoglobin. Further additions were made as indicated. (A), Effect of potassium chloride (10 mM) and VAL, valinomycin (6 µM) on methylammonium uptake by sucrose-grown cells of *A.vinelandii* (O-O), and succinate-grown cells (●-●); (B), effect of ammonium chloride (2 mM) on methylammonium uptake by sucrose-grown cells of *A.vinelandii* (O-O), and succinate-grown cells (●-●); (C), effect of NIG, nigericin (0.16 µM) on methylammonium uptake by sucrose-grown cells of *A.vinelandii* (O-O); (D), effect of ammonium chloride (2 mM) on methylammonium uptake by bacteroids of *R.leguminosarum*. (▲-▲), Control experiment carried out with *A.vinelandii* cells pretreated (5 min) with 1 mM potassium cyanide.

The data presented in Fig. 2 illustrate typical flow dialysis experiments. Shortly after the addition of radioactive methylammonium to the upper chamber, radioactivity appears in the dialysate (lower chamber), increases linearly for about 6 fractions (2 min) and reaches a maximum, which then decreases at a slow rate. In the latter part of the curve, the amount of radioactivity appearing in the dialysate is directly correlated with the concentration of free label in the upper chamber. It is observed that the concentration of methylammonium in the upper chamber does not change significantly by the addition of *A.vinelandii* cells pretreated with cyanide (Fig. 2A, B and C triangles). This indicates that methylammonium is not taken up by these cells. However, when respiring *A.vinelandii* cells were added to the upper chamber, the amount of methylammonium in the dialysate decreases relative to the level observed in the control experiment (circular symbols). This indicates that methylammonium is taken up by respiring *A.vinelandii*. It should be mentioned that the methylammonium concentration used (0.23 mM) was high enough to inhibit 90% of the nitrogenase activity of succinate-grown cells.

In sucrose-grown cells of *A.vinelandii*, steady-state levels of uptake were reached at 5 nmoles methylammonium per mg cell protein (Fig. 2A, B and C, open circular symbols). This corresponds to an internal methylammonium concentration of 1.0 mM which is equivalent to a 9-fold accumulation. Even higher levels of accumulation were observed in succinate-grown cells; at steady-state a 20-fold accumulation of methylammonium could be calculated (Fig. 2A, B and C, closed circular symbols). To study the effect of ionophores on the uptake of methylammonium, cells were pretreated with EDTA, in order to remove the outer membrane [31]. Addition of valinomycin into the incubation mixture results in an efflux of the accumulated label (Fig. 2A). Valinomycin facilitates the electrogenic movement of potassium ions across the membrane, causing dissipation of the membrane potential ($\Delta\Psi$)[32]. Addition of nigericin results in an enhanced uptake of methylammonium (Fig. 2C). Nigericin at low concentrations dissipates the transmembrane pH-difference (ΔpH) and enhances the $\Delta\Psi$ [32]. Thus, these results suggest that methylammonium is taken up as a cation in response to $\Delta\Psi$.

Fig. 2A and B also demonstrates the effect of potassium and ammonium on uptake of methylammonium. Ammonium was found to be a potent inhibitor of methylammonium uptake (Fig. 2B). Similar results were reported for the ammonium transport system in *Escherichia coli* [26]. In contrast, potassium only partially inhibits the uptake of methylammonium (Fig. 2A). In sucrose-grown cells, as in succinate-grown cells, the maximum inhibition appears to be about 50%, even when the concentration of potassium was increased from 50 µM up to 10 mM.

No accumulation of methylammonium was observed in isolated bacteroids (Fig. 2D). This observation is in agreement with the results presented in Fig. 1C; that is, ammonium has no effect on the nitrogenase activity of bacteroids.

5.4.3. Excretion of Ammonium

Indirect evidence suggests that most of the ammonium produced by bacteroids in their natural habitat is excreted and assimilated by plant enzymes [33-37]. The capacity of the pathways within the bacteroid was found to be insufficient to assimilate the ammonium produced by nitrogenase. Therefore, we investigated the efflux of ammonium by bacteroids, using the flow dialysis technique. Fig. 3

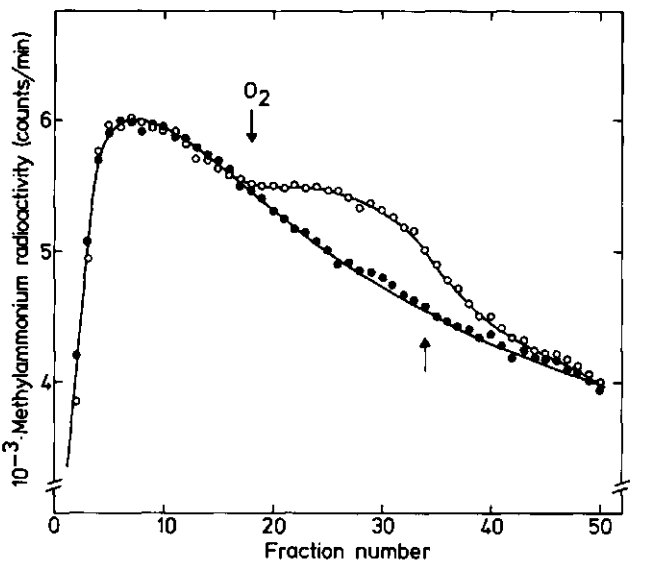

Figure 3. Excretion of methylammonium by bacteroids of *R. leguminosarum*
Bacteroids were isolated and experiments were performed as described in Materials and Methods. The arrows indicate the addition of oxygen (100% in the gas phase) and toluene (final concentration 0.5%), respectively. (O-O), with oxygen; (●-●), control experiment without oxygen.

shows that when oxygen is added to an anaerobic suspension of bacteroids, which are preloaded with radioactive methylammonium, more label appears in the dialysate (open symbols) relative to the level observed in the absence of oxygen (closed symbols). This clearly indicates that bacteroids excrete methylammonium in response to respiration. After the addition of toluene, an influx of excreted

methylammonium is observed. It is known that toluene rapidly makes the membrane permeable to small molecules [38]; thus, it is clear that respiring bacteroids maintain a concentration gradient of methylammonium across the membrane. A steady-state concentration gradient of 2.8 could be calculated (2.8 times as much methylammonium outside than inside). Under the applied experimental conditions (with methylammonium inside and the outside acidic), weak bases, such as methylammonium, act as a ΔpH probe [39]. From the steady-state level of ammonium efflux, a ΔpH of 0.45 pH units can be calculated. Exactly the same value was found previously, using acetate as a ΔpH probe [12]. These results therefore indicate that bacteroids excrete (methyl)ammonium only in response to a ΔpH.

