AEROBIC NITROGEN FIXATION IN *AZOTOBACTER VINELANDII*

Dit proefschrift met stellingen van

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doctorandus in de Chemie, geboren op 24 juli 1946, te Badhoevedorp, is goedgekeurd door de promotor Dr. C. Veeger, hoogleraar in de Biochemie.

De Rector Magnificus van de Landbouwhogeschool,
J.P.H. van der Want

Wageningen, 17 december 1976.

H.B.C.M. Haaker

Aerobic nitrogen fixation in *Azotobacter vinelandii*

(with a summary in Dutch)

Proefschrift

ter verkrijging van de graad
van doctor in de Landbouwwetenschappen,
op gezag van de Rector Magnificus,
Dr. Ir. J.P.H. van der Want,
hoogleraar in de Virologie,
in het openbaar te verdedigen
op woensdag 16 februari 1977
des namiddags te vier uur in de Aula
van de Landbouwhogeschool te Wageningen

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STELLINGEN

1

Zonder het te beseffen laten Appleby *et al.* zien dat in *Rhizobium* bacteroïden het electronen transport naar de nitrogenase geremd wordt door een ontkoppelaar.

Appleby, C.A., Turner, G.L. and Macnicol, P.K. (1975) Biochim. Biophys. Acta, 387, 461-474.

2

De uitspraak dat de nitrogenase activiteit aanwezig in de wortelknolletjes van sojabonen gereguleerd wordt door ATP alsmede de energielading kan niet gedaan worden aan de hand van de gepresenteerde experimenten.

Ching, T.M. (1976) Life Sciences 18, 1071-1076.

3

De redoxpotentiaal van *Azotobacter vinelandii* $(4Fe\text{-}4S)_2$ ferredoxine I is ondanks de redoxpotentiaal metingen van Yoch en Arnon en Sweeny *et al.* nog steeds niet bepaald.

Yoch, D.C. and Arnon, D.I. (1972) J. Biol. Chem. 247, 4514-4520.

Sweeny, W.V., Rabinowitz, J.C. and Yoch, D.C. (1975) J. Biol. Chem. 250, 7842-7847.

4.

De door Abramovitz en Massey aangedragen experimenten leveren onvoldoende bewijs voor het bestaan van een charge-transfer complex tussen old yellow enzyme en fenolen.

Abramovitz, A.S. and Massey, V. (1976) J. Biol. Chem. 251, 5327-5336.

5

De opvatting van Edmondson en Tollin, dat de ionisatie toestand van het aan flavodoxine gebonden flavine hydrochinon bepaalt of volledige reductie van flavodoxine kan worden verkregen of niet, is niet bewezen.

Edmonson, D. E. and Tollin, G. (1971) Biochemistry 10, 133-145.

6

Song heeft zich de beperkingen van de gebruikte rekenmethode om de ligging van triplet niveaus te voorspellen niet gerealiseerd.

Song, P.S. (1969) J. Phys. Chem. 72, 536-542.

7

De verklaring gegeven door Dénairié *et al.* voor het feit dat adenine-behoeftige mutanten van *Rhizobium* zich niet tot stikstofbindende bacteroïden ontwikkelen, is hoogst speculatief.

Dénarié, J., Truchet, G. and Bergeron, B.: In: Symbiotic nitrogenfixation in plants (P.S. Nutman, ed.) 47-61 Cambridge University Press, Cambridge 1976.

8

Het invoeren van kabel-t.v. kan de democratisering op plaatselijk niveau sterk bevorderen en het ware gezicht van plaatselijke hoogwaardigheidsbekleders duidelijk doen uitkomen.

9

De huidige mogelijkheid rente onbeperkt aftrekbaar te doen zijn van het belastbaar inkomen is onrechtvaardig en werkt denivellerend.

Proefschrift van H. Haaker

Voor niets gaat de zon op.

Aan Ursula
mijn ouders
Maruscha
Mischa

Voorwoord

Het werk beschreven in dit proefschrift is uitgevoerd in het Laboratorium voor Biochemie van de Landbouwhogeschool te Wageningen en mogelijk gemaakt door financiële steun van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.).

Iedereen zal begrijpen dat zonder de directe hulp van velen ik niet instaat zou zijn geweest dit proefschrift voort te brengen.

Om het risico te vermijden, dat ik iemand zou vergeten te noemen, wil ik volstaan met bij deze iedereen hartelijk te bedanken, die heeft bijgedragen tot de verwezenlijking van dit proefschrift.

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Contents

List of abbreviations

ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
CoA	coenzyme A
DEAE	diethylaminoethyl
EDTA	ethylene-diaminetetra-acetate
NAD^+	oxidized nicotinamide-adenine dinucleotide
NADH	reduced nicotinamide-adenine dinulceotide
$NADP^+$	oxidized nicotiamide-adenine dinucleotide phosphate
NADPH	reduced nicotiamide-adenine dinucleotide phosphate
P	phosphate residues
Tes	N-tris[hydroxymethyl] methyl-2-amino ethane
TPP	thiamine pyrophosphate
tricine	N-[tris(hydroxymethyl)-methyl]-glycin
tris	tris[hydroxymethyl]-aminomethane

List of enzymes

In this thesis the non-systematic names of enzymes are used. This list includes the trivial and the systematic names of the enzymes; also is included the enzyme number according to the Report of the Commission for Enzymes of the International Union of Biochemistry.

EC number	Systematic name	Trivial name
3.6.1.3	ATP phosphohydrolase	ATPase
4.1.2.14	3-deoxy-2-keto-6-phosphogluconate glyceraldehyde-3-phosphate-lyase	
1.1.3.4	β-D-glucose: oxygen 1-oxido-reductase	glucose oxidase
1.1.1.49	D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase	glucose-6-phosphate dehydrogenase
1.11.1.6	hydrogen-peroxide: hydrogen peroxide oxidoreductase	catalase
1.1.1.44	6-phospho-D-gluconate: $NADP^+$ 2-oxidoreductase (decarboxylating)	6-phosphogluconate dehydrogenase
4.2.1.12	6-phosphogluconate hydro-lyase	
1.2.4.1	pyruvate: lipoate oxido-reductase (acceptor acylating)	pyruvate dehydrogenase complex
1.6.1.1	Reduced-$NADP^+$: NAD^+ oxido-reductase	$NAD(P)^+$ transhydrogenase
		nitrogenase

Introduction

GENERAL INFORMATION ABOUT NITROGEN FIXATION

The reduction of molecular nitrogen to ammonia has long been known to be a property of microorganisms present in the root nodules of legumenes. Hellriegel & Withfarth (1) were the first ones to demonstrate biological nitrogen fixation back in 1880, but in the following 60 years only four organisms were shown to carry out this reaction: the free-living bacteria *Azotobacter spp* and *Clostridium spp*; the obligatory symbiotic *Rhizobium spp*-legume associations and the blue-green algae *Nostoc*. In the past 20 years there has been a great upsurge of research into many aspects of nitrogen fixation, a change that has been due largely to the development of new methods for measuring the process. In the early studies, nitrogen reduction was detected by using the insensitive Kjehldahl method to measure the total nitrogen content of microorganisms or plants before and after growth in media in which the sole source of nitrogen was molecular nitrogen. The introduction by Burris and Miller in 1942 of isotopic methods provided a more sensitive and convenient way of following the reaction. Work on the biochemistry of the process started, following the demonstration in 1960 that cell-free extracts of *Clostridium pasteurianum* produce ammonium ions from nitrogen (2).

The enzyme responsible for the catalysis of nitrogen reduction to ammonia has been termed nitrogenase. The discovery that the enzyme will also reduce acetylene to ethylene, a compound which can rapidly and sensitively determined by gas chromatographic techniques, ushered in the explosion of research on nitrogen fixation that exists at the present time. A historical review of the developments in the field between 1920 and 1970 has been written by Burris(3).

Nitrogen fixation research can roughly be devided into the following directions. Recent reviews, books or articles are given.

a. biology	(6,7)
b. molecular biology	(8-10)
c. enzymology	(11-15)
d. physiology	(14-17)
e. chemical nitrogen fixation	(14,15,18,19)

INTRODUCTION TO AEROBIC NITROGEN FIXATION

The main aim of this thesis is to describe how obligate aerobes such as *Azotobacter vinelandii*, are able to carry out the very reductive process of nitrogen fixation in an aerobic environment. The problem is divided into two parts: 1) Generation of reducing equivalents for nitrogenase; 2) protection of nitrogenase against inactivation by oxygen.

1. GENERATION OF REDUCING EQUIVALENTS

In anaerobic nitrogen-fixing organisms reduced ferredoxin is the physiological electron donor for nitrogenase. Ferredoxin can be reduced in different ways: by pyruvate in the so-called phosphoroclastic reaction, an activity which has been detected in *Clostridium pasteurianum* (20), *Bacillus polymyxa*(21), *Klebsiella pneumoniae*(22); and by other strong reducing agents such as H_2 or formate (22,23).

Ferredoxin is also the electron donor for nitrogenase in photosynthetic bacteria and blue-green algae, the ferredoxin in turn being reduced in a light reaction with photosystem I or by pyruvate and pyruvate dehydrogenase. It was shown that NADPH via ferredoxin:$NADP^+$ oxidoreductase, which enzyme was demonstrated to be present in these organisms and ferredoxin can act as an electron donor for nitrogenase (24-28).

Although low potential electron carriers which will react with nitrogenase have been isolated from aerobic organisms (20,29), the physiological electron donor and the ultimate source of reducing equivalents for N_2 reduction in such organisms as *Azotobacter* spp, *Mycobacterium flavum* and *Rhizobium* spp is still unknown. Ferredoxins have been isolated from *Azotobacter* (31), *Rhizobium* bacteriods (32) and *Mycobacterium flavum*. The low potential flavoprotein electron carrier, flavodoxin, has been isolated from *Azotobacter* (34,35) and *Rhizobium* bacteriods (36,37). Both types of carriers were found to mediate the transfer of electrons from illuminated spinach chloroplasts to a nitrogenase preparation. Much lower activities are found when the reducing equivalents are obtained from oxidizable organic compounds, such as glucose-6-phosphate or isocitrate and coupled to nitrogenase through $NADP^+$, spinach ferredoxin-$NADP^+$ oxidoreductase and ferredoxin.

Enzymes which couple the oxidation of pyruvate or NAD(P)H to the reduction of ferredoxin or flavodoxin have not yet been detected in obligate aerobic nitrogen-fixing organisms. Nevertheless an electron transport chain to

nitrogenase in *Azotobacter vinelandii* or *Rhizobium* bacteriods has been postulated (29,30). It was proposed that a high NADPH/$NADP^+$ ratio provides the reducing power and that reducing equivalents from NADPH are transfered to nitrogenase via a heat-labile factor, ferredoxin and flavodoxin (29). In paper I however it will be shown that in intact *Azotobacter vinelandii* electron transport to nitrogenase is regulated not by the NAD(P)H/$NAD(P)^+$ ratio but by the energized state of the cytoplasmic membrane. This conclusion suggests that the electron transport chain to the nitrogenase may be localized within the cytoplasmic membrane. In paper III it will be shown that an intact cytoplasmic membrane is necessary, for coupling between pyruvate oxidation and nitrogenase activity. In addition it will be shown, that reduced flavodoxin can act as a reductant for nitrogenase and that reduced ferredoxin cannot. Furthermore it will be shown that flavodoxin in intact structures from the cell is not soluble, but is more-or-less buried within the membrane.

In paper IV the presence of a membrane-bound NAD(P)H: flavodoxin oxidoreductase is demonstrated. In that paper the role of the cytoplasmic membrane in electron donation to nitrogenase of *Azotobacter* is summarized.

2. OXYGEN PROTECTION OF NITROGENASE

Nitrogen fixation is an anaerobic process; the arguments for this postulate are reviewed by Yates and Jones (38). For aerobically nitrogen-fixing organisms several protection mechanisms against oxygen have been proposed.

1. In nodules of legumes, leghemoglobin plays an important role in nitrogen fixation. a. Leghemoglobin has a high affinity for oxygen (39). This facilitates a high flux of oxygen, at a low concentration, to the bacteroids. b. Oxyleghemoglobin increases the efficiency of oxidative phosphorylation in intact bacteroids (40,41).
2. Most blue-green algae which fix N_2 in air possess characteristic cells, called heterocysts. It is thought that this type of cell protects the nitrogenase against oxygen in the following ways. a. The thick-wall of the heterocysts presents a physical barrier for oxygen (42). b. The absence of the oxygen-evolving photosystem II in heterocysts (43), together with a high respiratory activity in the heterocyst (44) may aid in keeping the oxygen concentration low.

3. Free living bacteria which fix N_2 in air also have protecting mechanisms against oxygen. Slime production may play a role in reducing the diffusion of O_2 to the inside of the bacteria (45,46). In addition respiratory as well as conformational protection mechanisms have been proposed (47).

a. RESPIRATION PROTECTION

It was observed that when *Azotobacter* was grown at a low oxygen input, the cells were not able to fix nitrogen immediately after an increase of the oxygen input. Following a period of adaptation, however, in which the oxidation velocity increased, the cells were able to fix nitrogen. This phenomenon was called respiration protection of nitrogenase. Beside the influence of oxygen during growth on the capacity to fix nitrogen at a high oxygen input, other effects of oxygen on the physiology of *Azotobacter* are known. When *Azotobacter* cells are switched from low to high aeration, the following phenomenon are observed.

1. The whole cell respiration increases (48,49).
2. The ratio N_2 fixed/carbon source oxidized decreases (48).
3. The composition of the respiratory chain changes to give higher levels of cytochromes and NAD(P)H oxidase activity (48,49).
4. The oxidative phosphorylation efficiency at site I and III decreases (49).

These data were used in proposals to clarify the nature of respiration protection in *Azotobacter* (38,49,50). Under highly aerobic conditions nitrogenase is inhibited and respiration is promoted via an electron pathway to oxygen that has a low phosphorylation efficiency. These events cause a loss of respiratory control associated with the NADH oxidation and produce a decreased ratio of ATP/ADP.P_i in whole cells. Since key enzymes in *A.vinelandii* catabolism are stimulated by ADP and AMP or inhibited by ATP, the expected lower intracellular energy charge would lead to the observed increase in whole cell respiration.

Paper II deals mainly with the question of respiration protection. In that paper it will be shown that the proposed hypothesis (50) is not correct. The composition of the respiratory chain do not show a correlation with the intracellular energy charge. It is also possible to show that *Azotobacter* has respiration protection even when the respiratory membranes are not adapted to high oxygen concentrations. The experiments indicate that the increase of

whole cell respiration in increased oxygen input during growth is due mainly to an increase in sugar uptake and not due to a change in intracellular energy charge or the ratio of $NAD(P)H/NAD(P)^+$.

b. CONFORMATIONAL PROTECTION

It has long been known that nitrogenase in crude extracts of *Azotobacter* is oxygen-tolerant whereas in crude extracts from anaerobic nitrogen fixers, the enzyme is oxygen-sensitive (51,52). All purified nitrogenases, that have been tested sofar, are oxygen sensitive. Yates (53) showed that DEAE-cellulose-purified nitrogenase becomes oxygen sensitive, and showed further that a crude preparation with NADH dehydrogenase activity, can restore the oxygen stability of purified enzyme. Oppenheim et al. (54) showed that nitrogenase obtained from *A. vinelandii* by an osmitic shock was free from membranes with NADH oxidase activity and more oxygen-sensitive than nitrogenase obtained by disrupting the cells with a French pressure cell. Their results suggest that the cytoplasmic membrane is in some way concerned with the oxygen protection. Paper III presents results which indicate that nitrogenase in *Azotobacter* is always protected against oxygen, independent on the cell rupture method. Purification of the nitrogenase complex till DEAE cellulose chromatography gives an oxygen-stable nitrogenase complex. Following DEAE-cellulose-treatment nitrogenase is found to be oxygen-sensitive and it can not be protected against oxygen by cytoplasmic membranes which have high NADH dehydrogenase activity. Paper IV shows that the rate of sedimentation of nitrogenase is independent of the cell rupture method and is the same as the soluble pyruvate dehydrogenase complex. It is also demonstrated that a Fe-S protein stabilizes within the crude nitrogenase complex against oxygen inactivation.

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I Regulation of dinitrogen fixation in intact *Azotobacter vinelandii*

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SUMMARY

1. In intact *Azotobacter vinelandii* the influence of oxygen on the levels of oxidized nicotinamide adenine dinucleotides and adenine nucleotides in relation to nitrogenase activity was investigated.

2. The hypothesis that a high (NADH+NADPH)/(NAD^++$NADP^+$) is the driving force for the transport of reducing equivalents to nitrogenase in intact *A. vinelandii* was found to be invalid. On the contrary, with a decreasing ratio of reduced to oxidized pyridine nucleotides, the nitrogenase activity of the whole cells increases.

3. By measuring oxidative phosphorylation and using 9-amino acridine as a fluorescent probe, it could be demonstrated that respiration-coupled transport of reducing equivalents to the nitrogenase requires a high energy level of the plasma membrane or possibly coupled to it, a high pH gradient over the cytoplasmic membrane. Furthermore nitrogen fixation is controlled by the presence of oxygen and the ATP/ADP ratio.