5.4.4. Effect of Ammonium Uptake on $\Delta\tilde{\mu}_{H^+}$, *ΔpH, ΔΨ and Internal pH*

Since uptake of ammonium in *A.vinelandii* is driven by the ΔΨ across the membrane, we investigated the effect of ammonium uptake on the protonmotive force and its components under conditions optimal for dinitrogen fixation. Recently, accurate techniques for measuring ΔΨ and ΔpH have been developed. The method for determining the ΔΨ is based on the property of lipophilic cations, such as tetraphenylphosphonium (Ph_4P^+), to become distributed across the membrane according to the ΔΨ [40-42]. The ΔΨ can then be calculated from the concentration gradient of Ph_4P^+ by means of the Nernst equation [42]. The other parameter of the protonmotive force, the ΔpH, can be approximated from the transmembrane distribution of weak acids, which are membrane permeable in the undissociated form [16-18]. Weak acids, such as acetate and 5,5-dimethyloxazolidine-2,4-dione, become distributed across the cytoplasmic membrane according to the pH gradient. We used 5,5-dimethyloxazolidine-2,4-dione as a ΔpH-probe, since acetate is rapidly metabolized in whole cells of *A.vinelandii* and 5,5-dimethyloxazolidine-2,4-dione not at all.

Table 1 summarizes the results obtained by means of flow dialysis with succinate-grown cells. It should be noted that these experiments were carried out in the presence of 1 mM potassium chloride. In order to function optimally, *A.vinelandii* absolutely requires potassium in its medium [12]. Without potassium, the rate of respiration decreases about 2-fold, and little if any nitrogenase activity can be observed. In addition, the nitrogenase activities and the ATP/ADP ratios are given. Ammonium at concentrations less than 0.2 mM hardly affects $\Delta\tilde{\mu}_{H^+}$, since the decrease in ΔΨ is compensated by an increase in ΔpH. Under optimal conditions for dinitrogen fixation the steady-state level of 5,5-dimethyloxazolidine-2,4-dione uptake corresponds to a ΔpH of about 0.45 pH units. The internal pH is therefore approximately 8. As the concentration of ammonium is increased to 0.2 mM, the

Table 1. Effect of ammonium chloride on $\Delta\tilde{\mu}_{H^+}$, $\Delta\Psi$, ΔpH, internal pH, nitrogenase activity and the ATP/ADP ratio in succinate-grown *A.vinelandii*.
Data were obtained from flow dialysis experiments as described in Materials and Methods. Cells, 5,5-dimethyloxazolidine-2,4-dione and Ph_4P^+ were added to the upper chamber at final concentrations of 4.2 mg protein/ml, 30 µM and 24 µM, respectively. When steady-state levels of Ph_4P^+ or 5,5-dimethyloxazolidine-2,4-dione uptake were reached, ammonium chloride was added to the incubation mixture at the concentrations given below. After approximately 5 min, when a new steady-state level of Ph_4P^+ or 5,5-dimethyloxazolidine-2,4-dione uptake was reached, uncoupler $(Cl_4CF_3Bza)^-$ was added up to a final concentration of 10 µM. See Fig. 1A for the nitrogenase activities and the ATP/ADP ratios.

ammoniumchloride (mM)	$\Delta\tilde{\mu}_{H^+}$	$\Delta\Psi$ (mV)	ΔpH	internal pH	nitrogenase activity (% remaining)	ATP/ADP ratio
--	132	106	26	8.05	100	1.8
0.06	133	95	38	8.25	40	1.9
0.10	128	90	38	8.25	10	2.0
0.20	120	82	38	8.25	0	3.0
0.50	105	75	26	8.05	0	2.5

internal pH rises to a fairly constant level of 8.25. At the same time, the nitrogenase activity is inhibited completely, and the ATP/ADP ratio is enhanced. In our opinion, the rise in the internal pH cannot explain the depressing effect of ammonium on dinitrogen fixation by *A.vinelandii*, since the nitrogenase activity further declines to zero after the internal pH has reached a constant level of 8.25. In short, our results indicate that ammonium uptake specifically switches off the flow of reducing equivalents to nitrogenase by lowering the $\Delta\Psi$. At higher ammonium concentrations, side effects were observed; both the values of $\Delta\Psi$ and ΔpH decrease. Similar effects are reported to occur when uncoupler is added to respiring cells of *E.coli* [43]. This indicates that ammonium chloride at concentrations higher than 0.2 mM acts as an uncoupler. This contention is further substantiated by the observation that ammonium, under such conditions, depresses oxidative phosphorylation (Fig. 1A).

Fig. 4 shows that there is a relationship between the nitrogenase activity (flow of reducing equivalent) and $\Delta\Psi$ in *A.vinelandii*. This figure also presents the results obtained from experiments with sucrose-grown cells. The $\Delta\tilde{\mu}_{H^+}$, $\Delta\Psi$ and ΔpH generated under optimal conditions for dinitrogen fixation by sucrose-grown cells appear to be the same as in succinate-grown cells. However, the $\Delta\Psi$-lowering capacity of the same amount of ammonium in sucrose-cells is less than in succinate-grown cells. This phenomenon parallels the effect of ammonium on the nitro-

genase activity (Fig. 1A and B), and furthermore, the amounts of methylammonium taken up are smaller in sucrose-grown cells than in succinate-grown cells (Fig. 2A and B). Fig. 4 demonstrates that the electron transfer to nitrogenase in *A.vinelandii*, as in bacteroids [12] is extremely sensitive towards changes in $\Delta\Psi$.

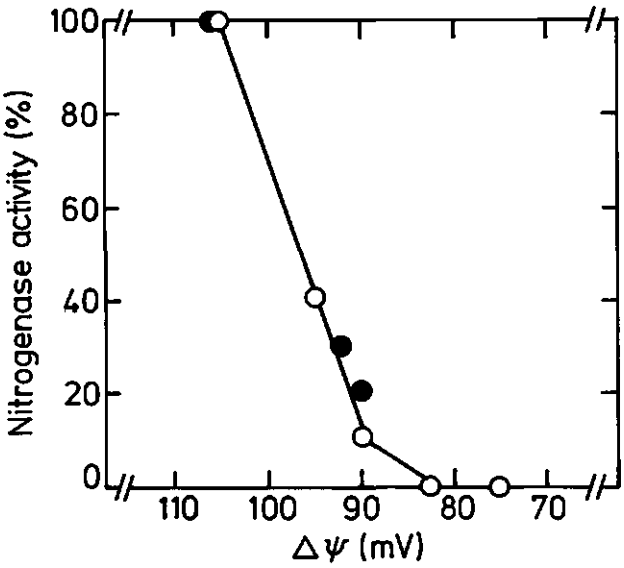

Figure 4. Relationship between nitrogenase activity and $\Delta\Psi$ in *A.vinelandii*, as affected by ammonium chloride.
Data are obtained from flow dialysis experiments, performed with succinate-grown cells (see Table 1) and with sucrose-grown cells of *A.vinelandii*. The nitrogenase activity depends on the flow of reducing equivalents to nitrogenase. (O-O), Succinate-grown cells; (●-●), sucrose-grown cells.