INTRODUCTION

Since Mortenson [1] showed how reducing equivalents are transfered to the nitrogenase of *Clostridium pasteurianum*, it is generally accepted that all anaerobic or photosynthetic nitrogen-fixing organisms use reduced ferredoxin as electron donor for dinitrogen reduction. In these organisms ferredoxin is reduced by respectively the phosphoroclastic and photoreceptor systems. Since that time many investigators searched for the physiological electron donor in aerobic dinitrogen fixers [2–4]. In the current concept, proposed by Benemann et al. [5, 6], a high NADPH/$NADP^+$ ratio is the driving force for the transfer of electrons through an electron carrier chain, thus obtaining the low-potential donor system needed to reduce Component II of the nitrogenase. Benemann et al. [5] in their experiments used cell-free extracts supplemented with isolated electron carriers.

This proposal was tested in intact bacteria by measuring the amounts of NAD^+

Abbreviations: TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazol; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide.

and $NADP^+$ in relation to the nitrogenase activity under a variety of conditions. The relation between nitrogenase activity and energy level of the cytoplasmic membrane was simultaneously studied.

METHODS

Growth conditions of the bacteria

Azotobacter vinelandii ATCC Strain OP was grown on a Burk's nitrogen-free basic salt medium [7]. The sugar oxidation of *Azotobacter* depends strongly upon the oxygen input during growth [8]. During this investigation bacteria grown in batch cultures were used. The cultures became oxygen-limited during growth. Bacteria grown oxygen-limited had a low rate of sucrose oxidation (0.1–0.5 μmoles oxygen $\cdot$ $min^{-1} \cdot mg^{-1}$ protein), but a high rate of acetate oxidation (1 μmole oxygen $\cdot$ $min^{-1} \cdot mg^{-1}$ protein). These cells, refered to as "oxygen-limited" cells were not able to reduce dinitrogen with oxygen present in the medium, with sucrose as carbon source. This type of cell can be converted into "oxygen-adapted" cells by exposing them to oxygen in the medium for a period of 3 h. This was done with sucrose as carbon source, in a New Brunswick Type C30 growth vessel, supplied with a standard New Brunswick oxygen probe. During the adaptation the sulphite oxidation rate was 110 mmoles oxygen $\cdot$ $l^{-1} \cdot h^{-1}$ at a bacterial protein concentration of 0.5 mg $\cdot$ ml^{-1}. "Oxygen-adapted" cells had a rate of sucrose oxidation of 0.8–1.3 μmoles oxygen $\cdot$ $min^{-1} \cdot mg^{-1}$ protein (cf. ref. 9).

The cells were harvested at 20 °C and then washed twice with distilled water; the cells were suspended in water at a protein concentration of 30–60 mg/ml. Each batch was tested for sucrose oxidation and dinitrogen reduction activity. The maximum oxidation velocity of a cell suspension and the influence of additions on the oxygen consumption were measured with an oscillating platinum electrode from Aminco Instruments Company. In all experiments the temperature was 24 °C.

Nitrogenase activity assay

The standard nitrogenase activity incubation mixture contained: 25 mM Tris–HCl, 5 mM phosphate, 2 mM EDTA, final pH 7.6. At $t = 0$ the cells were added to the standard incubation mixture in the cuvette and after 2 min 3 mM $MgCl_2$. A closed gas phase consisting of 70 % argon, 10 % acetylene and 20 % oxygen was pumped with an adjustable airpump through the incubation mixture. When necessary, the oxygen and argon concentrations were varied. The total volume of the gas phase varied between 100 and 160 ml, in order to limit the decline in oxygen consumption of the gas phase to less than 1 %. The oxygen concentration in the incubation medium was measured with a standard galvanic oxygen electrode and oxygen analyser of the New Brunswick Scientific Company. In all experiments the oxygen concentration in the medium was measured. At suitable time intervals aliquots were removed from the gas phase to analyze for acetylene reduction with a Pye 104 gas chromatograph on a porapack R column.

Oxygen input assay

The oxygen input into the reaction cuvette was either determined according to Cooper [10] or calculated from the oxidation time of a known amount of dithionite.

For this determination the reaction cuvette contained a mixture of 25 mM Tris–HCl (pH 7.6), 0.1 mM benzylviologen, 40 μg/ml catalase (Boehringer). The volume varied between 4 and 10 ml. A known amount of neutralized dithionite was added, and the benzylviologen coloured the solution blue. The gas was bubbled through the solution till the blue colour disappeared. Then a further known amount of dithionite was added and the time of de-colourization measured. It was checked that in the ranges used, the oxygen input was linear with the partial oxygen tension in the gas phase and the gas flow through the cuvette.

Determination of cofactors

The cells were fixed by rapidly adding $HClO_4$ to the incubation mixture up to a final concentration of 4 % (w/v). After 10 min at 0 °C the samples were neutralized with solid $KHCO_3$ and stored at −20 °C. The levels of ATP, ADP, AMP, NAD^+ and $NADP^+$ were determined according to Williamson and Corkey [11]. Control experiments show that no non-enzymatic breakdown occurs under these conditions (less than 5 %). Within 3 h NAD^+ and $NADP^+$ were determined; ATP, ADP and AMP within 30 h. Enzymatic assays were performed with an Aminco–Chance dual wavelength spectrophotometer.

Fluorescence assays

The total reduced pyridine nucleotide fluorescence was measured by placing the cuvette in an Eppendorf fluorimeter (filters: excitation, 313 and 366 nm; emission, an interference filter of 460 nm). By measuring the excitation and emission spectra of the oxidized and reduced cells, it was checked that the fluorescence changes were due to reduced pyridine nucleotides. In these experiments the oxygen input was changed by variation of the oxygen tension in the gas phase by a constant gas flow through the incubation mixture. The 9-aminoacridine fluorescence was measured with a primary filter 405+436 nm and a secondary filter of 500−3000 nm. Because the fluorescence of oxidized flavoproteins (transhydrogenase, lipoamide dehydrogenase) was also detected with the filter combination used, the changes in the bacteriological flavin fluorescence were registrated by experiments without the dye 9-aminoacridine. Where necessary the fluorescence emission was corrected as indicated. The fluorescence emission spectrum of 9-aminoacridine in the energized system was identical with that of the non-energized system. The difference was the much higher quantum yield in the energized system.

Protein was determined with the biuret method as modified by Cleland and Slater [12].

Chemicals and gasses

Acetylene and ethylene were purchased from Matheson, argon and oxygen from Loosco Amsterdam, 9-aminoacridine from Fluka and 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) from Sigma. 4,5,6,7-Tetrachloro-2-trifluoromethylbenzimidazol (TTFB) was a gift from Dr R. B. Beechey, Woodstock Agricultural Research Centre, Sittingbourne, Kent (U.K.).

RESULTS

NAD^+, $NADP^+$ levels and nitrogenase activity

Fig. 1 shows the effect of an increasing oxygen input on the levels of NADH

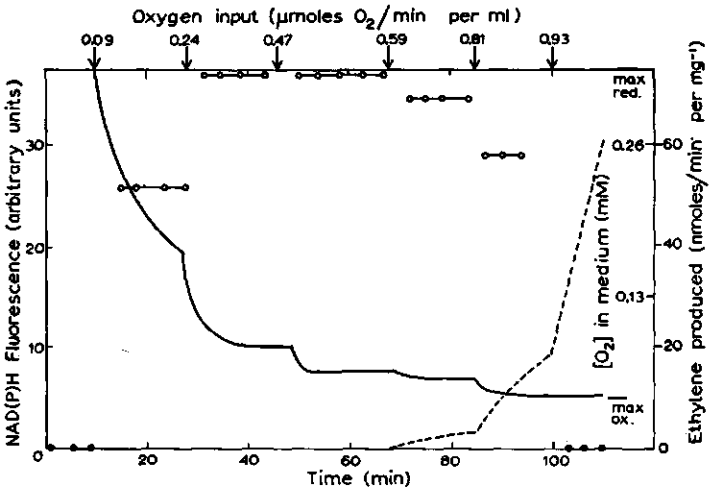

Fig. 1. The effect of increasing oxygen input on the NAD(P)H level and nitrogenase of whole *A. vinelandii* cells. "Oxygen-adapted" cells (0.42 mg/ml; sucrose oxidation, 1.3 μmoles oxygen · min^{-1} · mg^{-1}) were suspended in 7 ml standard incubation mixture with sucrose (20 mM) as substrate. 10 min after constant rates of acetylene reduction and oxygen consumption were reached, the oxygen input into the reaction vessel was changed by altering the oxygen concentration in the gas phase as indicated. At the end of the experiment the lowest (max. ox.) and highest (max. red.) possible level of NAD(P)H in the cells were determined by flushing with pure oxygen or argon, respectively. ———, NAD(P)H fluorescence; ○–○, acetylene reduction; – – –, oxygen input.

plus NADPH compared with nitrogenase activity of whole *A. vinelandii* cells. This figure shows clearly that at a higher oxygen input into the cell suspension, the amount of intracellular NADH plus NADPH decreases. Even at a low NADH plus NADPH level the cells are able to reduce acetylene. When the oxygen input into the cell suspension increases beyond the maximum oxidation capacity, oxygen becomes detectable in the medium and with a higher oxygen concentration in the medium the nitrogenase switches off, using the terminology of Dalton and Postgate [13]. With fluorimetric methods one cannot discriminate between NADH and NADPH levels.

The acid-stop method was used to determine the levels of the oxidized pyridine nucleotides and adenine nucleotides in separate experiments with varying oxygen input (Fig. 2). This figure clearly demonstrates that in whole bacteria the amounts of NAD^+ and $NADP^+$ increase with increased oxygen input. Thus under these conditions the intrabacterial ratios NADH/NAD^+ and NADPH/$NADP^+$ decrease parallel with increased nitrogen-fixing activity of the whole cells. Between an oxygen input of 0.14 and 0.54 μmoles oxygen · min^{-1} · ml^{-1} incubation mixture there is an increase of 300 % in nitrogenase activity, but the ratio NADPH/$NADP^+$ decreases from the maximum to almost the minimum value. In the same oxygen-input traject the intracellular ATP concentration increases from approx. 1.1 to 1.8 mM, assuming 5–6 μl intracellular water per mg protein (cf. ref. 14). Since no oxygen was detectable in the medium, it means that oxygen was used totally by the respiratory system(s) of the bacteria.

The flow rate of electrons through the respiratory chain can be influenced by a number of inhibitors such as HQNO (refs 15–17) and CN^- (ref. 18). HQNO alone

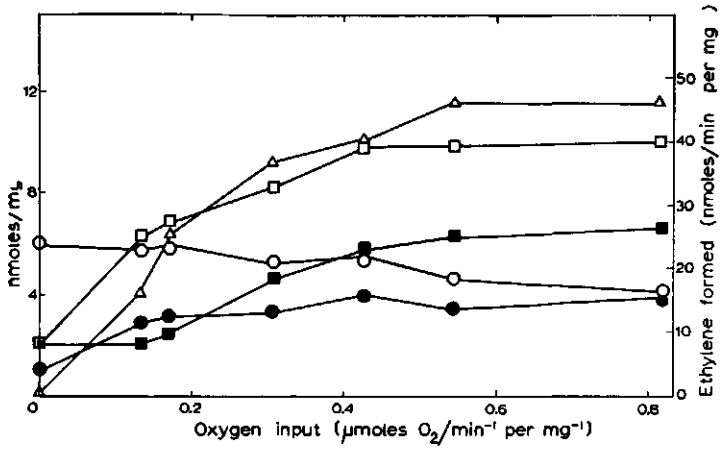

Fig. 2. Levels of ATP, ADP, NAD^+, $NADP^+$ and nitrogenase activity in intact cells of *A. vinelandii* with different oxygen supply. "Oxygen-adapted" cells (0.8 mg/ml; sucrose oxidation; 1.1 μmoles oxygen · min^{-1} · mg^{-1}) were suspended in 6 ml standard incubation mixture with sucrose (20 mM) as substrate. 10 min after constant rates of acetylene reduction and oxygen consumption were reached at the oxygen input as indicated, the total incubation mixture was fixed with $HClO_4$. After neutralization the cell extract was analyzed. □–□, ATP; ○–○, ADP; ●–●, NAD^+; ■–■, $NADP^+$ ×10; △–△, nitrogenase activity.

inhibits the sucrose oxidation only at high concentrations, 50 % at a concentration of 100 μM. Low concentrations of CN^- (10 μM) enhance the inhibitory effect of HQNO, for instance 85 % inhibition at 10 μM HQNO plus 10 μM CN^-. The inhibition by HQNO and CN^- (refs 18, 19) as observed with isolated membrane vesicles of *A. vinelandii*, is the same as observed here with intact bacteria. Due to the strong inhibition by the combined action of HQNO plus CN^-, it was impossible to control the rate of oxidation by the cells. Therefore high concentrations HQNO without CN^-

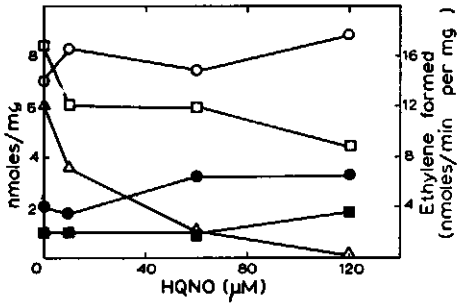

Fig. 3. The effect of HQNO on ATP, ADP, NAD^+, $NADP^+$ levels and nitrogenase activity of intact *A. vinelandii* cells under constant oxygen supply. "Oxygen-limited" cells (1.4 mg/ml; sucrose oxidation, 0.19 μmoles oxygen · min^{-1} · mg^{-1}) were suspended in 10 ml standard incubation mixture with sucrose (20 mM) as substrate. The oxygen input was 0.15 μmoles oxygen · min^{-1} · ml^{-1}. The maximal oxygen consumption was 0.26 μmoles oxygen · min^{-1} · ml^{-1}. 10 min after constant rates of acetylene reduction and oxygen consumption were reached at the HQNO concentrations as indicated. the total incubation was fixed with $HClO_4$ and neutralized. □–□, ATP; ○–○, ADP; ●–●, NAD^+; ■–■, $NADP^+$ ×10; △–△, nitrogenase activity.

TABLE I

INFLUENCE OF UNCOUPLER TTFB ON ATP, ADP AND AMP LEVELS IN *A. VINELANDII* CELLS OXIDIZING SUCROSE AND ACETATE

"Oxygen-adapted" cells (4.5 mg/ml; sucrose oxidation, 0.82 μmoles oxygen · min^{-1} · mg^{-1}) were suspended in 4 ml standard incubation mixture with sucrose (20 mM) or acetate (20 mM). 10 min after a constant rate of oxygen consumption as indicated was reached, the total incubation was fixed with $HClO_4$. After neutralization the cell extract was analyzed.

Substrate	TTFB (μM)	Oxydation velocity (μmoles oxygen·min^{-1}·mg^{-1})	ATP (nmoles·mg^{-1})	ADP (nmoles·mg^{-1})	AMP (nmoles·mg^{-1})	Sum of adenine nucleotides (nmoles·mg^{-1})
Sucrose	0	0.82	8.0	4.7	2.8	15.5
Sucrose	1	0.83	8.5	4.1	4.4	17.0
Sucrose	2	0.08	0.1	2.6	11.1	13.8
Acetate	0	1.42	8.2	4.6	4.2	17.0
Acetate	1	1.25	8.0	3.9	5.1	17.0
Acetate	2	1.28	7.7	3.8	5.5	17.0

TABLE II

EFFECT OF OXYGEN AND UNCOUPLER ON ATP, ADP, AMP, NAD^+, $NADP^+$ AND NITROGENASE ACTIVITY OF WHOLE *A. VINELANDII* CELLS

"Oxygen-limited" cells (1 mg/ml; sucrose oxidation, 0.16 μmoles oxygen · min^{-1} · mg^{-1}) were suspended in 6 ml standard incubation mixture containing acetate (20 mM). 10 min after constant rates of acetylene reduction and oxygen consumption were reached at the oxygen input as indicated, the total incubation was fixed with $HClO_4$. After neutralization the cell extract was analyzed.

Oxygen input (μmoles oxygen · min^{-1})	Maximum oxidation velocity (μmoles oxygen · min^{-1} · mg^{-1})	TTFB (μM)	ATP (nmoles · mg^{-1})	ADP (nmoles · mg^{-1})	AMP (nmoles · mg^{-1})	Sum of adenine nucleotides (nmoles · mg^{-1})	NAD^+ (nmoles · mg^{-1})	$NADP^+$ (nmoles · mg^{-1})	Nitrogenase activity (percentage of maximum activity*)
0	1.02	0	1.7	7.3	24.3	33.3	0.7	0.07	0
0.35	1.02	0	13.5	8.7	9.4	31.6	5.5	0.28	97
1.21**	1.02	0	17.9	8.3	8.0	34.2	8.3	0.80	0
0.43	1.02	2	13.9	9.4	12.1	35.4	6.8	0.21	56
0.43	1.00	3	12.5	10.1	9.4	32.0	6.8	0.21	14
0.43	0.99	4	13.3	8.3	12.5	34.1	6.6	0.35	3

* Maximum nitrogenase activity 34.6 nmoles ethylene formed · min^{-1} · mg^{-1}.

** "Switch off" condition (cf. ref. 13).

were used to study the influence of inhibition of the respiration on the ATP, ADP, NAD^+ and $NADP^+$ levels and the nitrogenase activity (Fig. 3). At a constant oxygen input, inhibition of the electron flow affects the nitrogenase activity considerably, while the ATP and ADP levels are much less influenced. Under the different conditions decribed in Fig. 3 no oxygen was detectable in the medium. Therefore the decrease in nitrogenase activity could not be due to "switching off conditions" (cf. ref. 13).