The flow of reducing equivalents, as measured by the rate of acetylene reduction, is maximal at a $\Delta\Psi$ of -110 mV, while at values less than 80 mV, interior negative, no functional reducing equivalents for nitrogenase are generated.

Bacteroids of *R.leguminosarum*, under optimal conditions for dinitrogen fixation, generate a $\Delta\tilde{\mu}_{H^+}$ of approximately -140 mV, which consists of a $\Delta\Psi$ of about -110 mV and a ΔpH of -30 mV [12]. In contrast to *A.vinelandii*, ammonium does not affect the energized state of the bacteroidal membrane. Exactly the same values for $\Delta\tilde{\mu}_{H^+}$, $\Delta\Psi$ and ΔpH are obtained when 5 mM ammonium chloride is present during flow dialysis.

5.5. DISCUSSION

In principle, ammonium can enter biological systems in two different ways. Firstly, ammonium or its analogues passively diffuse across the cytoplasmic membrane in their electroneutral form [39]. Secondly, ammonium or derivatives thereof are taken up as a cation. By means of flow dialysis, it can be demonstrated that *A.vinelandii* accumulates externally added (methyl)ammonium as a cation in response to a $\Delta\Psi$. In view of the chemiosmotic theory this is not surprising, since other positively charged species such as lysine and potassium are also accumulated by employing a $\Delta\Psi$ [44]. The ammonium transport system of *A.vinelandii* can therefore be regarded as an uniport process. In contrast, no inside-directed ammonium transport system could be detected in bacteroids. Our results indicate that bacteroids excrete newly-fixed ammonium in response simply to the ΔpH, presumably without the involvement of a translocator.

At this stage, the nature of the ammonium translocator in *A.vinelandii* is still unclear. To reveal this extensive kinetic studies would have to be performed. However, preliminary results indicate that potassium interferes with the uptake process of ammonium. Since both ions are assumed to be simple charged spheres of approximately the same diameter [32], we believe the potassium and ammonium transport systems to have similar features. This idea is further substantiated by the observation that, as was found for ammonium (Fig. 2A and B), the amount and rate of potassium uptake [45,46] in succinate-grown cells is higher than that in sucrose-grown cells of *A.vinelandii*.

It has been suggested that ammonium directly depresses dinitrogen fixation in *A.vinelandii* by decreasing the supply of ATP (lower ATP/ADP ratio) for nitrogenase, since the conversion of ammonium into glutamine via glutamine synthetase requires ATP [11]. However, our results clearly indicate that the ATP supply to nitrogenase is not affected, and is even enhanced by the addition of ammonium to respiring cells of *A.vinelandii*. In addition, we have shown that $\Delta\tilde{\mu}_{H^+}$ is not influenced by low concentrations of ammonium and that the observed changes in ΔpH and in the internal pH cannot be correlated with the decrease in nitrogenase activity. Based on these facts, we conclude that the uptake of ammonium in *A.vinelandii* specifically switches off the flow of reducing equivalents to nitrogenase by lowering the $\Delta\Psi$. The increase in the ΔpH after the addition of ammonium to a final concentration of 0.5 mM can explain the observed increase of the ATP/ADP ratio.

A possible explanation for the depressing effect of ammonium on $\Delta\Psi$ might be that ammonium, once accumulated, binds and neutralizes anionic sites induced by

energization of the cytoplasmic membrane. This mechanism is based on the observation that quaternary ammonium compounds such as 9-amino-substituted acridines show an energy-dependent electrostatic binding to negatively charged sites on the membrane [47]. At higher concentrations, ammonium starts to act as a classical uncoupler [48].

In *A.vinelandii*, a decrease in $\Delta\Psi$ of about 30 mV was found to be sufficient to switch off the flow of reducing equivalents to nitrogenase completely. At $\Delta\Psi$-values less than 80 mV, interior negative, no functional reducing equivalents are generated; while at $\Delta\Psi$-values >90 mV, nitrogenase is supplied with reducing equivalents. Exactly the same results were obtained with bacteroids of *R.leguminosarum* [12]. This implies that these aerobic organisms are able to control the flow of reducing equivalents to nitrogenase simply by regulating $\Delta\Psi$. Only in *A.vinelandii* can $\Delta\Psi$ be influenced by ammonium. Therefore, *Azotobacter* possesses a very fine regulatory mechanism for rapidly switching on/off the energetically expensive nitrogenase reaction. Moreover, by slightly lowering $\Delta\Psi$ with ammonium, essential metabolic processes, such as oxidative phosphorylation are not influenced.

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6. DISCUSSION

The central issue of this thesis deals with the relation between energy metabolism and dinitrogen fixation in two aerobic bacteria; *Azotobacter vinelandii* and bacteroids of *Rhizobium leguminosarum*. As was mentioned before, dinitrogen is only reduced when enough energy, in the form of ATP and in the form of a strong reductant, is generated. During the last decade, much progress has been made in understanding the energy-generating and energy-consuming systems in bacteria. In the light of these new developments, the coupling between energy-providing mechanisms and dinitrogen fixation in several groups of bacteria will be discussed.

6.1. ATP-GENERATION

The need for ATP in the nitrogenase reaction has been well established (see review [1]). An indication of the ATP demand for dinitrogen fixation in various organisms can be obtained from whole cell studies by measuring the molecular growth yield of cells growing with different nitrogen sources and with molecular nitrogen as sole nitrogen source [2]. In model studies, about 15 molecules of ATP are hydrolized by nitrogenase per molecule of dinitrogen fixed. In *A.vinelandii*, as well as in bacteroids of *R.leguminosarum*, the ATP requirement for nitrogenase is mainly met by oxidative phosphorylation. Some ATP is formed on substrate level, but this barely contributes to the total ATP production of the cell. Although the precise molecular mechanism of oxidative phosphorylation is still a topic of debate, it is generally accepted that electron transfer through the respiratory chain generates a protonmotive force across the cytoplasmic membrane, which in turn drives the synthesis of ATP *via* a membrane bound Mg-ATPase (see Chapter 1).

In *A.vinelandii* the process of oxidative phosphorylation has been studied extensively both in whole cells and in isolated membrane vesicles [3-7]. However, all measurements were performed in the presence of free oxygen and it is doubtful that these conditions are physiological for *Azotobacter*. In growing cultures of *Azotobacter* the environment of nitrogenase is kept free of oxygen by rapid removal of excess oxygen *via* the respiratory chain. The electron transfer systems in this organism therefore have a dual function; viz. the generation of metabolic

energy, a part of which is ATP, and the removal of excess oxygen. We therefore measured the efficiency of oxidative phosphorylation (P/O ratios) under oxygen-limited conditions; not only in membrane vesicles of *Azotobacter*, but also in vesicles of *R. leguminosarum* bacteroids (Chapter 3). Several established techniques are available to determine P/O ratios in isolated membrane systems [8]. However, all these methods have in common the fact that the measurements are performed in the presence of free oxygen. To circumvent this, we devised a technique which mimics the situation of these dinitrogen fixers *in vivo* (Chapter 3). This system made it for example possible to measure the phosphorylation efficiency with hydrogen as an electron donor. By means of the conventional methods this is not possible, since hydrogenase is rapidly inactivated by free oxygen.

From these results and others (see discussion in Chapter 3), it was concluded that hydrogen donates its electrons at a fourth proton-translocating site. The results further indicate that the respiratory systems of *A.vinelandii* and of bacteroids are capable of considerable flexibility regarding the efficiency of ATP synthesis. Two regulatory mechanisms can be distinguished:

1. As shown in Fig. 1 and 2 of Chapter 3, the efficiency of ATP synthesis is regulated by oxygen. The results for *A.vinelandii* can be explained as follows: When the oxygen is limited, oxygen will be consumed predominantly by the phosphorylating cytochrome o pathway. However, when more oxygen becomes available, the respiration rate increases and the cytochrome o pathway becomes saturated. Under these conditions, an increasing part of the total oxygen uptake will be consumed by the non-phosphorylating, or uncoupled, cytochrome d pathway. It should be emphasized that this is only possible when the terminal cytochromes differ in their affinity for oxygen. The results of Kauffman *et al.* [9] and of Hoffman *et al.* [10] suggest that this is indeed the case. In the latter paper, an apparent K_m of 18 μM for cytochrome d was reported and a value of 3.1 μM for cytochrome o.

   Although little is known about the composition of the branched respiratory chain of *R. leguminosarum* bacteroids, our results indicate that the regulation of oxidative phosphorylation in bacteroids is similar to the regulation in *A.vinelandii*. The regulatory mechanism outlined above makes it thus possible for both bacteria to synthesize ATP most efficiently when the ATP requirement for nitrogenase is at a maximum.
2. The efficiency of ATP synthesis can be further regulated by influencing the proton-translocating activity of site I. This can take place in two ways. Firstly, the efficiency of site I in *A.vinelandii* depends on the

oxygen input during growth. When cells are grown at low aeration (oxygen-limited), site I functions efficiently. In contrast, energy conservation at site I is low or absent in cells grown at high aeration (nitrogen-limited). Under these circumstances the activity of sites II, III and the proposed site IV are hardly influenced. Secondly, the efficiency of site I is determined by the availability of oxidizable substrates, such as NAD(P)H, malate and hydrogen. This is based on the observation that the uncoupling of site I in membranes isolated from cells grown nitrogen-limited can be abolished by the addition of an extra electron donor (see Hill coefficients in Chapter 3).

In aerobically grown *Parracoccus denitrificans*, energy conservation at site I can also be influenced [11]. In this organism a clear correlation was found between the efficiency of site I, the rotenone-sensitivity of the respiratory chain and the presence of an iron-sulphur center in complex I. The loss of site I phosphorylation, as well as the loss of rotenone-sensitivity could be explained by the disappearance of iron-sulphur center 2.

It is, however, doubtful whether the same explanation is valid for *A.vinelandii*, since a possible loss of an iron-sulphur center in complex I of *Azotobacter* would imply that the efficiency of site I cannot be restored by the addition of an extra electron donor. At present, it is difficult to offer an alternative explanation; but, a possible answer to this intriguing problem may be found in the mode of action of the central pathway in the respiratory chain, the ubiquinone-cytochrome b complex. Our results indicate that the reduction state of this complex modulates the efficiency at site I. The higher its degree of reduction the better site I functions. This contention is further substantiated by the observation that the P/O ratio increases when the reduction state of the respiratory chain is increased by the addition of low concentrations of the inhibitor 2-heptyl-4-hydroxyquinone-*N*-oxide (HQNO) [5]. In contrast to *A.vinelandii*, there are no indications that bacteroids can regulate their proton-translocating activity of site I.

One way or another, it is clear that the flexibility of oxidative phosphorylation in both organisms is due to the considerable flexibility of the proton-translocating activity of the respiratory chain.

In metabolizing cells, ATP is constantly synthesized and subsequently converted into ADP or AMP by a variety of reactions. A living cell contains, there-

fore not only ATP but also ADP and AMP. AMP does not influence the nitrogenase activity, whereas ADP, a product of nitrogenase function, strongly inhibits the actual nitrogenase reaction [12,13]. For this reason, the intracellular ATP/ADP-ratio may be an important regulator of the nitrogenase activity in whole cells. Throughout this investigation, the levels of ATP, ADP and AMP in *A.vinelandii* and bacteroids were determined (Chapter 2, 4 and 5). It became apparent that the internal ATP/ADP-ratio in these bacteria is fairly constant under different conditions, whereas the nitrogenase activity varies dramatically. This indicates that the rate-limiting step in aerobic dinitrogen fixation is at the level of the generation of reducing equivalents.

6.2. GENERATION OF REDUCING EQUIVALENTS TO NITROGENASE

The need for a strong reductant ($E_h \sim$ -460 mV) in the nitrogenase reaction has long been recognized (see review [14]). In fermenting bacteria, several enzyme systems are known to catalyze reactions that produce reducing equivalents at a sufficiently low potential for nitrogenase (Chapter 1). However, none of these scalar reactions occur in *A.vinelandii* or in bacteroids of *R.leguminosarum*. While searching for a mechanism which generates enough reducing power for nitrogenase in aerobes, it was suggested that a high intracellular ratio of reduced to oxidized pyridine nucleotides generates the necessary reducing power for nitrogenase [15,16]. The most direct evidence against this proposal is the observation that under dinitrogen-fixing conditions the ratios of $NADPH/NADP^+$ and $NADH/NAD^+$ in *A.vinelandii* are too low to serve as a reductant for nitrogenase [17]. A second criticism of this hypothesis is that such high ratios would inhibit several key enzymes of catabolism; not only in *Azotobacter*, but in any living cell [18]. Furthermore, Haaker observed that proton-conducting uncouplers inhibit electron transport to nitrogenase in *A.vinelandii* [17]. It is therefore unlikely that a possible answer to this fascinating problem can be found in the traditional biochemistry of scalar reactions. From the results of Haaker *et al.*, it can be concluded that electron transport to nitrogenase in *Azotobacter* behaves like a reversed electron flow which is driven by the $\Delta\tilde{\mu}_{H^+}$ [17]. From the results described in Chapter 2, a similar conclusion can be drawn for bacteroids of *R.leguminosarum*. In the following papers (4 and 5) the involvement of $\Delta\tilde{\mu}_{H^+}$ in the generation of reducing requivalents for nitrogenase in these aerobes has been studied in more detail. According to the chemiosmotic hypothesis (see Chapter 1), the $\Delta\tilde{\mu}_{H^+}$ is composed of an electrical ($\Delta\Psi$) and an osmotic parameter (ΔpH). For bacteroids