Influence of uncoupler on nitrogenase activity

In the obligate aerobe *A. vinelandii* sugar is not taken up with group translocation, but is associated with the oxidatively energized cytoplasmic membrane [20]. It is clear that de-energization of the cytoplasmic membrane by uncoupler TTFB [21], has a direct effect on the sucrose and therefore on the oxygen uptake and intracellular ATP concentration (Table I). The same phenomenon has been observed by Postma [22] with the uptake and oxidation of succinate by *A. vinelandii*. The uptake of acetate is not energy-linked [23], thus an uncoupler will have less effect on the rate of oxidation of acetate. From the data presented in Table I it is clear that the acetate oxidation by the cells is not influenced at the concentrations of the uncoupler TTFB used. Table I also shows that the steady-state levels of ATP, ADP and AMP of cells which oxidize acetate are much less influenced by the uncoupler than those which oxidize sucrose. Therefore acetate was used as substrate to test the effect of low concentrations of uncoupler on the nitrogenase activity of whole cells. The results are shown in Table II. Notwithstanding the ATP and ADP levels are more or less constant, a dramatic decrease in nitrogenase activity with increasing uncoupler concentration is observed at a constant oxygen input. Since the concentrations of ATP and ADP, being substrate and inhibitor of the nitrogenase respectively, are practically constant, it means that at the saturating concentrations of acetylene used, the flow of reducing equivalents to the nitrogenase is inhibited by uncoupler.

Furthermore the experiments show that this inhibition is accompanied by relative small changes of the $NADPH/NADP^+$ and $NADH/NAD^+$ ratios. The uncoupler, whose only known effect is to lower the energized state of the cytoplasmic membrane, inhibits the transfer of reducing equivalents indicating a direct relation between nitrogenase activity and energized state of the cytoplasmic membrane. Control experiments have shown in accordance with previous work of Hardy et al. [24] that in the concentration range used uncoupler has no effect on the nitrogenase activity with $Na_2S_2O_4$ as electron donor.

9-Aminoacridine fluorescence and nitrogenase activity

Fluorescence probes have been used to detect changes in energy levels of energy-transducing membranes [25–28]. The probe 9-aminoacridine itself has a low uncoupling capacity and does not influence nitrogen reduction of whole bacteria within the concentration range used. The mitochondrial inner membrane and the bacterial cytoplasmic membrane are thought to have the same polarity, while the chloroplast grana membrane is opposite in polarity (cf. ref. 29). Uptake [25] or binding [27] of the acridine dyes upon energization in chloroplasts causes a decrease of fluorescence. Therefore an increase in fluorescence, corresponding to a release of the probe upon energization of the bacterial cytoplasmic membrane is expected. That this is the case

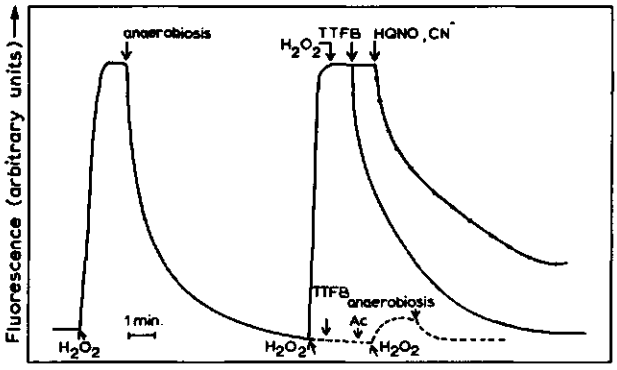

Fig. 4. Enhancement of 9-aminoacridine fluorescence by whole *A. vinelandii* cells upon energization. "Oxygen-limited" cells (0.33 mg/ml; sucrose oxidation, 0.35 μmoles oxygen · min^{-1} · mg^{-1}) were suspended in 2 ml standard incubation mixture with sucrose (20 mM), 9-aminoacridine (2 μM), catalase (Boehringer, 20 μg/ml). At the arrows the following additions were made: H_2O_2 (1.0 μmole), TTFB (2 μM), acetate (20 mM), CN^- (10 μM), HQNO (10 μM). ———, standard incubation; ●–●, standard incubation plus HQNO and CN^-; – – –, standard incubation plus acetate and TTFB.

is shown in Fig. 4. Oxygen induces energization of the membrane indicated by a release of the probe which is seen as an enhancement of the fluorescence. De-energization of the membrane by anaerobiosis results in a fluorescence quenching, caused by uptake of the probe or a more polar environment. Addition of TTFB inhibits the sucrose oxidation leading to a de-energization of the membrane. The same effect is

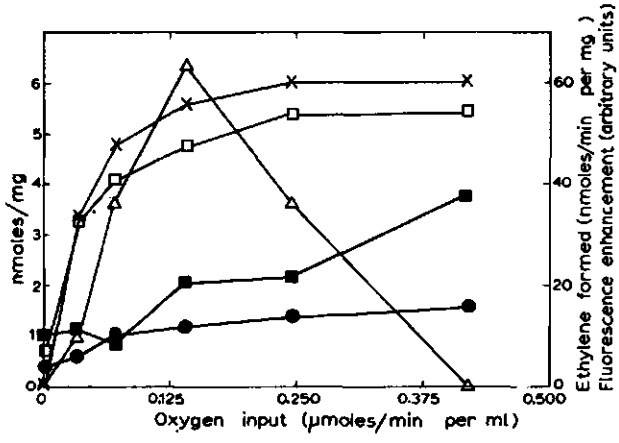

Fig. 5. Enhancement of 9-aminoacridine fluorescence, ATP, NAD^+, $NADP^+$ levels and nitrogenase activity of whole *A. vinelandii* cells on increasing oxygen input. "Oxygen-limited" cells (0.8 mg/ml; sucrose oxidation, 0.46 μmoles oxygen · min^{-1} · mg^{-1}) were suspended in 8 ml standard incubation mixture containing sucrose (20 mM) and 9-aminoacridine (1 μM). The 9-aminoacridine fluorescence enhancement is corrected for the bacterial fluorescence emission, as described in Methods. 10 min after constant rates of acetylene reduction and oxygen consumption were reached at the oxygen input as indicated, the total incubation was fixed with $HClO_4$. After neutralization the cell extract was analyzed. □–□, ATP; ●–●, NAD^+; ■–■, $NADP^+ \times 10$; △–△, nitrogenase activity; ×–×, fluorescence emission.

seen upon inhibition of the sucrose oxidation by HQNO plus CN^-. Because in these experiments the sucrose oxidation was inhibited, oxygen was present during the whole experiment. Thus the observed phenomena are not due to anaerobiosis. TTFB inhibits the formation of an energized state or pH gradient. Upon the addition of TTFB and acetate (Fig. 4) to an anaerobic cell suspension, the oxidation is normal (seen from the oxidation time of the H_2O_2) but the fluorescence enhancement is lower. The changes as given by the dotted line of Fig. 4 are the sum of the bacterial fluorescence changes plus those of the dye emission. In other words much less than the changes under energized conditions.

If the oxygen input is lower than the oxidation capacity of a cell suspension, the fluorescence emission is not maximal. The amount of fluorescence emission was thus used as indicator for the energy level of the cytoplasmic membrane. Fig. 5 shows the dependence of the ATP level and nitrogenase activity on the energy level of the cytoplasmic membrane measured with the energy-induced fluorescence enhancement of 9-aminoacridine. It is clear from this figure that a relative high energy level of the membrane stimulates the nitrogenase activity of whole cells. When the oxygen input into the cell suspension gets near or exceeds the maximum oxidation velocity (0.31 μmoles oxygen $\cdot$ min^{-1} $\cdot$ ml^{-1}), the "switch off" phenomenon [13] is seen. The oxygen electrode registrates free oxygen in the medium (not shown). Under these conditions the ATP and either pH gradient or energized state are high. The data presented here cannot discriminate between inactivation of the nitrogenase by a conformational change or the oxidation of an electron donor or carrier. Fig. 6 shows the influence of uncoupler on the fluorescence of 9-aminoacridine in cells with an active nitrogenase. The initial maximum in the fluorescence emission in the presence of 9-aminoacridine is probably due to the emission of oxidized flavoproteins. This peak is also seen in cell suspensions without 9-aminoacridine. That the flavoproteins of the

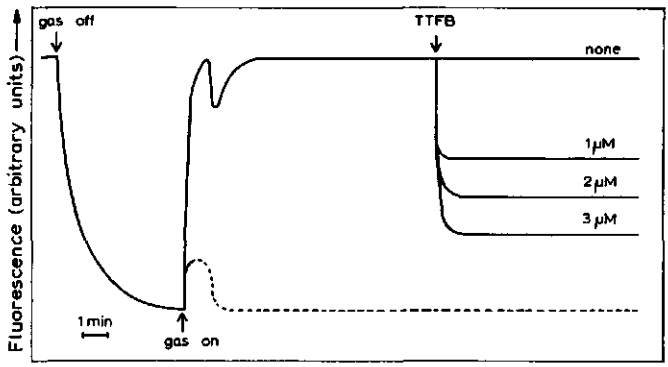

Fig. 6. The effect of TTFB on the fluorescence emission of 9-aminoacridine. "Oxygen-limited" cells (0.38 mg/ml; sucrose oxidation, 0.25 μmoles oxygen $\cdot$ min^{-1} $\cdot$ mg^{-1}) were suspended in 4 ml standard incubation mixture with acetate (20 mM) and 9-aminoacridine (2 μM). 10 min after a constant rate of oxygen consumption was reached (not shown), the gas-flow stopped. After anaerobiosis for 5 min the gas-flow restarted. TTFB was added in the concentration indicated. 8 min after constant rates of oxygen consumption and acetylene reduction were reached, the total incubation was fixed with $HClO_4$. After neutralization the cell extract was analyzed. The results are given in Table III. The dotted line represents the fluorescence emission changes without added 9-aminoacridine.

TABLE III

INFLUENCE OF TTFB ON NITROGENASE ACTIVITY AND LEVELS OF THE ADENINE NUCLEOTIDES IN *A. VINELANDII*

Conditions are described in Fig. 6.

Oxygen input (μmoles oxygen · min^{-1} · ml^{-1})	TTFB (μM)	Nitrogenase activity (percentage of maximum activity*)	ATP (nmoles · mg^{-1})	ADP (nmoles · mg^{-1})	AMP (nmoles · mg^{-1})	Sum of adenine nucleotides (nmoles · mg^{-1})
0.13	0	100	9.9	5.5	5.1	20.0
0.13	1	28.3	9.6	5.2	4.7	19.5
0.13	2	20.0	9.4	5.8	3.7	18.9
0.13	3	0	9.6	5.2	4.7	19.5
0.58	0	0**	11.9	5.5	1.4	18.8

* 100 % nitrogenase activity is 19.8 nmoles ethylene formed · min^{-1} · mg^{-1}.

** "Switch off" condition (cf. ref. 13).

bacteria are oxidized during the first minute is in agreement with a lag that is seen in the oxidation rate. This is also found by Postma and Van Dam [14], who concluded that during the first minute of succinate oxidation the cells had build up a steady-state concentration of Krebs-cycle intermediates. As indicated in Fig. 6, TTFB lowers the fluorescence of 9-aminoacridine. Table III shows the nitrogenase activity and adenine nucleotide levels of the incubations of Fig. 6. It is clearly shown that there is a close relation between nitrogenase activity and height of 9-aminoacridine fluorescence emission. TTFB, at the concentrations used, does not influence the adenine nucleotide levels.

DISCUSSION

In the past decade several proposals have been put forward for the mechanism of electron donation to the nitrogenase in obligate aerobic organisms such as *A. vinelandii*. According to the recent hypothesis of Benemann et al. [5, 6], a high ratio $NADPH/NADP^+$ is the driving force for electron transport to the nitrogenase in an electron transport chain consisting of NADPH ferredoxin oxidoreductase (added from spinach), ferredoxin and Shethna flavoprotein (azotoflavin).

These experiments were done with cell-free extracts from *A. vinelandii*. From our experiments it is clear that an increase in nitrogenase activity in whole cells coincides with an increase in $NADP^+$ content and thus with a lower ratio $NADPH/NADP^+$ (Figs 2 and 5). Since in some of these experiments the ATP level increases concomitantly, it could be argued that ATP, being a substrate for nitrogenase, is the cause of the increased activity. From the value of 5–6 μl intracellular water per mg protein [14], it can be calculated that the ATP concentration in the respiring cells varies between 1.0 and 2.0 mM, well above the K_m value of 0.1–0.3 mM (refs 30 and 31) of the isolated enzyme. Assuming a similar cooperative inhibitory rate of ADP as observed with the purified nitrogenase from *C. pasteurianum* [30], the large increase in nitrogenase activity (300 % or more) can be expected as consequence of the regulation by the intracellular ATP/ADP ratio.

In these experiments acetylene is added in saturating amounts, therefore it can be concluded that under these conditions three possibilities exist: 1. During the whole oxygen-input traject the nitrogenase activity is regulated by the ATP/ADP ratio. Under these conditions a lowering of the $NADPH/NADP^+$ has no effect on the total nitrogenase activity. But according to the simple system proposed by Benemann et al. [5, 6], a high ratio $NADPH/NADP^+$ is required to decrease the redox potential of the electron carriers enough to give nitrogenase activity. Our results indicate that even at a very unfavourable ratio $NADPH/NADP^+$, the nitrogenase activity is maximal. Therefore it is unlikely that this simple system operates as the driving force for the donation of reducing equivalents to the nitrogenase in *A. vinelandii*. 2. During the whole oxygen-input curve the overall nitrogenase activity is rate-limited by the generation of reducing equivalents. This possibility is also unlikely, because where initially the cells are in a highly reduced state a lag in nitrogenase activity is observed. 3. Upon increase of the oxygen input the overall nitrogenase activity is initially regulated by the ATP/ADP ratio, but at the end of the curve where the nitrogenase activity is maximal, the generation of reducing equivalents is rate limiting. An increase in electron transport through the respiratory chain (Figs 2 and 5) stimulates the

overall nitrogenase activity, while a decrease in electron transport (Fig. 3) inhibits the nitrogenase activity. During an oxygen-input curve there is an increase in metabolic activity or possibly a change in metabolic pattern and an increase in electron transport associated with the energized state of the cytoplasmic membrane. In order to discriminate between the production of a metabolite acting as electron donor for nitrogenase and a process connected with the driving force of the energized membrane, the uncoupler TTFB was used. The oxidation velocity of acetate by *A. vinelandii* is hardly influenced by addition of TTFB (Tables I and II) which creates a low energy state (Figs 4 and 6) together with a relative high ATP/ADP level (Tables I, II and III).

Under these conditions where the metabolic activity and the ATP/ADP ratio are constant, the nitrogenase activity is totally inhibited by TTFB. Because no other effects of TTFB than uncoupling have been found, it means that TTFB inhibits the nitrogenase activity by lowering the energized state of the membrane. This is seen in Fig. 6. From this figure and Table III we can conclude that there is a direct relationship between the generation of reducing equivalents and oxidatively generated membrane energy. Whether dinitrogen reduction is directly coupled with the membrane potential or is coupled to an outwards proton movement through the membrane (pH gradient) cannot be derived from these data.

In addition to regulation by the ATP/ADP ratio and electron transfer connected with membrane energization a third mechanism of switch off by oxygen (cf. ref. 13) is clearly distinguishable. So far this process does not seem to be related with any of the parameters measured in this study. Work is in progress to characterize the interaction between the energized membrane and generation of reducing equivalents for the nitrogenase in *A. vinelandii*.

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II Regulation of respiration and nitrogen fixation in different types of *Azotobacter vinelandii*

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The levels of the adenine nucleotides, pyridine nucleotides and the kinetical parameters of the enzymes of the Entner-Doudoroff pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) were determined in *Azotobacter vinelandii* cells, grown under O_2- or N_2-limiting conditions. It was concluded that the levels of both the adenine nucleotides and pyridine nucleotides do not limit the rate of sucrose oxidation.

Experiments with radioactive pyruvate and sucrose show that the rate of sucrose oxidation of *Azotobacter* cells is associated with an increase in the rate of sucrose uptake. The sites of oxidative phosphorylation and the composition of the respiratory membranes with respect to cytochromes $c_4 + c_5$, *b* and *d* differ in cells growth either O_2- or N_2-limited. It was possible to show that the respiration protection of the nitrogen-fixing system in *Azotobacter* is mainly independent of the oxidation capacity of the cells. The oxidation capacity intrinsically depends on the type of substrate and can be partly adapted. The maximum activity of the nitrogenase in *Azotobacter* depends on the type of substrate oxidized. Although the level of energy charge is somewhat dependent on the type of substrate used, no obvious relation can be derived between changes in energy charge and nitrogenase activity. An alternative proposal is given.

Obligate aerobic organisms which fix dinitrogen need a device to protect their nitrogen fixing system against oxygen. According to Dalton and Postgate [1] two protecting mechanisms operate in whole cells of *Azotobacter*: firstly, a so-called switch-on/switch-off mechanism giving short time protection against moderate oxygen concentrations; secondly, adaptation of the oxidation velocity to the O_2 input. Drozd and Postgate [2] reported that the Q_{O_2} and cytochrome *d* content increase when the pO_2 is increased during growth. Jones and coworkers [3,4] investigated the dependence of cytochrome content, oxidative phosphorylation efficiency and whole cell respiration on the O_2 input during growth. Isolated membranes from cells grown at high oxygen input, showed a decrease in phosphorylation efficiency, which correlated with a decrease in ATP levels in whole cells. The increased sugar respiration was attributed to this effect, namely an activation of the key enzymes in sugar catabolism by the lower ATP level. In many cases however the concentrations of all three adenine nucleotides, interrelated by the adenylate kinase reaction, the so-called energy charge (*cf.* [5]), determines the regulation.

Since Jones and coworkers measured only ATP levels in intact cells, we decided to check their proposal by determining the levels of the adenine and pyridine nucleotides in relation to the ability of the growing cells to protect the nitrogen-fixing system against oxygen. Because chemostat-grown cells are better defined than batch-grown cells we used a chemostat for culturing the bacteria. Two types of cells were used; cells whose growth rate was limited by O_2 (cells grown O_2-limited), and cells grown with excess oxygen and substrates, whose growth rate was limited by the nitrogen fixation (cells grown N_2-limited). Some properties of the two types of cells were compared in relation with the adaptation of the *A. vinelandii* respiration to the oxygen concentration during growth and the cytochrome composition. In addition the oxidative phosphorylation of respiratory membranes isolated from the types of cells were compared.

Enzymes. Glucose-6-phosphate dehydrogenase or D-glucose-6-phosphate : $NADP^+$ oxidoreductase (EC 1.1.1.49); 6-phosphogluconate dehydrogenase or 6-phosphogluconate : $NADP^+$ oxidoreductase (EC 1.1.1.44); 6-phosphogluconate hydro-lyase (EC 4.2.1.12); 3-deoxy-2-keto-6-phosphogluconate aldolase is 3-deoxy-2-keto-6-phosphogluconate glyceraldehyde-3-phosphate-lyase (EC 4.1.2.14).

MATERIALS AND METHODS

Growth Conditions of the Bacteria

A. vinelandii ATCC strain O.P. was grown on a Burk's nitrogen-free basic salt medium [6] in a New Brunswick type C30 chemostat supplied with an oxy-

gen electrode. Two types of cells were used; cells whose growth rate was limited by O_2 (oxygen input 32 mmol $O_2 \cdot l^{-1} \cdot h^{-1}$; dilution rate 0.1 h^{-1}), and cells whose growth rate was limited by the N_2-fixation with O_2 present in the medium, as discussed by Dalton and Postgate [7] (oxygen input 90 mmol $O_2 \cdot l^{-1} \cdot h^{-1}$; dilution rate 0.1 h^{-1}). Cells were harvested from the bulk of the growth medium in the chemostat rather than taking cells from the effluent. The culture was centrifuged (10000 × *g*, 10 min) at 20 °C to maintain respiratory protection of nitrogenase. After washing the cells twice with distilled water, they were suspended in water at 0 °C at a protein concentration of 20–60 mg/ml. In all experiments the temperature was 25 °C.

Nitrogenase Activity Assay and Determination of Nucleotides

The standard incubation mixture contained: 25 mM Tris-HCl, 1 mM KCl 5 mM $MgCl_2$ and 10 mM substrate final pH 7.6. The cells were preincubated for 5 min at an oxygen input of about 1/3 of the maximum oxidation capacity (independently determined) to restore the intracellular cofactor concentration. The oxygen input was then adjusted to the required level and 10 min after constant rates of acetylene reduction and oxygen consumption were reached at the oxygen input indicated, the total incubation mixture was fixed by rapidly adding $HClO_4$ up to a final concentration of 4% (w/v). After neutralization with $KHCO_3$ the adenine nucleotides and pyridine dinucleotides were determined as described earlier [8]. When the oxygen input was zero the incubation mixture was fixed 2 min after anaerobic conditions had been reached. The time sequence is important, firstly to enable the anaerobic equilibrium to be reached and secondly to avoid the breakdown of the adenine nucleotides [9].

Measurements of Oxygen Consumption

Oxygen consumption was measured with a Clark type electrode (Yellow Spring Instruments). Bacterial cells were suspended in the standard incubation mixture with 10 mM substrate at a protein concentration of 0.01–0.1 mg/ml. Oxygen consumption was measured during at least 10 min. All rates are corrected for endogenous respiration. Oxygen input into the vessel was calculated according to Cooper [10]. Protein was determined with the biuret method as modified by Cleland and Slater [11].

Preparation of Respiratory Membranes and Assay of Oxidative Phosphorylation

The respiratory membranes from the chemostat-grown cells were prepared exactly as described by Ackrell and Jones [12]. The membrane preparation was used immediately. The assay mixture contained at a final pH of 6.8: 40 mM piperazine *N*,*N*′-bis-2-ethane sulphonic acid (PIPES) buffer, 10 mM $MgCl_2$, 0.5 mM ADP, 5 mM glucose, 5 mM phosphate and 10 U dialysed yeast hexokinase (Boehringer). Respiratory membranes (maximum oxygen consumption 20 mmol O_2/min at maximum amount of 20 µg, final volume 1.6 ml) were incubated 3 min in the assay mixture.

The reaction was started by adding substrate. Final concentrations: 1 mM NADH, 4 mM D,L-malate, 4 mM ascorbate plus 0.1 mM 2,6-dichloroindophenol or 0.1 mM tetramethyl-phenylene-diamine. The reaction was stopped after 5 min by transferring 1 ml of the reaction mixture into 0.1 M ice-cold $HClO_4$ 40% (w/v). After removal of the protein by centrifugation (3000 × *g*, 20 min) the aliquot was neutralized and its content of glucose 6-phosphate was determined. In the control experiments the substrate was omitted.

Cytochromes

Cytochromes $c_4 + c_5$, $b_1 + o$, a_1 and d in membranes were analysed by measuring difference spectra between oxidized membranes and membranes reduced with dithionite; cytochrome o was analysed by measuring difference spectra between membranes reduced with dithionite plus CO and membranes reduced with dithionite. Because of the contribution of cytochromes $c_4 + c_5$ to the spectrum of cytochrome $b_1 + o$, it was necessary to use a small slit (<0.6 nm bandwith). The amounts of cytochrome c and $b_1 + o$ were calculated from the spectra according to Sinclear and White [13]. Because cytochrome b_1 and o have their maximum absorbance at the same wavelength, the net cytochrome b_1 content was determined by subtracting the concentration of cytochrome o from $b_1 + o$. The molar absorption coefficients of cytochrome $c_4 + c_5$, $b_1 + o$, d and o were used as reported by Jones and Redfearn [14] respectively by Sinclear and White [13]. Cytochrome concentrations were expressed as nmol/mg protein. The spectra were scanned on an Amino-Chance dual wavelength spectrophotometer.

Preparation and Assays of Soluble Enzymes

Freshly harvested cells (160–210 mg protein in 25 mM Tris-HCl and 1 mM dithiothreitol, final volume 10 ml, pH 7.6) were sonicated for 90 s in a MSE sonifier. After centrifugation of the broken cells (20000 × *g* for 30 min), the supernatant was centrifuged 120 min at 200000 × *g*. The resulting supernatant was dialysed for 2 h against 2 l 25 mM Tris-HCl and 1 mM EDTA pH 7.6 at 4 °C. After dialysis,

1 mM dithiothreitol was added. Glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, 6-phosphogluconate hydro-lyase and 3-deoxy-2-oxo-6-phosphogluconate aldolase were determined according to Senior and Dawes [15].

Incorporation of Radioactive Sucrose or Pyruvate and $^{14}CO_2$ Assay

The incubation mixture contained at a final pH 7.6: 25 mM Tris-HCl, 10 mM substrate ([U-^{14}C]-sucrose or [2-^{14}C]pyruvate). The reaction was started by adding *A. vinelandii* cells, 0.3–0.6 mg protein, final volume 0.5 ml. Air was bubbled through the solution to keep it aerobic and the reaction was terminated by adding an 0.1 ml aliquot of the reaction mixture to 5 ml 10 mM Tris-HCl and 10 mM dithionite, final pH 7.5. The cells were collected on a millipore filter (pore size, 0.45 μm) and washed on the filter with 5 ml of the Tris-dithionite mixture. Postma [16] has shown that under anaerobic condition no leakage of Krebs-cycle intermediates occurs from the cells, although they are taken up energy dependently. The filters were transfered to 0.5 ml soluene (Packard) and after adding scintillation liquid the radioactivity was counted.

Assays involving the liberation of $^{14}CO_2$ were carried out in Warburg vessels. The reaction mixture in the main vessel contained 25 mM Tris-HCl, 10 mM labeled substrate and 0.3–0.6 mg protein in a final volume of 0.2 ml and a final pH 7.6. The reaction was started by addition of cells and stopped by adding 0.1 ml 2.5 M H_2SO_4 from the side-arm. The centre wall contained 0.05 ml 3.5 M KOH on a strip of Whatmann paper. After shaking the mixture for 1 h the filter paper was transfered to 0.5 ml soluene (Packard) and counted as described above. The respiration activity of the cells was determined under the same conditions in parallel experiments with an Clark type oxygen electrode.

Special Chemicals

[U-^{14}C]Sucrose and [2-^{14}C]pyruvate were obtained from the Radiochemical Centre (Amersham).

RESULTS

The rate of sucrose oxidation of cells grown O_2-limited is 20–140 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$; the oxidation rate pyruvate is considerably higher 300–600 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$. The large fluctuations in rate of sucrose oxidation depend strongly on small variations in growth conditions. In contrast cells grown N_2-limited oxidize sucrose and pyruvate at about the same rate (300–600 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$). The observed differences in sucrose oxidation might be due to either a direct effect on metabolic rate or an effect on the rate of uptake of this substrate. It is well known that ATP, ADP, NADH and NADPH affect the activity of the enzymes of both the Entner Doudoroff and oxidative pentose phosphate cycle pathways in *Azotobacter beijerinkii* [15]. Still and Wang [17] showed that the Entner Doudoroff pathway is the most important pathway for the degradation of sugar in *Azotobacter* species. Though changes in metabolic rate may be induced by these nucleotides, other possibilities are changes in concentration of the enzymes catalysing rate-limiting steps or a change in kinetic pattern of individual enzymes in the two kinds of cells used. Therefore we studied the influence of growth conditions on the kinetic parameters of the key enzymes of the Entner Doudoroff pathway glucose-6-phosphate dehydrogenase, 6-phosphogluconate hydro-lyase and 6-phosphogluconate aldolase and the first enzyme of the oxidative pentose phosphate cycle, 6-phosphogluconate dehydrogenase (Fig. 1, Table 1).

The glucose-6-phosphate dehydrogenase in extracts of *A. vinelandii* has unusual properties. Is has about the same maximum velocity with NAD^+ and $NADP^+$ but in contrast to other glucose-6-phosphate dehydrogenases which are also active with both pyridine nucleotides [18–21] the plot of rate *versus* $NADP^+$ concentration is not sigmoidal but biphasic (Fig. 1). The double reciprocal plot shows two linear regions, one presenting a site with a high affinity for $NADP^+$ ($K_{m(1)} \approx 16$ μM) and one a site with a low affinity ($K_{m(2)} \approx 0.17$ mM). The double reciprocal plot of velocity versus NAD^+ concentration is linear and gives K_m (NAD^+) of 0.12 mM.

The specific activities of two of the enzymes of the Entner-Doudoroff pathway are the same in extracts from cells grown O_2- or N_2-limited (Table 1). However the K_m (gluconate) of 6-phosphogluconate dehydrogenase is three times larger in cells grown N_2-limited. In addition the specific activity of glucose-6-phosphate dehydrogenase, the first enzyme of both the Entner-Doudoroff pathway and the oxidative pentose phosphate cycle, shows a 3–4 fold increase in extracts of cells grown N_2-limited.

Senior and Dawes [15] studied the regulation of glucose metabolism in *A. beijerinckii* and observed inhibition by ATP of glucose-6-phosphate dehydrogenase, 6-phosphogluconate hydro-lyase and 6-phosphogluconate aldolase. Rather than using ATP alone, we studied the effect of the energy charge (*cf.* [5]) on the activities of these three enzymes. Fig. 2 shows the influence of glucose 6-phosphate on the activity of glucose-6-phosphate dehydrogenase at a saturating concentration of $NADP^+$ and at energy charges of 0.3 and 0.9. Similar results were obtained when NAD^+ was substituted for $NADP^+$. It is clear that at concen-

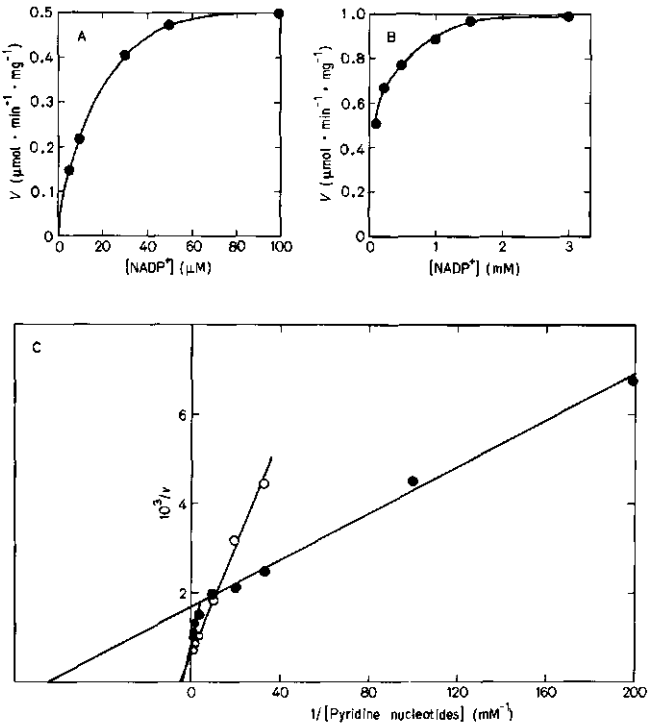

Fig. 1. *Dependence of the activity of glucose-6-phosphate dehydrogenase from* A. vinelandii *on the concentration of pyridine nucleotides.* (A) Plot of initial velocity *versus* $NADP^+$ concentration for glucose-6-phosphate dehydrogenase in the presence of saturating concentrations (10 mM) of glucose-6-phosphate. Assays were carried out as described in Materials and Methods. Protein concentration of 2.8 μg/ml. (B) As in (A), but with higher $NADP^+$ concentrations. (C) Lineweaver-Burk plots of the effect of $NADP^+$ and NAD^+ concentrations on the glucose-6-phosphate dehydrogenase activity in the presence of saturating concentrations of glucose-6-phosphate (10 mM). The protein concentration was 2.8 μg · ml^{-1}. (O——O) NAD^+, (●——●) $NADP^+$

trations of glucose 6-phosphate lower than 0.7 mM the enzyme is inhibited at this concentration of the adenine nucleotides however the energy charge has little effect. Similar observations were made with saturating concentrations of glucose 6-phosphate and varying concentrations of $NADP^+$ (Fig. 3). Again the enzyme is inhibited by adenine nucleotides, the inhibition is especially marked at low concentrations of $NADP^+$, but does not depend on the energy charge. When NAD^+ is substituted for $NADP^+$, adenine nucleotides inhibit the enzyme competitively and no effect of energy charge is observed. The results show that the $NADP^+$-dependent glucose-6-phosphate dehydrogenase shows two distinct types of binding sites. The low-affinity site is mainly influenced by adenine nucleotides; the inhibition however completely disappears, similarly as with NAD^+, at saturating $NADP^+$ concentration. Furthermore positive cooperativity with respect to glucose 6-phosphate is enhanced by adenine nucleotides.

No influence of energy charge was observed on the activity of 6-phosphogluconate hydro-lyase and 6-phosphogluconate aldolase. In a typical experiment the activity of the two enzymes was inhibited 10% at an energy charge 0.3 and 11% at an energy charge 0.9, at a total adenine nucleotide concentration of 1 mM; at a concentration of 2 mM the activity was inhibited 18% at an energy charge 0.3 and 23% at an energy charge 0.9).