(Chapter 4), as well as for *A.vinelandii* (Chapter 5), evidence was obtained that the reversed electron flow to nitrogenase depends on the $\Delta\Psi$ component of the $\Delta\tilde{\mu}_{H^+}$. This evidence showed that the nitrogenase activity of these aerobes could be varied considerably by changing the $\Delta\Psi$ in such a way that the intracellular ATP/ADP ratio was hardly influenced. In the case of bacteroids, the ionophores valinomycin and nigericin proved to be very useful tools in modulating the magnitude of $\Delta\Psi$. In *A.vinelandii* these ionophores were useless in attempting to vary the electron transport to nitrogenase, since both the nitrogenase activity, as well as the ATP/ADP-ratio, dropped considerably when low concentrations of valinomycin or nigericin were added. A possible explanation for this observation is that the uptake process of potassium in *A.vinelandii* might be ATP dependent. ATP-dependent potassium transport has already been indicated in *Escherichia coli* and *Streptococcus faecalis* (see discussion [19]). Upon addition of valinomycin or nigericin to respiring *A.vinelandii*, the natural-existing potassium gradient across the cytoplasmic membrane is disturbed, and consequently, the ATP/ADP-ratio collapses due to the induced ATPase activity.

Although valinomycin and nigericin could not be used for *A.vinelandii* to vary the electron flow to nitrogenase, it was found that instead of these ionophores, ammonium at relatively low concentrations can be used to switch off the electron flow very specifically (Chapter 5). At higher concentrations, ammonium was found to act as an uncoupler, since the ATP/ADP-ratio declined as well. The inhibitory short-term effect of ammonium at relatively low concentrations on electron transport to nitrogenase could be attributed to a decrease in $\Delta\Psi$. The results described in Chapters 4 and 5 therefore indicate that both in bacteroids and in *A.vinelandii*, the electron transport to nitrogenase is dependent on $\Delta\Psi$. In both organisms, a $\Delta\Psi$ value of >80 mV, interior negative, was found to be necessary for electron transport to nitrogenase. Especially small changes of $\Delta\Psi$ centered around 100 mV are accompanied by marked changes in the flow of reducing equivalents to nitrogenase. However, from these results it cannot be concluded that only a $\Delta\Psi$ > 80 mV is necessary for electron transport to nitrogenase; namely under the applied experimental conditions, there was always some ΔpH present. It remains plausible that $\Delta\tilde{\mu}_{H^+}$ is the driving force for reversed electron flow to nitrogenase, and that the activity of this system is regulated by the $\Delta\Psi$.

Fig. 1 visualizes one possible way as to how $\Delta\Psi$ can regulate the activity of a membrane-bound NAD(P)H flavodoxin or ferredoxin oxidoreductase. The driving force for the thermodynamically unfavourable reduction of flavodoxin or ferredoxin by NAD(P)H, *via* the proposed oxidoreductase, is supposed to be the $\Delta\tilde{\mu}_{H^+}$. In re-

spiring cells of *A.vinelandii* and bacteroids, the internal pH is maintained at a value of around 8.0. At this pH, the midpoint potential of the NAD(P)H/NAD(P)$^+$ redox couple is -350 mV ($E_m(1)$), whereas the midpoint potential of the flavodoxin hydroquinone/flavodoxin semiquinone redox couple is -495 mV ($E_m(2)$). A potential difference of at least 145 mV has to be bridged by the $\Delta\tilde{\mu}_H^+$ before flavodoxin is fully reduced. At equilibrium this can be formulated as follows:

$$E_m(2) = E_m(1) + n\,\Delta\tilde{\mu}_H^+$$

where n is the number of protons involved. It should be noted that this equation is also valid when ferredoxin instead of flavodoxin is supposed to be the ultimate electron donor for nitrogenase. Both in *Azotobacter*, and in bacteroids, the $\Delta\tilde{\mu}_H^+$ amounts approximately 150 mV. This implies that the influx of one proton is just sufficient to drive one electron from NAD(P)H to ferredoxin or flavodoxin. How the $\Delta\Psi$ can act as a regulator of the electron transport system to nitrogenase can be visualized as follows (Fig.1): Upon energization of the membrane by respiration, anionic sites are created at the inner site of the cytoplasmic membrane. Due to electrostatic interactions, the proposed flavodoxin or ferredoxin oxidoreductase could be forced to change its conformation. In this bended conformation, proton(s) can flow back through the enzyme system, and electron transfer occurs. In this way the $\Delta\Psi$, in combination with a proton influx, can perform work. It is beyond any doubt that energization of the membrane leads to a conformational change of certain membrane-bound or linked proteins. A well-known example of this phenomenon is the conformational change of the proton-translocating Mg^{2+}-ATPase upon energization [20]. The molecular mechanism is, however, still obscure. It should be noted that the model presented in Fig. 1 is based completely on the results obtained from studies performed with whole cells. To test the validity of this model, extensive studies would have to be performed with isolated membrane vesicles and isolated enzymes.

How can the effect of ammonium on electron transport to nitrogenase in *Azotobacter* and the absence of such an effect in bacteroids be explained? In the first place, it has been shown that (methyl)ammonium is taken up by *Azotobacter* but not by bacteroids. Furthermore, ammonium affects the $\Delta\Psi$ in *Azotobacter* and not in bacteroids (Chapter 5). These observations can be explained by the following model (Fig. 2). Once ammonium accumulates in the cell, it binds and neutralizes anionic sites induced by energization. This mechanism is based on the observation that quaternary ammonium compounds show an energy-dependent electrostatic

binding to negatively charged sites on the membrane [21]. In this way, ammonium can prevent the proposed conformational change of the flavodoxin or ferredoxin oxidoreductase and, thereby, also prevent electron transport to nitrogenase. Apart from the depolarizing effect of ammonium specifically on $\Delta\Psi$ (Fig. 2A), ammonium can also act as a classical uncoupler (Fig. 2B). This latter phenomenon is known from the literature [22] and occurs only at relatively high concentrations of ammonium.

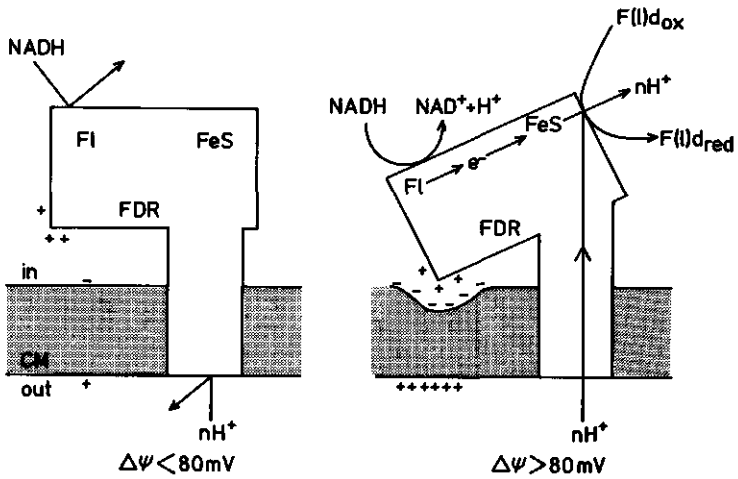

Figure 1. Proposal for electron transport to nitrogenase in *A.vinelandii* and bacteroids of *R.leguminosarum*. CM, cytoplasmic membrane; FDR, ferredoxin or flavodoxin oxidoreductase; Fl, flavin molecule; FeS, iron-sulphur center; $F(l)d_{ox}$, oxidized ferredoxin or flavodoxin semiquinone; $F(l)d_{red}$ reduced ferredoxin or flavodoxin hydroquinone; n, number of protons.

In Chapter 5 it was demonstrated that bacteroids do not accumulate (methyl)-ammonium. However, when ammonium is forced into the bacteroidal cell by prolonged incubation with ammonium under anaerobic conditions at $0^{o}C$, it is possible to inhibit the electron flow to nitrogenase. It appears that newly-fixed ammonium does not inhibit electron transport to nitrogenase in bacteroids, since it is excreted simply in response to the ΔpH across the membrane. Once ammonium is excreted to the outside of the cell, it can be assimilated by plant enzymes [23].

The depressing effect of ammonium on reversed electron flow to nitrogenase in *A.vinelandii* indicates that this organism possesses a very fine regulatory

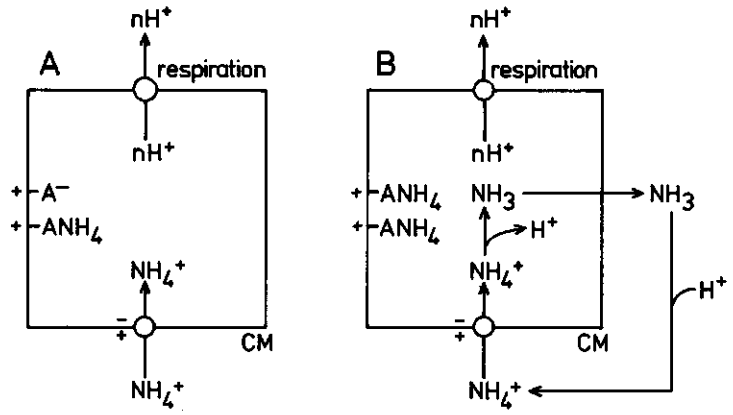

Figure 2. Proposal for the mode of action of ammonium on the energized state of the cytoplasmic membrane of *A.vinelandii*. CM, cytoplasmic membrane; A⁻, anionic sites on the membrane. (A) Depolarizing effect of ammonium; (B), depolarizing plus uncoupling effect of ammonium.

mechanism for rapidly switching on and off the energetically expensive nitrogenase reaction. By slightly lowering the $\Delta\Psi$ with ammonium, no functional reducing equivalents for nitrogenase are generated anymore, whereas essential metabolic processes are not influenced. Even the process of oxidative phosphorylation is only slightly influenced by ammonium concentrations that inhibit electron transport to nitrogenase completely. The observation that ammonium rapidly inhibits dinitrogen fixation in *A.vinelandii* is also found in other bacteria. Among these, are the photosynthetic bacteria such as *Rhodospirillum rubrum* [24], *Rhodopseudomonas capsulata* [25] and *Rps.palustris* [26]. In our opinion, the similarities of the short-term effect of ammonium on the nitrogenase activity of *Azotobacter* and photosynthetic bacteria, may suggest that the reducing power for dinitrogen fixation in photosynthetic bacteria, as in *A.vinelandii*, is generated by the $\Delta\tilde{\mu}_{H^+}$-driven reversed electron flow and regulated by $\Delta\Psi$ (see 1.2.2.2). At present work is in progress to investigate this possibility.

In addition to this short-term effect of ammonium, a so-called long-term effect of ammonium is also observed in most dinitrogen-fixing bacteria. The mechanism of this long-term effect of ammonium was recognized to be the repression of nitrogenase synthesis [27]. Dependent on the growth rate, the amount of nitrogenase decreases in the presence of ammonium. No short-term effect can be observ-

ed in fermentative dinitrogen-fixing bacteria such as *Klebsiella pneumoniae* [28] and *Clostridium pasteurianum* [29]. Since *C.pasteurianum*, like *Azotobacter*, contains an ammonium transport system [30], it seems reasonable to suggest that ammonium uptake by *C.pasteurianum* also results in a decrease in the $\Delta\Psi$. According to this view it might seem paradoxical that a lowered $\Delta\Psi$ inhibits dinitrogen fixation in *A.vinelandii*, but not in *C.pasteurianum*. However, in contrast to aerobes such as *Azotobacter*, reducing power for dinitrogen fixation in fermenting bacteria is generated on the substrate level; thus, without the involvement of $\Delta\Psi$ (see Chapter 1).

In cyanobacteria, the process of dinitrogen fixation is reserved to specialized cells, called heterocysts. The information concerning electron transport to nitrogenase in these cyanobacteria is considerable, but confusing. Recently, Haaker *et al.* reviewed the present state of knowledge and pointed out that the electron donating pathways for dinitrogen fixation found in isolated heterocysts or cell-free extracts are difficult to extrapolate to the situation *in vivo* [31]. Therefore, Haaker *et al.* has suggested that electron transport to nitrogenase in cyanobacteria might be a reversed electron flow driven by the $\Delta\tilde{\mu}_H^+$ [31]. A beneficial property of this alternative mechanism is, that it can operate equally well in the light or dark. In light $\Delta\tilde{\mu}_H^+$ is generated by cyclic electron flow and in the dark by respiration.