Jones *et al.* [4] studied the decrease in P/O ratio of isolated *A. vinelandii* membranes occurring in changing the growth conditions from low to high respiration. This was related to changes in cytochrome *d* level and ATP concentration in intact cells. The intracellular ATP concentration was determined by removing samples from the medium. Since the intracellular ATP concentration decreases very quickly after reaching anaerobiosis [22]; the possibility cannot be excluded that the suspension of the sample transfered becomes anaerobic. Thus in our studies, under

Table 1. *Kinetic parameters of enzymes of Entner-Doudoroff pathway and 6-phosphogluconate dehydrogenase measured in a crude extract from O_2- and N_2-limited grown* A. vinelandii

Crude extract was prepared as described in Materials and Methods. Activity of all enzymes was measured as described in Materials and Methods. Protein concentrations of the enzymatic assays: glucose-6-phosphate dehydrogenase, 5.7 μg/ml cell extract of cells grown O_2-limited, 2.8 μg/ml cell extract of cells grown N_2-limited. 6-phosphogluconate hydro-lyase dehydrogenase, 3-deoxo-2-oxo-5-phosphogluconate aldolase and 6-phosphogluconate hydrogenase: 17.1 μg/ml cell extract of cells grown O_2-limited, 14.0 μg/ml cell extract of cells grown N_2-limited. For the definition of $K_{m(1)}$ and $K_{m(2)}$ used by glucose-6-phosphate dehydrogenase see *text* and Fig. 1 C

Enzyme	Conditions of growth	
	O_2-limited	N_2-limited
Glucose-6-phosphate dehydrogenase	nmol NADH $\times min^{-1} \times mg^{-1}$	
V (NAD^+)	371	1260
V ($NADP^+$)	274	910
	mM	
$S_{(0.5)}$ glucose-6-P at NAD^+ 3 mM	0.25	0.25
$S_{(0.5)}$ glucose-6-P at $NADP^+$ 3 mM	0.25	0.30
$S_{(0.5)}$ glucose-6-P at $NADP^+$ 0.1 mM	0.5	0.4
K_m (NAD^+)	0.15	0.12
$K_{m(1)}$ ($NADP^+$)	0.011	0.016
$K_{m(2)}$ ($NADP^+$)	0.18	0.17
6-Phosphogluconate hydro-lyase dehydratase and 3-deoxo-2-oxo-6-phosphogluconate aldolase		
	nmol pyruvate $\times min^{-1} \times mg^{-1}$	
V (apparent)	163	188
6-Phosphogluconate dehydrogenase	nmol NADH $\times min^{-1} \times mg^{-1}$	
V (NAD^+)	23	15
V ($NADP^+$)	90	95
	μM	
K_m 6-phosphogluconate ($NADP^+$)	40	110
K_m (NAD^+)	90	100
K_m ($NADP^+$)	13	14

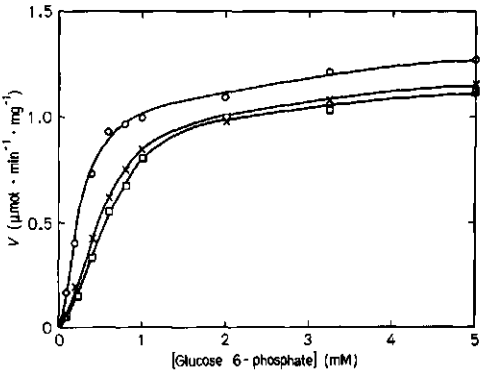

Fig. 2. *Effect of the energy charge on the activity of glucose-6-phosphate dehydrogenase from* A. vinelandii. Plot of velocity *versus* glucose-6-phosphate concentration in the presence of a saturating concentration (3 mM) of $NADP^+$ at a total adenine nucleotide concentration of 1 mM. Assays were carried out as described in Materials and Methods. Protein concentration of 4.5 μg/ml. (O——O) Control; (×——×) energy charge 0.3; (□——□) energy charge 0.9

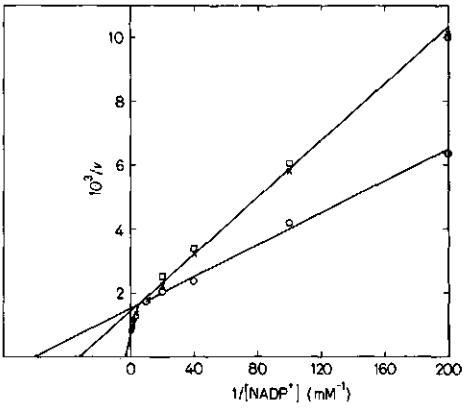

Fig. 3. *Effect of the energy charge on the activity of glucose-6-phosphate dehydrogenase from* A. vinelandii. Lineweaver-Burk plots of the effect of $NADP^+$ concentration on glucose-6-phosphate dehydrogenase activity at a total adenine nucleotide concentration of 1 mM in the presence of saturating concentrations (10 mM) of glucose-6-phosphate. Assays were carried out as described in Materials and Methods. Protein concentration of 2.3 μg/ml. Since at high $NADP^+$ concentrations the activities at an energy charge 0.9 are nearly the same as at an energy charge 0.3 these points were omitted from the figure. (O——O) Control; (×——×) energy charge 0.3; (□——□) energy charge 0.9

constant gas bubbling, to maintain the right oxygen input, $HClO_4$ was added to the cell suspension in order to guarantee that no changes in the ATP concentration occurred.

Fig. 4 shows the effect of different oxygen inputs on N_2-fixation, ATP, ADP, AMP, NAD^+ and $NADP^+$ levels in cells oxidizing sucrose or pyruvate and grown O_2- or N_2-limited. It is clear that no significant changes in mutual ratios of ATP, ADP and AMP occur upon increasing the oxygen input between the two types of cells when the same substrate is oxidized. When the intracellular ATP, ADP and AMP concentrations are interrelated as energy charge [5] it is found that the energy charge of cells grown O_2-limited is somewhat higher than that of cells grown N_2-limited (Fig. 5). However, the effect of the oxidizable substrate is much more important. The increase in the energy

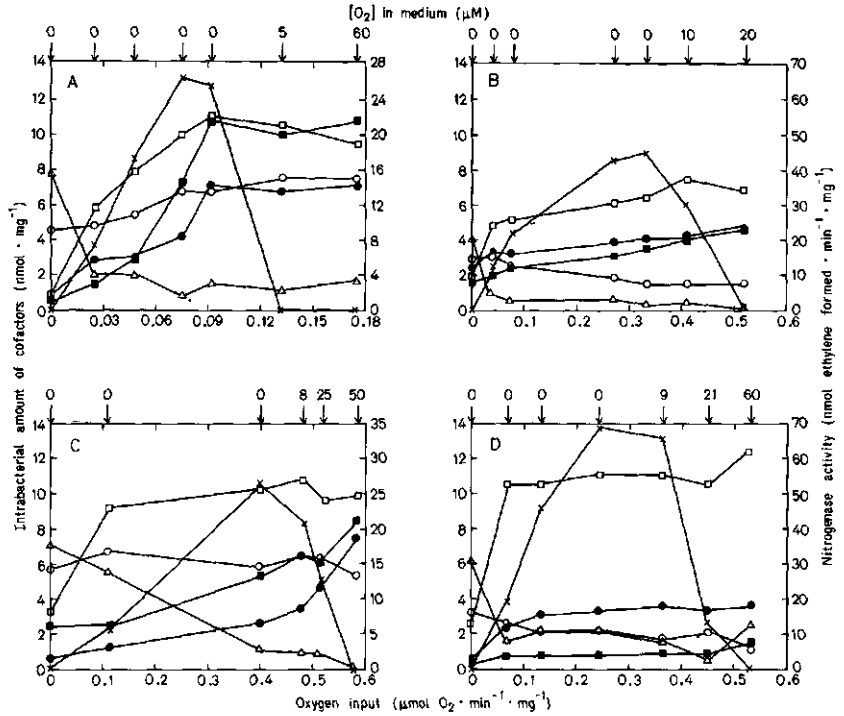

Fig. 4. *Effect of oxygen on the levels of adenine nucleotides, pyridine nucleotides and nitrogenase activity in intact cells of* A. vinelandii. Cells were suspended in 5 ml standard incubation mixture with substrate. 10 min after constant rates of acetylene reduction and oxygen consumption were reached at the oxygen input as indicated, the total incubation mixture was fixed with $HClO_4$. After neutralization the cell extract was analyzed. In all four figures the upper and lower horizontal axes represent the oxygen concentration in the medium and the oxygen input in the incubation respectively. The right and left vertical axes represent the nitrogenase activity and the intrabacterial amount of cofactors respectively. (□——□) ATP; (○——○) ADP; (△——△) AMP; (●——●) NAD^+; (■——■) $NADP^+ \times 10$; (×——×) nitrogenase activity. (A) Cells grown O_2-limited (sucrose oxidation 0.13 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$) were suspended at a protein concentration of 0.9 mg/ml with sucrose (20 mM) as substrate. (B) Cells grown O_2-limited (pyruvate oxidation 0.45 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$) were suspended at a protein concentration of 0.82 mg/ml with pyruvate (20 mM) as substrate. (C) Cells grown N_2-limited (sucrose oxidation 0.49 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$) were suspended at a protein concentration of 0.9 mg/ml with sucrose (20 mM) as substrate. (D) Cells grown N_2-limited (pyruvate oxidation 0.35 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$) were suspended at a protein concentration of 0.9 mg/ml with pyruvate (20 mM) as substrate. The arrows indicate the oxygen concentration detectable in the medium

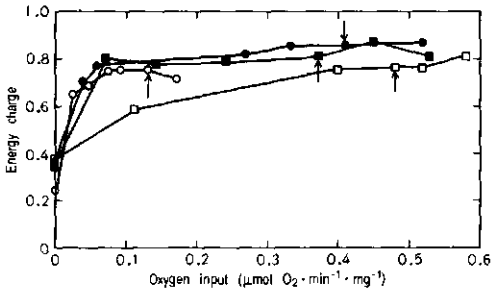

Fig. 5. *Influence of oxygen-input on the energy charge of different types of* A. vinelandii *cells.* The cells were oxidizing sucrose or pyruvate; conditions and data as described in Fig. 4. Cells grown O_2-limited: (○——○) sucrose; (●——●) pyruvate. Cells grown N_2-limited: (□——□) sucrose; (■——■) pyruvate. The arrows indicate the first measurements at which O_2 was detectable in the medium

charge with oxygen input is larger with pyruvate than with sucrose and the maximum value reached is higher. Regulation of sucrose degradation by energy charge thus seems possible only at low values of oxygen input.

The total amount of intracellular adenine nucleotides in *Azotobacter* varies with the conditions of incubation. Schramm and Leung [9] showed that after anaerobiosis AMP slowly degrades. Therefore in our experiments the cell suspension was first incubated aerobically during 10 min to restore the pool of adenine nucleotides after which it was further incubated for at least 10 min at the desired oxygen input. In the case of an anaerobic determination of cofactors the cell suspension was fixed 2 min after anaerobiosis to eliminate as much as possible the breakdown of the adenine nucleotides. We also found

Table 2. *Oxygen consumption, $^{14}CO_2$ production and incorporation of [U-^{14}C]sucrose or [2-^{14}C]pyruvate in O_2-or N_2-limited grown* A. vinelandii *cells*

Oxygen consumption, $^{14}CO_2$ production and incorporation of radioactive sucrose or pyruvate were measured as described in Materials and Methods. The reactions were started with cells which were grown as described in Materials and Methods. After 6 min the rate of $^{14}CO_2$ production, the oxidation velocity and amount of radioactivity incorporated were constant and were determined. For the calculation of the amount of oxygen necessary to oxidize sucrose or pyruvate completely to H_2O and CO_2 the following equations were used: Sucrose: $C_{12}O_{11}H_{22} + 12\,O_2 \rightarrow 12\,CO_2 + 11\,H_2O$; Pyruvate $2\,C_3O_3H_4 + 5\,O_2 \rightarrow 6\,CO_2 + H_2O$

Type of cells	Substrate	Oxidation velocity	CO_2 produced	Calculated O_2 consumption	Incorporated radioactive substrate
		nmol O_2 · min^{-1} · mg^{-1}	nmol · min^{-1} · mg^{-1}		nmol · mg^{-1}
Cells grown O_2-limited	sucrose	22.4	22.7	22.7	31.4
	pyruvate	380	235	196	45
Cells grown N_2-limited	sucrose	273	252	252	27.4
	pyruvate	475	438	365	35.5

that the pool size of the adenine nucleotides depends on additional factors. For example a cell suspension incubated under aerobic conditions without substrates, contained 8.2 nmol of adenine nucleotides (ATP + ADP + AMP)/mg, with sucrose 12.5 nmol/mg and with pyruvate 11.2 nmol/mg; under anaerobic conditions with sucrose or pyruvate 10.0 nmol/mg.

Fig. 4 shows that cells grown N_2-limited, thus with oxygen present in the medium during growth, are capable of fixing nitrogen with sucrose and pyruvate as substrate (Fig. 4C, D). In contrast, cells grown O_2-limited fix nitrogen with oxygen present in the medium only with pyruvate as substrate (Fig. 4A, B).

By extracting the cells with KOH instead of $HClO_4$, NADH and NADPH were determined. Combined KOH and $HClO_4$ extractions showed that under anaerobic conditions 5 – 30 % of the pyridine nucleotides are in the oxidized form and that at saturating oxygen concentrations no reduced pyridine nucleotides are detectable. It seems therefore that at saturating concentrations of oxygen the rate of sucrose oxidation in *Azotobacter* cells is not regulated by reduced pyridine nucleotides. Surprising are the lower pyridine nucleotide levels in both types of cells oxidizing pyruvate.

The change in concentration of one of the key enzymes in carbohydrate degradation, glucose-6-phosphate dehydrogenase (*cf.* Table 1) may account for part of the differences in oxidation rate of sucrose in the two types of *A. vinelandii* cells. Therefore the uptake and flux of [^{14}C]sucrose and [^{14}C]pyruvate were studied and compared. Both kinds of washed cells exhibit a lag period when they oxidize sucrose or pyruvate. The rates of oxidation and the amounts of radioactivity incorporated in the cell material and the $^{14}CO_2$ production were therefore determined after this lag, at linear rates (Table 2). With sucrose as substrate there is a good correlation between the rate of oxygen consumption, measured polarographically, and the rate of $^{14}CO_2$ production. However with pyruvate the correlation is poor. The measured rate of oxygen consumption exceeds that calculated from the rate of $^{14}CO_2$ production. This effect indicates that pyruvate stimulates the oxidation of endogenous substrates; for example a high intracellular concentration of pyruvate together with a high oxaloacetate concentration lowers the ratio acetyl-CoA/CoA, which stimulates the degradation of poly-(3-hydroxybutyrate) [23]. Table 2 shows that despite the much higher fluxes of substrates through the metabolic pathways of the higher respiring cells the amounts of radioactive sucrose or puruvate incorporated are nearly the same in cells grown O_2- and N_2-limited.

We were able to confirm the observation [3, 4, 12, 24] that membranes isolated from cells grown N_2-limited have a low phosphorylation efficiency at site I (Table 3). In spite of very careful attempts to prepare different cytochrome preparations of respiratory membranes under identical conditions the absolute P/O ratios varied between 0.1 and 0.4. On the other hand the mutual ratios of P/O between different substrates were always the same. Cells grown N_2-limited have no phosphorylation at site I (P/O with NADH minus P/O with malate). There is only phosphorylation between ubiquinone and oxygen. The phosphorylation as measured with ascorbate plus dichloroindophenol observed in cells grown O_2-limited is very low in the N_2-limited grown cells (Table 3). Ackrell and Jones [12] reported that oxygen uptake and oxidative phosphorylation increase linearly with respiratory membrane concentration over the range 0 – 0.50 mg/ml. We observed deviation from linearity with malate or ascorbate plus dichloroindophenol as substrates at membrane concentration higher than 50 µg/ml; at higher protein concentrations both the rate of oxygen uptake and the P/O ratio decrease. Therefore we used protein concentrations of 20 µg/ml or lower in our experiments. In contrast to Jones *et al.*

Table 3. *The effect of oxygen on the properties of* A. vinelandii

Respiratory membranes were prepared from cells grown O_2- or N_2-limited as described in Materials and Methods. P/O ratios, cytochrome content nmol/mg and dehydrogenase activities (μatom O · min^{-1} · mg^{-1}) of the respiratory membranes were measured as described in Materials and Methods. $Ph(NMe_2)_2$, tetramethylphenylenediamine

Respiratory membranes isolated from	Dehydrogenase activity				Cytochrome content				
	NADH	malate	ascorbate + dichloro-indophenol	ascorbate + $Ph(NMe_2)_2$	$c_4 + c_5$	$b_1 + o$	o	b_1	d
	μatom O × min^{-1} × mg^{-1}				nmol/mg				
Cells grown O_2-limited	1.7	1.1	2.3	3.0	4.9	3.7	4.3	–	0.9
Cells grown N_2-limited	4.7	0.95	0.41	0.44	3.3	5.8	2.6	3.2	3.6

	P/O			
	NADH	malate	NADH plus malate	Ascorbate plus dichloroindophenol
Cells grown O_2-limited (2.7 μg protein/ml incubation)	0.18	0.14	0.17	0.12
Cells grown N_2-limited (7.3 μg protein/ml incubation)	0.24	0.25	0.26	0.02

[4] we observed differences in the content of cytochromes $c_4 + c_5$ and b_1 in the different types of cells. Cytochrome b_1 is higher in cells grown N_2-limited while cytochrome $c_4 + c_5$ is lower. We confirmed that the amount of cytochrome d is higher in cells grown N_2-limited than in those than in those grown O_2-limited [2, 4]. Membranes isolated from cells grown N_2-limited have a higher NADH dehydrogenase activity and a higher content of cytochromes b_1 and d than membranes from cells grown O_2-limited. The reverse is observed for the ascorbate plus dichloroindophenol oxidase activity and the cytochrome $c_4 + c_5$ content (Table 3).

DISCUSSION

The data presented in this paper show that under saturating concentrations of O_2 in intact *A. vinelandii* cells the ATP levels are maximum while the intracellular pyridine nucleotides are almost completely oxidized (Fig. 4). Therefore an increase in respiratory chain dehydrogenase activity as was measured with isolated membranes does not contribute under these conditions to a lower intracellular level of reduced pyridine nucleotides because the generation of NAD(P)H by catabolic pathways is slower than the conversion of NAD(P)H into $NAD(P)^+$ by the respiratory chain dehydrogenases. Thus it is unlikely that an increase in activity of the respiratory chain dehydrogenases influences the activities of the enzymes of the Entner-Doudoroff pathway and the oxidative pentose phosphate cycle by lowering the intracellular NAD(P)H levels in N_2-limited grown cells. Thus the proposal [3, 4], that the increase in respiratory chain dehydrogenase activity and a decline in intracellular level of ATP is responsible for the increase in rate of sugar oxidation at high O_2 concentration can be excluded by the observation.

Similarly intracellular regulation by the energy charge can be excluded. Our results show that there is no significant difference in energy charge at saturating oxygen concentration between the different types of cells used. However, some differences in energy charge can be induced by a change in substrate; the energy charge of both types of cells is higher with pyruvate than with sucrose. The decrease in phosphorylation efficiency at sites I and III as measured with isolated membranes, might be the cause of a somewhat lower value of the energy charge in cells grown N_2-limited.

In addition, apart from a 4-fold increase in glucose-6-phosphate dehydrogenase activity and a 3-fold increased K_m (gluconate) for 6-phosphogluconate dehydrogenase no kinetic differences between the key enzymes of the Entner-Doudoroff pathway isolated from cells grown O_2- and N_2-limited are detectable. Changes in energy charge have little effect on the activity of glucose-6-phosphate dehydrogenase and the enzymes of the Entner-Doudoroff pathway. The total concentration of the adenine nucleotides is of more importance for the activity of these enzymes than the variation in energy charge at a constant level of

total adenine nucleotides. Since the amount of intracellular radioactive material in the different types of cells does not vary significantly under steady-state conditions of respiration, it is obvious that the sucrose translocator activity can be followed by the determination of the rate of product formation. It follows from our data that in *A. vinelandii* cells under the conditions of saturating amounts of oxygen the sucrose respiration is mainly determined by the activity of the sucrose translocator, possibly coupled to the glucose-6-phosphate dehydrogenase activity. Our experiments cannot exclude however that the absolute amount of sucrose translocator differs in the two types of cells. The possibility exists that the activity of the translocator is regulated by metabolites other than the adenine or pyridine nucleotides. The influence of the value of the energy charge and especially of the ratios $NAD(P)H/NAD(P)^+$ on the activities of glucose-6-phosphate dehydrogenase and the enzymes of the Entner-Doudoroff pathway can play an important role at non-saturating oxygen concentrations in preventing an accumulation of metabolites.

Cells grown on sucrose have a high nitrogenase activity at least when they oxidize pyruvate. One could argue that the high value observed for the energy charge under these conditions stimulates nitrogen fixation, but the increase in nitrogenase activity with increasing oxygen input at a nearly constant energy charge, as discussed previously [8], is difficult to explain in that case. A more likely explanation is that a more highly energized cytoplasmic membrane in cells respiring pyruvate stimulates the transport of reducing equivalents to the nitrogenase (*cf.* [8]). The observation that cells grown on sucrose under O_2 limitation and respiring pyruvate fix nitrogen with oxygen present in the medium, proves that the respiration protection of the nitrogen fixing system in *A. vinelandii* does not depend on the oxidation capacity of the cells but on the substrate used. Therefore it is not necessary to modify elements in the electron-donating or the nitrogenase system to fix nitrogen in the presence of oxygen. The experiment with cells grown O_2-limited show that the oxidation capacity is high with pyruvate, while the much lower rate of sucrose oxidation is probably due to limitations in translocation rate. The result of this limitation is a higher sensitivity of the nitrogenase to O_2 and can be overcome by adaptation of the sucrose metabolism.

One can only speculate on the relation between the variation of the amounts of the different cytochromes and the oxidative phosphorylation efficiency. Our data indicate that the composition of the respiratory chain as proposed by Jones and Redfearn [25] is at least doubtful. The composition of the chain depends on the growth conditions. Table 3 shows that cells grown O_2-limited mainly contain cytochromes $c_4 + c_5$ and cytochrome *o*. In cells grown N_2-limited apart from these cytochromes also cytochrome b_1 and cytochrome *d* are present. Nevertheless the problem of the composition of the branched respiratory chain and its involvement in oxidative phosphorylation in *A. vinelandii* needs further investigation.

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III Aerobic nitrogen fixation in *Azotobacter vinelandii*

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1. INTRODUCTION

Organisms which fix dinitrogen in the presence of oxygen have to overcome two difficulties: 1. Protect the oxygen-sensitive nitrogenase against irreversible inactivation. 2. Synthesize electron carriers with a low redoxpotential necessary from nitrogenase activity with oxygen present in the medium. With regard to the first problem Dalton and Postgate (1970) proposed a respiration and conformation protection. About the mechanisms of both protection systems some information is available (Drozd and Postgate, 1970; Jones et al., 1973; Haaker and Veeger, 1976; Yates, 1970). Electron carriers such as ferredoxin and flavodoxin, capable of donating reducing equivalents to the nitrogenase (Benemann et al., 1969; Yoch, 1969, 1972 a, b; Yates 1972), have been isolated from *Azotobacter*. Benemann et al. (1971) proposed and electron transport chain between NADPH via ferredoxin and flavodoxin to the nitrogenase. In our opinion some reservations must be made. 1. In whole cells nitrogen fixation takes place at different ratios of $NAD(P)H/NAD(P)^+$ (Haaker et al., 1974; Haaker and Veeger, 1976), while it is doubtful that the intracellular redoxpotential of pyridine nucleotides is low enough to generate reducing equivalents for the nitrogenase. 2. Ferredoxin-$NADP^+$ oxidoreductase has never been found in *Azotobacter*. In this report we present some recent results in relation to the oxygen protection of, and the electron donation to, the nitrogenase of *Azotobacter*.

2. MATERIALS AND METHODS

GROWTH CONDITIONS AND ENZYME PREPARATIONS

Azotobacter vinelandii OP was either grown in a batch culture, harvested during the logarithmic phase and stored as described earlier (Bresters et al., 1975) or grown in a chemostat N_2-limited with a dilution rate of 0.1 h^{-1}, harvested, washed and used directly (Haaker and Veeger, 1976).

Crude extracts were prepared in two ways. Mechanically, using a Manton Gaulin homogenizer (CEM) and by an osmotic (CEO) treatment as described by Shah et al. (1972).

Nitrogenase complex was purified as described by Bulen and Le Comte (1972); depending on the extract they were derived from, the terminology M and O was added to the terminology of Bulen and Le Comte. The following preparations were tested; crude extract (CEM or CEO) and C 42-I(M and O). The crude components I (crude I) and II (crude II) were prepared from C 42-I and separated on a DEAE cellulose column as described by Shah et al. (1972). C 42-I was also eluted from a DEAE cellulose column as a single fraction with 90 mM $MgCl_2$ (C-I-90). Chemostat-grown cells were used to prepare a Tris-EDTA-toluene preparation. 100-200 mg bacterial protein was suspended in 50 mM Tris-HCL, 10 mM EDTA, 0.2 ml toluene, final pH 8.0, final volume 20 ml. The suspension was held anaerobically at 0^{o} for 15 min. After centrifugation (10,000 x g, 5 min.) at $4^{o}C$ the pellet was washed with 40 ml 25 mM Tris-HCL pH 7.4 and 5 mM $MgCl_2$ and centrifuged again. The pellet was resuspended in the same buffer and stored at $0^{o}C$. (TET preparation). A control preparation was prepared without adding toluene (TE preparation).

ANALYTICAL METHODS

Acetylene reduction assays were run at 30^{o}, shaking in 6.5 ml bottles, sealed with Subaseal, which contained a mixture of 25 mM Tricine buffer, 2 mM ATP, 4 $MgCl_2$, 10 mM, creatine phosphate and 5 U. creatine kinase final pH 7.5. This mixture was thouroughly flushed for at least 30 min. with purified argon which passed a heated BASF catalyst. The acetylene (10%) and 20 mM dithionite were added. The mixture was equilibrated for 10 min. with the gasphase to remove the last traces of oxygen, after which the nitrogenase preparation was added and the ethylene produced was measured as described earlier (Haaker et al., 1974). When other reducing agents instead of dithionite were used, the last traces of oxygen were removed by 10 mM glucose, 10 U glucose oxidase and 40 µg catalase/ml, which was added to the assay mixture.

Pyruvate dehydrogenase was measured in the assay mixture described by Bresters et al. (1975), but the activity was measured by following the oxygen consumption caused by the presence of excess NADH oxidase activity using 5 mM pyruvate plus 5 mM oxaloacetate. The NADH, NADPH and malate

oxidase activities were measured according to Ackrell and Jones (1971), glucose-6-phosphate dehydrogenase according to Senior and Dawes (1971). Acridine fluorescence was measured as described by Haaker et al. (1974) using 5 μM atebrin instead of 9-amino-acridine. For a TET preparation the fluorescence assay mixture contained a pyruvate dehydrogenase assay mixture plus 0.2 mg catalase, final volume 2 ml. The fluorescence of a TE preparation was performed in a mixture containing 20 mM Tris-HCL, 1 mM $MgCl_2$, 5 mM pyruvate and 0.2 mg catalase, final pH 7.4 final volume 2 ml. Photoreactions were performed in the nitrogenase assay mixture under the following additions: 10 or 40 μM deazaflavin (3,10 dimethyl-5-deazaisoalloxazine), flavodoxin purified according to Yoch (1972 a). The light intensity was 15 mW/cm^2 measured between 400-500 nm. Anaerobic spectrophotometry was performed with "Subaseal" stoppered cuvettes on a Cary-16 spectrophotometer final volume 1 ml. Oxygen inactivation was followed by exposing nitrogenase to air as described by Kelly (1969) in a medium containing 25 mM Tris-HCL pH 7.4 and 5 mM $MgCl_2$, final volume 2 ml. 0.3 ml samples were taken and examined for nitrogenase activity. Anaerobic gel electrophoresis was performed according to Kedinger et al. (1974) except that thioglycolic acid was omitted and 2 mM $Na_2S_2O_4$ was added to the upper buffer, after flushing with argon for 30 min. Samples were prior to application diluted in a medium containing: 25 mM Tris-HCL, 2 mM $Na_2S_2O_4$, 5 mM $MgCl_2$,20% sucrose and 0.04 mM indigosulfonic acid, final pH 7.6. About 50 μg of protein was applied/gel.

BIOCHEMICALS

All enzymes and cofactors were purchased from Boehringer. Flavodoxin from P. *elsdenii* and D. *vulgaris* were gifts from S.G. Mayhew; deazaflavin was a gift from V. Massey and S. Ghisla.

3. RESULTS AND DISCUSSION

OXYGEN PROTECTION

Oppenheim et al. (1970) and Drozd and Postgate (1970) suggested that a nitrogenase-cytoplasmic membrane complex or the presence of cytoplasmic membranes is responsible for an oxygen-tolerant nitrogenase. When *Azotobacter* cells are ruptured by osmotic shock, the cell free extract is relatively free of cytoplasmic membranes and the nitrogenase was oxygen sensitive (Oppenheim et al. 1970). this contrasts with a cell

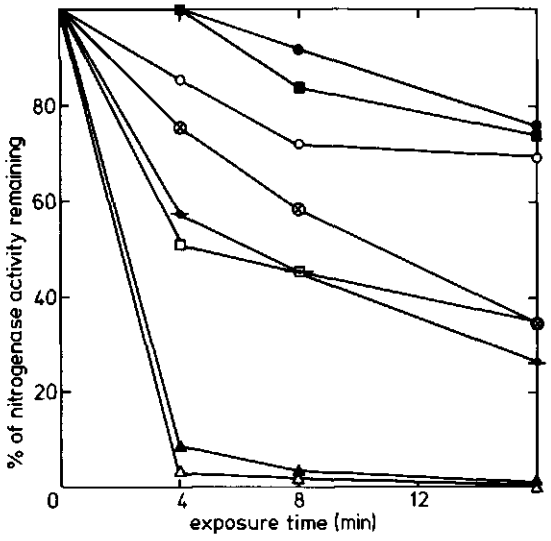

Fig. 1 Oxygen stability of different nitrogenase preparations of *A. vinelandii*. Conditions as described in Materials and Methods. For every tested preparation a nitrogenase activity of about 100 nmoles ethylene produced per min. was used.
●—●, CEM; O—O, CEO; ⊗—⊗, CEO+ 0.1 M KCL
■—■, C 42-I-M; □—□, C 42-I-O; ◐—◐ C 42-I-90;
Δ—Δ, crude I + crude II (1:6); ▲—▲ Crude I + crude II (1:6) plus cytoplasmic membranes 3.6 mg

extract prepared after disruption with a French pressure cell. Fig. 1 gives a comparison of the oxygen sensitivity of nitrogenase preparations prepared by different cell-lysis methods. Addition of o.1 M KCL to the cell-free extract of osmotically lysed cells increases the oxygen-sensitivity considerably. Since in the experiments presented by Oppenheim et al. (1970) in the osmotically lysed cell extract 0.1M KCL was present, we think that the observed oxygen-sensitivity is due to the ionic strength and not to the method of cell-rupture. Fig. 1 also shows that during the purification of the nitrogenase complex according to Bulen and Le Comte (1972) the oxygen stability is relatively constant. After eluting the nitrogenase as a single fraction with 90 mM $MgCl_2$ from a DEAE-cellulose column, the nitrogenase is more oxygen sensitive than the original complex. The nitrogenase complex prepared by mixing the crude components is extremely oxygen sensitive and only a slight stabilization against oxygen inactivation can be obtained by adding washed cytoplasmic mem-

branes. This in contrast with the effect of a crude or purified NADH dehydrogenase reported by Yates (1970). Fig. 1 also shows that the oxygen inactivation is bi-phasic. The first phase shows a rapid inactivation; the extent depends on the type of preparation. The second phase is slower and occurs in all preparations.

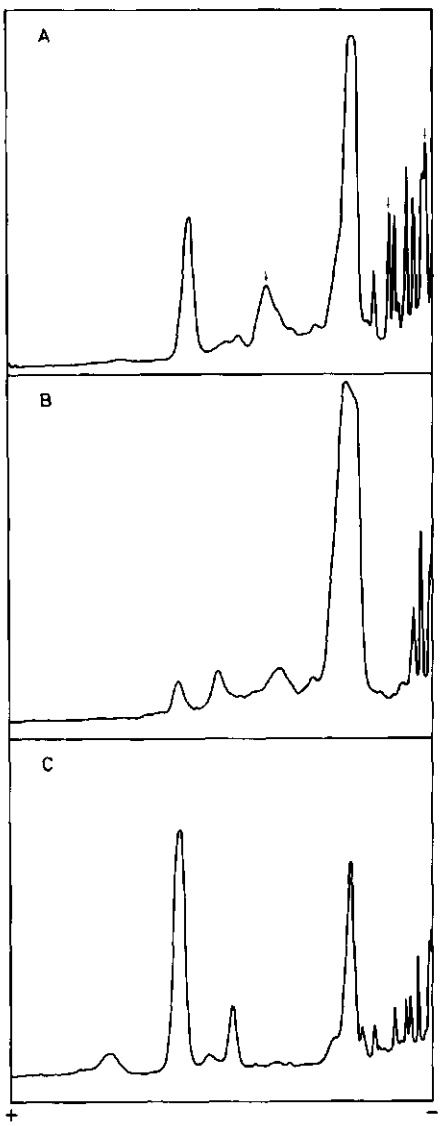

Fig. 2

Anaerobic non-denaturating polyacrylamide gel electrophoresis of "C 42-I" nitrogenase preparations. Gels were prepared and run as described in Materials and Methods. Patterns are from the following preparations: A, C 42-I; B. crude component I; C. Crude component II.

Fig. 2 shows anaerobic polyacrylamide gel electrophoresis of different nitrogenase preparations. The nitrogenase components are easily detectable; these main bands were shown to be Fe-containing by reaction with dipyridyl reagent according to Brill et al. (1974). In no case protein was retained on the stacking gel, therefore the oxygen-protecting proteins must be detectable on the gels. The first pattern is that of a nitrogenase complex purified according to Bulen and Le Comte (1972) up to the second Mg precipitation, which has high NADH dehydrogenase activity (NADH oxidase activity also was measurable). The activity of the complex is stable for at least 3 months at 0°C under Ar. When this preparation was applied to a DEAE-column and eluted succesively with 0.25 M and 0.35 M NaCL, the eluates showed patterns 2 and 3. These eluates or combinations have no NADH oxidase or NADH dehydrogenase activity and upon storing the components 2-3 weeks separately, no nitrogenase activily could be induced by combining them. Arrows in Fig. 2A indicate the proteins which are only present in the oxygen stable complex and the proteins may be involved in stabilization and oxygen protection of the nitrogenase. The results presented in Fig. 1 and 2 allow us to conclude that oxygen stability of *Azotobacter* nitrogenase is not explained by the presence of cytoplasmic membranes but is caused by a high molecular weight protein factor that forms more or less a complex with the nitrogenase. The method of cell-rupture determines only partly the formation of such a complex.

PROPERTIES OF TOLUENE-TREATED AZOTOBACTER CELLS

Jackson and Demoss (1965) showed that low concentrations of toluene makes bacterial membranes permeable to small molecules. Since the possibility exists that toluene attacks the permeation barrier and does not influence the original structure, such a preparation may be useful for studying the generation of reducing equivalents for the nitrogenase in *Azotobacter*.

Table I gives some information about the influence of toluene on the permeation of important metabolites and cofactors. All cofactors tested like ATP, TPP, CoASH, NAD^+, $NADP^+$ permeate into a TET preparation. Probably the diffusion limit for molecules with a mol. wt. less than 1000 is relatively small; however, since cytoplasmic enzymes do not leak into the medium, enzymes which are added externally probably do

Table I.