Last, but not least, the energetics of hydrogen oxidation by *A.vinelandii* should be discussed. It has been suggested that hydrogen oxidation via hydrogenase recovers some of the energy lost during wasteful production of hydrogen by nitrogenase [32]. In Chapter 3, evidence was presented supporting the idea that hydrogen oxidation provides ATP. In fact, the amount of ATP produced by hydrogen oxidation is in the same order of magnitude as produced by NADH-oxidation. However, Walker and Yates showed that the nitrogenase activity of *Azotobacter* is still low during hydrogen respiration [33]. We therefore proposed that hydrogen oxidation only recovers the ATP lost during wasteful production of hydrogen by nitrogenase and that hydrogen oxidation cannot generate enough reducing power for nitrogenase. It was suggested, for this reason, that reducing equivalents are generated at the level of NADH dehydrogenase. Since there are few experimental data about this subject more explanations can be offered. Firstly, it is uncertain whether or not hydrogen oxidation generates a $\Delta\Psi > 80$ mV. Secondly, hydrogen oxidation does not generate NAD(P)H. Therefore, the NAD(P)H concentration is probably low during hydrogen oxidation. Due to the absence of an electron donor (see Fig. 1), no electron transport to nitrogenase occurs, even when the $\Delta\Psi$ appears to be sufficient.

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SUMMARY

The central issue of this thesis is how obligate aerobes, such as *Rhizobium leguminosarum* bacteroids and *Azotobacter vinelandii*, generate and regulate the energy supply (in the form of ATP and reducing equivalents) for nitrogenase.

In an effective *Rhizobium*-legume symbiosis, the actual reduction of dinitrogen into ammonia is carried out in the root nodules by bacteroids. In order to study this intriguing process on the level of the bacteroids, it is clear that bacteroids have to be isolated from the root nodules. The first task to be achieved was, therefore, to develop an isolation procedure for bacteroids with a nitrogenase activity which is comparable with that found in whole plants. Chapter 2 describes such a procedure. The only difference in comparison with conventional procedures is that bacteroids were treated with fatty acid-free bovine serum albumin. By means of this extra treatment, the dinitrogen-fixing capacity of bacteroids could be improved considerably. Using these highly active bacteroids, an introductory survey was performed of the factors controlling nitrogenase activity in this organism. It was observed that aerobic dinitrogen fixation by pea nodule bacteroids in many respects resembles that of the free-living dinitrogen fixer *A.vinelandii*. As in *A.vinelandii*, dinitrogen fixation by bacteroids might be controlled by the intracellular ATP/ADP-ratio. Furthermore, evidence was found which suggests that, as in *A.vinelandii*, the energized state of the cytoplasmic membrane of bacteroids regulates the supply of reducing equivalents to nitrogenase. In addition, the dinitrogen-fixing system of bacteroids, as in *Azotobacter*, could be inhibited by free oxygen.

In aerobic organisms, membranes are energized by respiration *via* the electron transfer chain. Thus, the ultimate source of energy for the generation of a low potential reductant in *A.vinelandii* and bacteroids is provided exclusively by the respiratory chain. However, the so-called energized state of the membrane is a rather unmanageable term. Since the time, it has become known that respiration is accompanied by proton movement across the membrane; the energized state is recognized as a protonmotive force, and can be expressed in real physical parameters, such as $\Delta\Psi$ (membrane potential) and ΔpH (pH difference across the membrane) (Chapter 1). In Chapters 4 and 5, these parameters were measured under

dinitrogen-fixing conditions. It was observed that both in bacteroids (Chapter 4), as well as in *A.vinelandii* (Chapter 5), the flow of reducing equivalents to nitrogenase is regulated exclusively by the $\Delta\Psi$-component of the protonmotive force ($\Delta\tilde{\mu}_{H^+}$). At this stage of investigation it is difficult to describe a mechanism that explains the $\Delta\Psi$-induced formation of reducing equivalents, nevertheless, one attempt is presented in Chapter 6.

It has been known for some time that ammonium, when added to cultures of *A.vinelandii*, rapidly inhibits the nitrogenase activity. To date, no reasonable explanation has been given for this phenomenon. In Chapter 5, evidence is presented which shows that uptake of ammonium by *A.vinelandii* specifically switches off the flow of reducing equivalents to nitrogenase by lowering the $\Delta\Psi$. In contrast, ammonium had no effect on the rate of dinitrogen fixation by bacteroids. It has been demonstrated that bacteroids do not accumulate added ammonium, but on the contrary excrete the ammonium produced by the nitrogenase system (Chapter 5). Furthermore, it appears that newly-fixed ammonium is excreted in response simply to the ΔpH across the bacterial membrane.

In aerobes, the respiratory chain not only provides energy for the generation of reducing equivalents, but also for the synthesis of ATP *via* a process called oxidative phosphorylation. During this investigation it became apparent that, in comparison with the ATP-synthesizing system, more respiratory energy is necessary for the generation of reducing equivalents. The rate-limiting step in aerobic dinitrogen fixation therefore seems to be at the level of electron transport to nitrogenase. In Chapter 3, some factors controlling the efficiency of oxidative phosphorylation in *A.vinelandii* and bacteroids are considered. It was observed that these bacteria are capable to adjust their ATP-synthesizing capacity to the availability of oxygen in their environment. In this way, they are capable of producing ATP most efficiently when the ATP cost for dinitrogen fixation is maximal. In Chapter 6, the flexibility of the ATP-synthesizing system, which results from the considerable flexibility of the proton-translocating activity of the respiratory chain, is discussed.

It is generally accepted that the respiratory chain of *A.vinelandii* contains three proton-translocating sites. In Chapter 3 evidence is presented which suggests that protons are also translocated at the level of hydrogenase. This implies that *A.vinelandii* contains four instead of three proton-translocating sites. In contrast, no respiratory chain-linked hydrogenase could be detected in bacteroids of *R.leguminosarum*.

SAMENVATTING

Van alle bekende organismen zijn slechts een beperkt aantal micro-organismen in staat om het inerte stikstofgas uit de lucht via het enzym nitrogenase om te zetten, te fixeren, in een voor de plant bruikbare vorm van stikstof, ammoniak. Deze, voor de landbouw, zo belangrijke omzetting vindt echter alleen plaats, wanneer de cel voldoende energie kan produceren in de vorm van ATP en reductie-equivalenten. Daarnaast dient het stikstoffixerende systeem beschermd te worden tegen zuurstof inactivatie. Afhankelijk van het type micro-organisme zijn er verschillende manieren te onderscheiden waarop een bacterie aan deze metabole energievormen kan komen. Hoofdstuk 1 geeft hierover een algemeen overzicht. De rest van dit proefschrift spitst zich toe op de vraag, hoe in de aerobe stikstofbinders *Azotobacter vinelandii* en *Rhizobium leguminosarum* de benodigde hoeveelheden ATP en reductie-equivalenten voor het nitrogenase worden gegenereerd.