Some enzymatic properties of A.vinelandii cells after a toluene treatment. TET preparation was prepared and enzyme activities were measured as described in the text.
The activity is expressed as nmoles min^{-1}. mg^{-1}.

nitrogenase	TET	sonicated TET
complete	1.6	3.0
no-ATP regenerating system	0.3	0
no-dithionite	0	0
pyruvate dehydrogenase complex		
complete	105	66
no-pyruvate	4.0	7.3
no-TPP	6.0	7.8
no-CoASH	5.3	6.9
no-NAD^+	0	0
glucose-6-phosphate dehydrogenase		
complete	410	440
no-NAD^+, $NADP^+$	0	0
no-glucose-6-P, NAD^+	13	12
no-glucose-6-P, $NADP^+$	17	4
no-glucose-6-P	30	100
oxidases		
NADH-oxidase	347	659
NADPH-oxidase	95	280
malate-oxidase	200	350

not pass the membranes. Since the diffusion limitation for small molecules is also occurring with the products, one must be careful with the interpretation of the observed velocities in a TET preparation. Nitrogenase activity produces ADP, a strong inhibitor (Ljones, 1973). Since ATP has to be regenerated on the other side of the membrane, the diffusion of ADP out of the TET vesicle probably determines the maximum nitrogenase activity in a TET preparation. The increase in oxidase activity is due to a membrane destruction by sonication. Freezing and thawing of *Azotobacter* membranes also increases the oxidase activities (Jones and Redfearn, 1966). As shown in Table I glucose-6-phosphate and other substrates like isocitrate and $NAD(P)^+$ have no diffusion limit into a TET preparation in spite of the very high enzyme activities. Another aspect of a TET preparation is the enzyme organization. In a TET preparation one can expect a higher degree of organization than in ruptured cell preparations. The pyruvate dehydrogenase overall reaction, measured by the oxygen uptake via NADH oxidase, is an example of this higher degree of enzyme organization in a TET preparation. Because NADH and especially AcCoA strongly inhibit the overall reaction (Bresters et al., 1975 b), it is necessary to generate CoASH and NAD^+ efficiently to obtain a maximum steady-state activity. Because it is unlikely that enzymes such as the pyruvate dehydrogenase complex or citrate synthase are inactivated by sonication, a higher degree of enzyme organization must be the reason for the higher activity of the combined action of pyruvate dehydrogenase complex, citrate synthase and NADH oxidase in a TET preparation as compared with a sonicated TET preparation. However, one cannot exclude the possibility that, in the case of nitrogenase, the higher degree of organization within the membrane leads to lower activity, as discussed on theoretical grounds by Goldman and Katchalski (1971).

REACTION OF FLAVODOXINS WITH NITROGENASE

Complete reduction of flavodoxins, especially the flavodoxin of *Azotobacter*, has long been a problem. Photoreduction of flavodoxin in the presence of EDTA, acetate and Tricine was not possible but with catalytic amounts of deazaflavin it is possible to photoreduce flavodoxins fast and completely (V. Massey, personal communication). With this system, we were able to reduce different types of flavodoxins completely and studied their reactivities as electron donor for the nitrogenase.

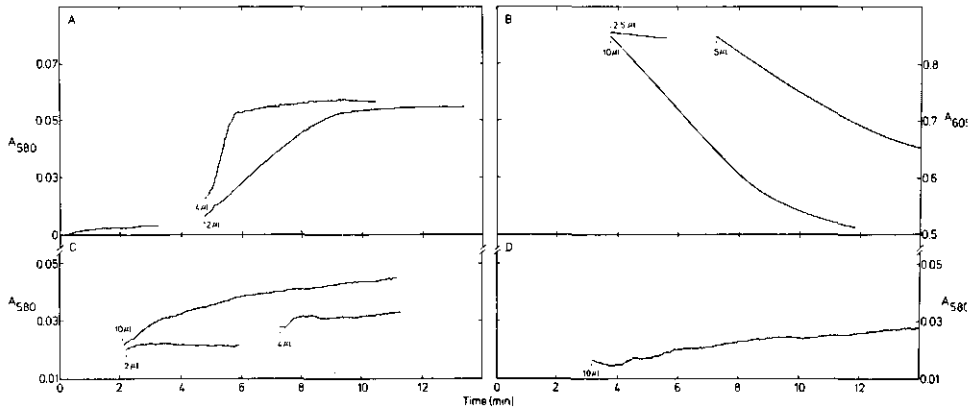

Fig. 3 Time course of enzymatic and non-enzymatic oxidation of photo-reduced electron carriers.
Flavodoxins are from the following organisms A, *A.vinelandii* (0.3 mg/ml); C, *P.elsdenii* (0.2 mg/ml); D. *D.vulgaris* (0.2 mg/ml; B, of methylviologen (0.2 mM). Reactions were performed with 10 µM deazaflavin as described in the text. Different amounts of enzyme C 42-I, 12.5 mg/ml were added at the times indicated.

Reduced flavodoxins from *A.vinelandii*, *P.elsdenii* and *D.vulgaris* were treated with different amounts of partly purified nitrogenase and rates of electron transfer were measured spectrophotometrically as shown in Fig. 3. These rates differ widely and one can observe that of the three types of flavodoxin the *Azotobacter* flavodoxin is by far the best electron donor. Continuous illumination with a fixed limiting amount of carrier, depending on the mol. wt. 21-32 µg/ml a non-rate-limiting amount of 1.4 nmol./ml nitrogenase complex (0.24 mg/ml) and 20% C_2H_2 present gave rates of 2, 0.3 and 0.2 nmoles ethylene produced min^{-1}. $nmol^{-1}$ of flavodoxin, respectively. On the other hand we have observed that when *P.elsdenii* flavodoxin (0.42 mg/ml) was continuously photo-reduced by chloroplasts and a cellfree extract of *A.vinelandii* was added (with endogenous electron carriers present) rates of C_2H_2-reduction approached that of the dithionite-driven reaction. *A.vinelandii* flavodoxin 0.55 mg/ml is hardly active (10%) in this system because it

is not reduced beyond the semiquinone state (c.f. Benemann et al., 1969). So presumably the redox potential as well as the "fit" with the nitrogenase are important in determining the rate of electron transfer mediated by a flavodoxin. Lastly, the importance of redox potential is especially clear when the extent of oxidation of reduced methylviologen by nitrogenase was compared with that of reduced benzylviologen. Benzylviologen, photoreduced as far as possible with our system, can be oxidized by nitrogenase only slightly whereas Fig. 3B shows that reduced methylviologen can be oxidized to a high extent. By continuous illumination, however, no difference in reaction rates with either methylviologen or benzylviologen as carriers can be observed.

LOCALISATION OF FLAVODOXIN IN A TET PREPARATION

As shown in Fig. 3A fully-reduced flavodoxin from *Azotobacter* acts as a very good electron donor for purified nitrogenase. Under constant illumination photo-reduced flavodoxin has for at least 20 min. the same activity as dithionite as electron donor for the nitrogenase; also in a crude extract (Fig. 4B).

Fig. 4A,B shows the low nitrogenase activity with photochemically reduced deazaflavin in a TET preparation in contrast with the activity of a sonicated TET preparation. With a purified nitrogenase complex, photochemically reduced deazaflavin has an activity of about 10% of the dithionite activity. This means that the observed nitrogenase activity with deazaflavin in a sonicated TET preparation, about 50% of that of the dithionite activity, is due to the presence of endogenous flavodoxin, ferredoxin or another unknown low-potential electron donor.

As already shown in Tabel I dithionite can act as electron donor for nitrogenase in a TET or sonicated TET preparation (Fig. 4). The same is true for photochemically reduced benzylviologen. In a TET preparation the rate-limiting step in the overall nitrogenase activity is probably not the donation of electrons by the reducing agent but the diffusion of the strong inhibitior ADP out of the vesicle, because the rate of ethylene production is nearly the same with dithionite as with reduced benzylviologen. With a purified nitrogenase complex the rate of ethylene production with reduced benzyl- or methylviologen (see also Fig. 3) is about 1/3 of that of the dithionite- or reduced flavodoxin driven nitrogenase activity. The observed activity with benzylviologen in a soni-

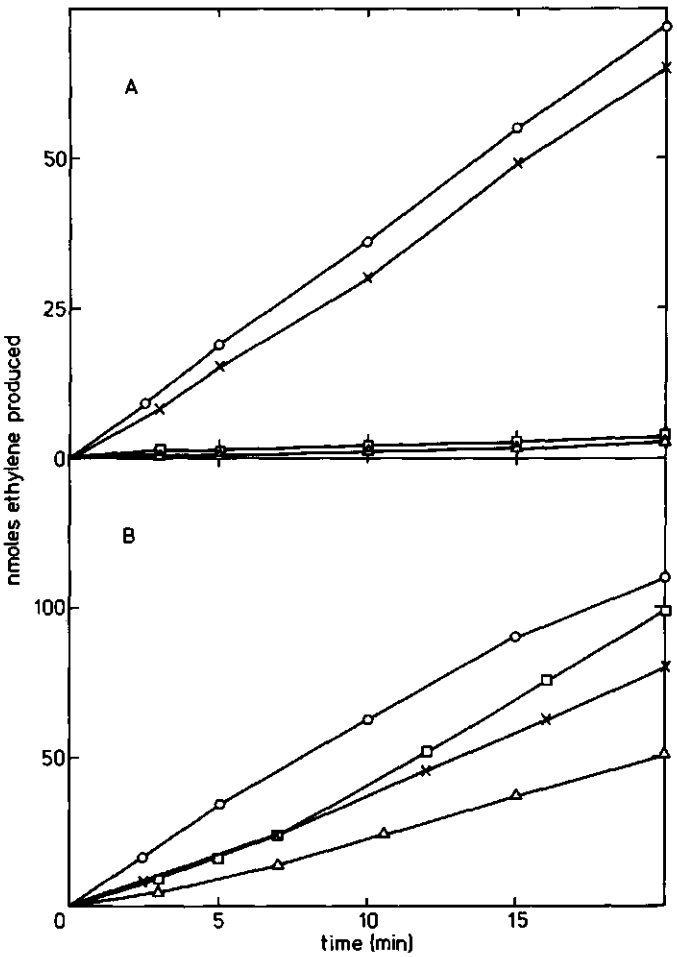

Fig. 4 Effect of different electron donors on the nitrogenase activity in toluene-treated *A.vinelandii* cells or sonicated toluene-treated *A.vinelandii* cells. Preparations were made and activities measured as described in Material and Methods. A, 0.93 mg TET preparation was suspended in the acetylene assay mixture. Electron donors: O——O, dithionite; X——X, light, 40 µM deazaflavin, 40 µM benzylviologen; Δ——Δ, light, 40 µM deazaflavin; □——□ light, 40 µM deazaflavin, 3.8 nmoles *A.vinelandii* flavodoxin. B, 1.1 mg sonicated TET preparation was suspended in the acetylene assay mixture same symbols as A.

cated TET preparation is of course the sum of the activity with photochemically reduced benzylviologen plus that with the photochemically reduced endogenous electron carriers.

The results indicate that benzylviologen and dithionite react in the TET preparation directly with the nitrogenase or a site close to it. Furthermore the lack of activity of the TET preparation with deazaflavin indicates that flavodoxin and ferredoxin are buried inside the membrane and thus are not reducable. The lack of activity of a TET preparation in the presence of deazaflavin plus flavodoxin is explained by the fact that the flavodoxin does not penetrate through the membrane.

ELECTRON TRANSPORT SYSTEM TO NITROGENASE IN *AZOTOBACTER*

Benemann et al. (1971) suggested that reduced pyridine nucleotides donate electrons via ferredoxin and flavodoxin to the nitrogenase. We tried to repeat their experiments with extracts of mechanically-ruptured cells, but where unsuccessful. The reason for the failure of these experiments in our hands is not known, but it might be connected with the fact that the redox properties of flavodoxin from the strain of *Azotobacter* used by Benemann et al. (1974) differ from those of flavodoxin from the strain used by us and others (Yoch, 1975).

Because a TET preparation certainly has organized structures which could provide an electron donating system for nitrogenase, we tested such preparations for acetylene reduction with organic substrates as the ultimate source of reducing equivalents. However, in the presence of the substrates and cofactors used by Benemann et al. (1971) the rate of ethylene production was very low (0.1 nmoles. min^{-1}. mg^{-1}). In earlier publications (Haaker et al., 1974; Haaker and Veeger 1976) we proposed that so called energized state of the cytoplasmic membrane is important in nitrogen fixation by whole cells and possibly coupled to the generation of reducing equivalents. We therefore investigated whether it is possible to energize the cytoplasmic membrane in a TET preparation by using the change of acridine fluorescence as a probe (Haaker et al., 1974). Intact cells (TET preparation) show a high nitrogenase activity with pyruvate as substrate (Haaker and Veeger, 1976) and under aerobic conditions they cause a large increase in atebrin fluorescence indicating that the cytoplasmic membrane is highly energized (Fig. 5).

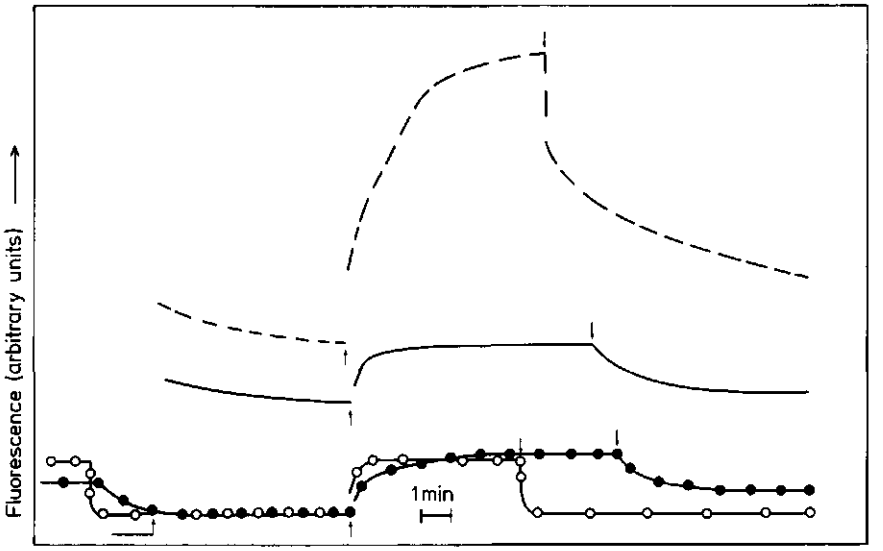

Fig. 5 Effect of toluene on the enhancement of the atebrin fluorescence upon energization of *A. vinelandii* cells. *A. vinelandii* cells were treated with toluene and fluorescence was measured as described in Materials and Methods. O——O, 8 mg TET preparation in 2 ml medium; ●——●, 8 mg TET preparation in 2 ml medium. At the arrow ——↑ 5 µM atebrin was added, and fluorescence followed ——, TET preparation; ----, TE preparation. At arrow ↑ 0.34 µmole H_2O was added to a TET preparation and 3.4 µmole H_2O to a TE preparation. At ↓ anaerobiosis was reached. The fluorescence of both preparations in the absence of atebrin are given in the corresponding ●——● and O——O curves. The corresponding arrows indicate the time of H_2O addition (corresponding amounts) and anaerobiosis.

Table I and Fig. 5 show that TET preparations also oxidize pyruvate, but that there is no concomitant energization of the membrane. This lack of energization is not surprising since TET preparations are not selectively permeable for substrates and are thus permeable for protons.
This could be the reason why no nitrogen fixation can be observed aerobically or anaerobically in a TET preparation while the conditions for nitrogen fixation are optimal: organic substrates which introduce high rations of reduced to oxidized pyridine nucleotides, together with a high ratio of ATP to ADP. Such conditions are not possible even in whole cells.

A further attempt was made to obtain a preparation in which the oxidation of organic substrates could be coupled to nitrogenase. Since it was observed that crude nitrogenase preparations become very sensitive the oxygen in the presence of reducing agents (dithionite or oxidizable substrates), we worked under anaerobic conditions. Pyruvate is chosen as substrate because it is found that under anaerobic conditions pyruvate oxidation can be coupled to nitrogenase, although the activity is low (1% of the activity with pyruvate under optimal aerobic conditions). Cells are broken gently by treating them with lysozyme in the presence of Tris-EDTA. During cell-lysis the activity with pyruvate decreases to zero indicating that even these mild conditions finally cause completely loss of coupling between pyruvate oxidation and nitrogenase activity. Coupling is not restored by adding of a variety of cofactors, but full nitrogenase activity is observed with dithionite and ATP. However, there is some evidence for intermediate structures which respond to substrates that are thought to influence the ratio Acetyl CoA/CoASH. During the course of the lysis reaction oxaloacetate cause a 2-fold stimulation of activity and β-hydroxy-butyrate and acetylphosphate cause an inhibition. None of these components has any effect on the activity of whole cells. It appears therefore that, at an intermediate stage of lysis, the cell becomes permeable to low mol. wt. compounds. These observation emphasise the strong dependence of nitrogenase activity with physological substrates on an intact cytoplasmic membrane and suggest that the ratio Acetyl CoA/CoASH can be important in the coupling with pyruvate oxidation.

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IV Involvement of the cytoplasmic membrane in nitrogen fixation by *Azotobacter vinelandii*

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SUMMARY

1. *Azotobacter vinelandii* cells were ruptured by using a French pressure cell, by lysozyme treatment, or by osmotic shock. The rates of sedimentation of nitrogenase from these extracts were compared and related to the rates of sedimentation of marker enzymes. No evidence was found for the so-called particulate nitrogenase or an interaction between nitrogenase and cytoplasmic membrane vesicles. Nitrogenase sediments as a complex and the rate of sedimentation is comparable with that of pyruvate dehydrogenase complex.