In tegenstelling tot de vrijlevende *Azotobacters* fixeren *Rhizobia* normaliter alleen stikstof in symbiose met een vlinderbloemige, zoals bijvoorbeeld de erwt. Het stikstoffixatie proces vindt plaats in the wortelknolletjes van de plant en wordt uitgevoerd door getransformeerde *Rhizobia* bacteriën, de *zgn.* bacteroïden. Het is duidelijk dat dit gecompliceerde proces het eenvoudigste bestudeerd kan worden aan geïsoleerde bacteroïden. In hoofdstuk 2 is beschreven hoe optimaal functionerende bacteroïden geïsoleerd kunnen worden uit de wortelknolletjes van de erwt. Een duidelijk verschil met de in de literatuur beschreven isolatieprocedure is, dat de bacteroïden nu met vetvrij runder serum albumine worden behandeld. Door deze extra behandeling kon het stikstoffixerend vermogen van bacteroïden aanzienlijk worden verhoogd. De aldus verkregen bacteroïden vertonen een stikstoffixerend vermogen dat vergelijkbaar is met de activiteit van bacteroïden in de hele plant. Gebruikmakend van deze zeer actieve bacteroïden werd onderzocht welke factoren de nitrogenase activiteit in dit organisme bepalen. De resultaten van experimenten welke beschreven zijn in hoofdstuk 2 laten duidelijk zien, dat het stikstoffixatie proces van bacteroïden in veel opzichten overeenkomt met dat van *A.vinelandii*. Net als in *Azotobacter* wordt de activiteit van het stikstoffixerende systeem in bacteroïden mogelijk bepaald door de intracellulaire ATP/ADP verhouding. Verder kon worden aangetoond, dat het electronentransport naar het

nitrogenase in bacteroïden op analoge wijze als in *A.vinelandii* wordt gereguleerd door de energisatiegraad van het cytoplasmatisch membraan. Voorts wordt het stikstofbindende systeem van bacteroïden, net als van *Azotobacter*, geremd door de aanwezigheid van teveel zuurstof.

Het is bekend, dat het cytoplasmatisch membraan in aerobe bacteriën alleen geënergiseerd wordt tijdens electronentransport via de ademhalingsketen. Nu is het begrip "energisatiegraad" een betrekkelijk vage term, welke nader omschreven dient te worden. Sedert de introductie van de chemiosmotische theorie is het mogelijk om de energisatiegraad van het membraan uit te drukken in de meetbare parameters $\Delta\Psi$ (membraan potentiaal) en ΔpH (pH verschil over het membraan). In de inleiding van dit proefschrift wordt deze theorie uitgebreid behandeld en in de hoofdstukken 4 en 5 zijn deze parameters onder stikstoffixerende condities gemeten. Zowel voor bacteroïden (hoofdstuk 4) als voor *A.vinelandii* (hoofdstuk 5) kon worden aangetoond, dat alleen de $\Delta\Psi$ component van de "protonmotive force" de toevoer van reductie-equivalenten naar het nitrogenase reguleert. Hoe men zich dat op moleculair niveau moet voorstellen is nog niet geheel duidelijk. Een mogelijk mechanisme wordt besproken in hoofdstuk 6. In dit hoofdstuk wordt tevens gespeculeerd over de mogelijkheid dat in fotosynthetische stikstofbinders de $\Delta\Psi$ betrokken is bij het electronentransport naar het nitrogenase.

Het is bekend, dat *A.vinelandii* direct stopt met stikstofbinden, indien ammonium aan het groeimedium wordt toegevoegd. Tot nu toe kon geen afdoende verklaring worden gegeven voor dit verschijnsel. De resultaten van experimenten, vermeld in hoofdstuk 5, laten echter zien dat het remmingsmechanisme van ammonium betrekkelijk simpel is. Er kon namelijk worden aangetoond, dat ammonium specifiek het electronentransport naar het nitrogenase remt doordat de actieve opname van ammonium door *A.vinelandii* gepaard gaat met een kleine verlaging van de $\Delta\Psi$. Op deze manier is *Azotobacter* dus in staat om de energetisch-kostbare nitrogenase reactie zeer specifiek uit te schakelen. Met andere woorden: zonder dat het onderhoudsmetabolisme van de cel wordt aangetast. Aan de andere kant bleek ammonium geen direct effect te hebben op de nitrogenase activiteit van bacteroïden. Aangetoond kon worden dat bacteroïden ammonium uitscheiden in plaats van opnemen (hoofdstuk 5). Verder bleek de mate van ammonium excretie af te hangen van de ΔpH over het membraan.

In aerobe stikstofbindende bacteriën levert de ademhalingsketen niet alleen energie voor de productie van reductie-equivalenten maar ook voor de synthese van ATP. Het laatstgenoemde proces staat bekend onder de naam: oxidatieve fosforylering. Uit de resultaten van verschillende experimenten, welke beschreven zijn in

dit proefschrift kon worden geconcludeerd, dat het genereren van reductie-equivalenten voor de bacterie lastiger is dan de synthese van ATP. De snelheidsbeperkende stap in het aerobe stikstoffixatie proces ligt dus waarschijnlijk op het niveau van het electronentransport naar het nitrogenase. In hoofdstuk 3 is onderzocht welke factoren de efficientie van het ATP synthetiserend systeem in *A.vinelandii* en bacteroïden bepalen. Ondubbelzinnig kon worden aangetoond, dat de activiteit van het ATP synthetiserend systeem in beide bacteriën voornamelijk bepaald wordt door de hoeveelheid zuurstof, die tijdens de groei wordt aangeboden. Op deze manier zijn ze in staat om ATP het meest efficient te produceren als het ATP-verbruik van het nitrogenase complex maximaal is. In hoofdstuk 6 wordt nader ingegaan op het feit, dat de flexibiliteit van het ATP synthetiserend systeem in deze bacteriën een gevolg lijkt te zijn van een aanzienlijke flexibiliteit wat betreft de protontranslocerende activiteit van de ademhalingsketen.

Tot nu toe werd aangenomen, dat de ademhalingsketen van *Azotobacter* drie protontranslocerende plaatsen bezit. In hoofdstuk 3 zijn aanwijzingen gevonden dat er nog een protontranslocerende plaats is op het niveau van hydrogenase. Daarentegen kon geen membraan gebonden hydrogenase worden aangetoond in bacteroïden van *R. leguminosarum*.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 28 november 1952 te Roermond. In 1970 behaalde hij het HBS-B diploma aan het Triniteits Lyceum te Haarlem. In hetzelfde jaar begon hij zijn studie scheikunde aan de Rijks Universiteit van Groningen. In 1973 werd het kandidaats examen behaald en in december 1976 het doctoraalexamen (hoofdvak biochemie, bijvak chemische oceanografie).

Vanaf 15 januari 1977 tot heden is de auteur werkzaam bij het laboratorium voor Biochemie van de Landbouwhogeschool te Wageningen. Eerst als promotie-assistent, vanaf 1 januari 1980 als wetenschappelijk medewerker.