2. The oxygen stability of nitrogenase from *Azotobacter* is not caused by the presence of cytoplasmic membranes in extracts but by a complexation of the nitrogenase components with an Fe-S protein. Recombination experiments showed that maximum stabilization against oxygen was only obtained at certain ratios of the Fe-S-protein and nitrogenase complex. It is proposed that the switch-off state in *Azotobacter* cells is caused by the oxidation of flavodoxin hydroquinone rather than by a reversible inactivation of the nitrogenase.

3. A membrane-bound NAD(P)H-flavodoxin oxidoreductase was detected. Evidence was obtained that flavodoxin might also be membrane-bound.

4. A proposal is given for electron donation to nitrogenase in *Azotobacter*. In this proposal the generation of reducing equivalents for nitrogenase is localized in the cytoplasmic membrane and mediated by the NADH-flavodoxin oxidoreductase. The pH gradient which is generated by membrane energization, is used to reduce flavodoxin to its hydroquinone form in order to achieve a redoxpotential low enough to reduce nitrogenase.

INTRODUCTION

It is generally accepted that nitrogen reduction is an anaerobic process, and that organisms which require oxygen to obtain energy for nitrogen fixation have mechanisms that protect nitrogenase from the deleterious effects of oxygen. Two protection mechanisms have been proposed for *Azotobacter* spp (1),

both involving the cytoplasmic membrane; augmented rates of respiration to scavenge excess oxygen (respiration protection) and a conformational state of nitrogenase that prevents damage by O_2 (conformational protection). Some information is available about respiration protection (2-5), but the literature about the conformational protection of nitrogenase is somewhat confusing. For example it has been assumed that after rupture of *Azotobacter* cells with a French pressure cell, the nitrogenase in the resulting crude extract is oxygen tolerant (6-8) and particle-bound (9,10). On the other hand Oppenheim et al. (11) showed that after rupture of the cells by osmotic shock, nitrogenase is oxygen-labile and soluble. These authors, as well as Drozd and Postgate (2), suggested that the particle-bound nitrogenase may represent the conformational protected nitrogenase. In contrast to Oppenheim et al. (11), Reed et al. (12), reported that the sedimentation behaviour of the nitrogenase is independent of the method of cell rupture. A further function of the cytoplasmic membrane in obligate aerobic nitrogen-fixing organisms may be the generation of reducing equivalents for the nitrogenase. Yates and Daniel (13) and Biggins ans Postgate (14) reported that in membrane preparations low nitrogenase activity in the absence of artificial electron carriers could be observed. We have shown (15) that the energized cytoplasmic membrane is involved in the generation of reducing equivalents for the nitrogenase in intact cells of *Azotobacter vinelandii*.

In this study we report on the interaction of the cytoplasmic membrane with the nitrogenase with respect to the localisation of nitrogenase and the role of the cytoplasmic membrane in the protection against oxygen and the electron donation to the nitrogenase.

MATERIALS AND METHODS

Growth conditions and enzyme preparations

Azotobacter vinelandii ATCC 478 was grown in a batch culture of 2500 liter, harvested during the logarithmic phase and stored as described earlier (16). Crude extracts were prepared in three ways.

1. Mechanically, using a Manton Gaulin homogenizer, a French pressure cell.
2. Enzymically by incubating 1.0 g of bacterial protein in 40 ml 10 mM EDTA-Tris, pH 8.0, with 40 mg lysozyme. After 120 min at 24° C, $MgCl_2$ was added to 11 mM. The pH, which dropped to 6, was brought with Trizma base (Sigma) to pH 7.4 and 0.1 mg/ml deoxyribonuclease was added. After a further 30 min the preparation was used for centrifugation experiments.

3. Osmotically by the method described by Shah et al. (17).

Centrifugation experiments were performed with an MSE 75 ultracentrifuge using an 8 x 35 ml rotor. After each centrifugation stap, the supernatant plus fluffy layer was used for the next following centrifugation step. The following centrifugation scheme was used: 20,000 x g, 30 min, P_1; 100,000 x g, 30 min, P_2; 200,000 x g, 60 min, P_3; 200,000 x g, 240 min, P_4 and S_4. All operations were done under argon, pellets were suspended in 25 mM N-tris [hydroxymethyl methyl-2-aminoethane] sulfonic acid-KOH (TES-KOH), 1 mM $MgCl_2$, pH 7.4. The P_1 pellets were washed twice with 25 mM TES-KOH, 1 mM $MgCl_2$, pH 7.4. Nitrogenase complex was purified as described by Bulen and Le Comte, till the second $MgCl_2$ precipation (C 42-I) (18). The nitrogenase complex, C 42-I, was separated into 3 fractions by DEAE cellulose chromatography. The first fraction was eluted with 0.15 M NaCl, the second fraction with 0.27 M NaCl and a third fraction with 0.4 M NaCl. Ferredoxin and flavodoxin were purified as described by Yoch and Arnon (19). Fresh grown cells were used to prepare toluene treated *Azotobacter* cells. 100-200 mg bacterial protein was suspended in 50 mM Tris-HCl, 10 mM EDTA, 0.2 ml toluene, final pH 8.0, final volume 20 ml. The suspention was held anaerobically at 0^oC for 15 min. After centrifugation (10,000 xg, 5 min.) at 4^0C, the pellet was washed with 40 ml 25 mM Tris-HCl pH 7.4 and 5 mM $MgCl_2$ and centrifuged again. The pellet was resuspended in the same buffer, stored at 0^oC, sonicated or treated with 1 mg/ml lysozyme at 30^oC for 60 min.

Analytical methods

Acetylene reduction assays were run at 30^o, shaking in 6.5 ml bottles sealed with Subaseal, which contained a mixture of 25 mM Tricine buffer, 1 mM ATP, 2 mM $MgCl_2$, 10 mM creatine-phosphate and 5 U creatine kinase final pH 7.5. This mixture was thorougly flushed for at least 30 min. with purified argon which passed a heated Basf catalyst. The acetylene (10%) and 20 mM dithionite were added. The mixture was equilibrated for 10 min. with the gasphase to remove the last traces of oxygen, after which the nitrogenase preparation was added and the ethylene produced was measured as described earlier (15). Several amounts of a nitrogenase preparation were analysed to exclude an underestimation of the nitrogenase activity caused by low nitrogenase concentrations.

Photoreactions were performed in the nitrogenase assay mixture with 40 μM deazaflavin (3,10 dimethyl-5-deazaisoalloxazine), 10 mM glucose, 10 U glucose oxidase and 40 μg catalase/ml. The light intensity was 20 mW/cm^2 measured between 400-500 nm. The NADH and NADPH oxidase activities were measured as

described by Ackrell and Jones (3). Acridine fluorescence was measured at pH 7.5 in a 2 ml incubation mixture containing 25 mM TES-KOH, 1 mM $MgCl_2$, 10 µg catalase, 2 mM NADH and 5 µM 9-amino-6-chloro-2-methoxyacridinehydrochloride. Per incubation 0.5 - 1.0 mg bacterial protein was added. The fluorescence measurements were performed with a Perkin Elmer fluorimeter, excitation wavelength 410 nm, emission wavelength 490 nm. The amount of quenching obtained upon energization of the cytoplasmic membrane was calculated from the difference between the fluorescence after all of the oxygen had been consumed, and the fluorescence under aerobic conditions. At the protein concentrations and wavelengths used, the contribution to the fluorescence of bacterial components was negligible.

A highly effective NAD(P)H regenerating system was obtained with 25 mM TES-KOH, 5 mM $MgCl_2$ 10 mM glucose-6-phosphate, 20 µg/ml glucose-6-phosphate dehydrogenase, 0.22 mg/ml transhydrogenase, 0.25 mM $NADP^+$ and 0.25 mM NAD^+, final pH 7.5. Pure transhydrogenase of *Azotobacter vinelandii* was used (21).

NADH-flavodoxin oxidoreductase was measured in 25 mM TES-KOH, 1 mM $MgCl_2$, 10 mM glucose, 0.5 mg/ml glucose oxidase, 10 µg/ml catalase, 2 mM NAD(P)H and 15 µM flavodoxin, final pH 7.4. The absorbance increase at 580 nm was monitored. For the calculations we used the following molar extinction coefficients for *Azotobacter vinelandii* flavodoxin: $E_{455}ox.= 10,600\ M\ cm^{-1}$, $E_{580}semiquinone= 5.540\ M\ cm^{-1}$ (22).

Transhydrogenase was measured according to Krul (21), pyruvate dehydrogenase according to Schwartz and Reed (23), glucose-6-phosphate dehydrogenase according to Senior and Dawes (24) and NADH dehydrogenase according to Yates (25) using benzylviologen as the electron acceptor.

Nitrogenase was exposed to air in 25 mM TES-KOH, pH 7.5. Prior to oxygen inactivation, crude component I and crude component II and stabilizing factor were brought together under argon. Complexation was complete after 30 min at room temperature. The incubation mixture was then rapidly aerated by using a whirl mixer. Tests showed that within 5 sec the incubation mixture was saturated with air. After 1 min, an 0.1 ml sample was examined for nitrogenase activity. Anaerobic DEAE cellulose chromatography was performed according to Shah et al. (17). The eluates were anaerobically concentrated and desalted with an Amicon ultrafiltration cell using a UM 10 filter. Anaerobic gel electrophoresis was performed as described previously (20) exept that thioglycolic acid was replaced by o.o4 mM indigosulfonic acid and 2 mM $Na_2S_2O_4$ was added to the upper buffer after flushing with argon for 30 min. About 50 µg of protein was applied/gel.

Biochemicals

All enzymes and cofactors were purchased from Boehringer. Deazaflavin was a gift from V. Massay and S. Ghisla.

RESULTS AND DISCUSSION

LOCALISATION OF NITROGENASE

The sedimentation behaviour of nitrogenase was determined after exposure *A. vinelandii* cells to three different breakage procedures and correlated with the sedimentation behaviour of a number of other enzyme activities (Fig 1). The known characteristics of the marker enzymes allowed the fragmentation, caused by each cell rupture method, to be assessed.

NADH oxidase was used as a marker enzyme for the cytoplasmic membranes. Its activity correlated well with the amounts of cytochromes present in a particulate preparation. The mutual ratios of the cytochromes were the same in all membrane preparations indicating that they are parts of the same membrane. Fig. 1 clearly shows that during breakage of the cells with a French pressure cell, the cytoplasmic membrane degrades into a large number of pieces, and that enzymic attack on the peptidoglycan layer has a similar effect. During lysozyme treatment no mechanical or osmotic forces are exerted on the cells, only the rigidity due to the peptidoglycan layer is lost. This effect is probably enough to cause the formation of vesicles and fragments from the cytoplasmic membrane. The osmotic shock is a relatively mild cell rupture method, which has little effect on the peptidoglycan layer, since most of the cytoplasmic membrane remains attached to it (11). These experiments show that the sedimentation behaviour of cytoplasmic membrane vesicles and fragments is determined by the efficiency of rupture of the peptidoglycan layer during cell breakage. The NADH oxidase activity observed in cytoplasmic membranes prepared after breakage of the cell with a French pressure cell is remarkably high. An explanation may be the higher degree of inside-out orientation of these membranes compared with the membranes prepared by lysozyme treatment or osmotic shock (26). Since the cytoplasmic membrane is impermeable for NADH, one can expect a higher NADH oxidase with the converted vesicles.

In general acridine fluorescence can be used as a probe for the energized state of a number of cytoplasmic membranes, including *Azotobacter* membranes (27). Upon energization, the dye binds to the membrane and the fluorescence

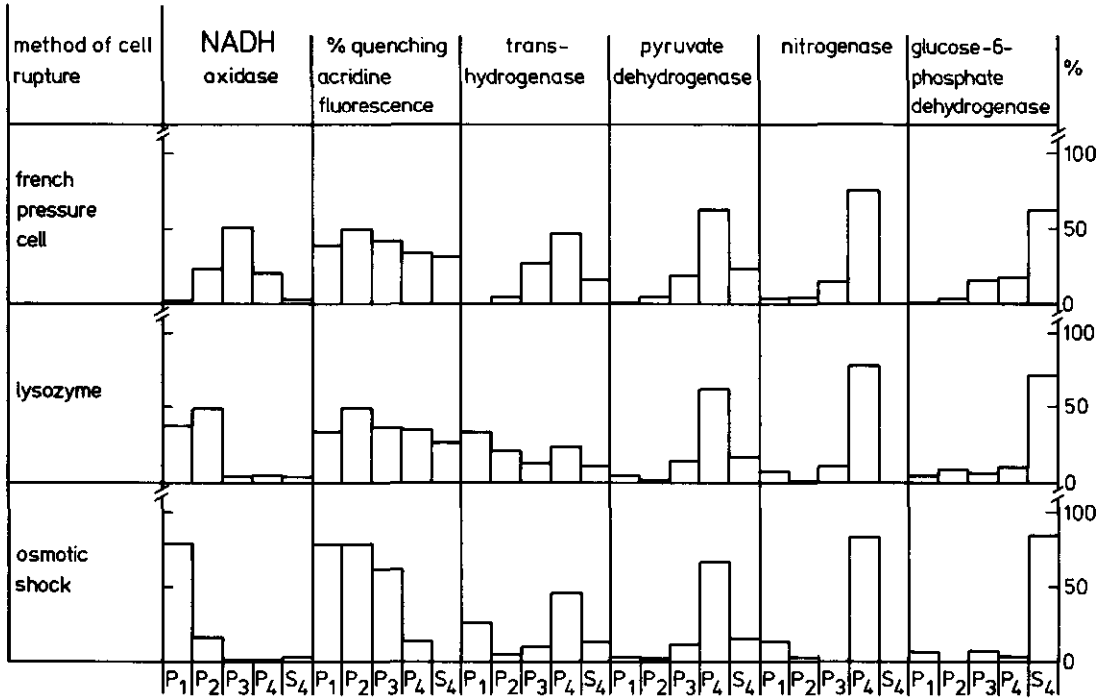

Fig. 1. Localization of nitrogenase after different cell rupture methods compared with marker enzymes. Cells were ruptured, 6.2 g protein was used, the centrifugation scheme was performed and enzyme activities were measured as described in Methods. 100% was taken as the sum of the activities present in the fractions, which was always in good agreement with the total activities present in the crude extracts. The total activities in the crude extracts were: in sequence, extracts prepared with a French pressure cell, with lysozyme and with an osmotic shock. NADH oxidase, 1183, 634, 605; transhydrogenase, 1251, 1165, 892; pyruvate dehydrogenase complex, 520, 406, 443; nitrogenase, 52, 35, 25; glucose-6-phosphate dehydrogenase, 1251, 1165, 892. The activities are expressed in $\mu moles.min^{-1}$. Each block represents the percentage of the total activity present in that fraction.

is quenched. We checked that under our conditions the quenching of the fluorescence is not dependent on the protein concentration, but on coupling between oxidation and energization; addition of a low concentration of uncoupler caused a return of fluorescence. Cytoplasmic membranes can be depleted of ATP-ase by incubating the membranes in a medium of low ionic strength. Such ATP-ase depleted membranes do not show quenching of acridine fluorescence upon energization; but this can be restored by adding crude ATP-ase (27, 28). We used this fluorescence method to determine whether the method of cell rupture removed tightly bound enzyme such as the membrane-bound ATP-ase and to demonstrate closed vesicles in membrane preparations in which oxidation can be coupled to energization. Control experiments show, ATP-ase activity in preparations where acridine fluorescence quenching is observed. ATP-ase remains bound to all cytoplasmic membranes preparations except P_4 and S_4 obtained from osmotic shocked cells; the P_1, P_2 and P_3 pellets obtained by this rupture method are, however, well coupled. Some small membrane vesicles are present in S_4 derived from a French pressure cell and lysozyme ruptured cell extract, since during centrifugation the fluffy layers are taken with the supernatants.

In most organisms transhydrogenase is membrane-bound and the production of NADPH from NADH can be driven by energy. It is possible to solubilize the enzyme but it is then unable to catalyse the energy-linked reduction of NADPH. In *Azotobacter*, transhydrogenase is readily solubilized, but when the cells are opened more gently (lysozyme and osmotic shock) transhydrogenase is found in P_1 and P_2 (Fig. 1). In these membrane preparations energy-linked transhydrogenase activity is measurable (29, H. Haaker unpublished observation). Purified transhydrogenase has a minimum molecular weight of 54,000 but in the presence of $CaCl_2$ the enzyme aggregates (21). Therefore it is not possible to discriminate between membrane bound transhydrogenase or highly polymerized transhydrogenase in the P_3 and P_4 sediments. From the results presented in Fig. 1 we propose to use transhydrogenase as a marker for loosely membrane-bound enzymes.

The isolated pyruvate dehydrogenase complex from *Azotobacter* (minimum mol. weight 1.10^6, c.f.ref. 16) sediments after 4 h. 200.000 xg. The sedimentation behaviour of the complex in our preparations is similar indicating that it does not form an association with the cytoplasmic membrane. Therefore the pyruvate dehydrogenase complex can be used as a marker for enzyme complexes.

The glucose-6-phosphate dehydrogenase sedimentation characteristics are those of a relatively small enzyme (mol. weight $\pm$ 100,000). Glucose-6-phos-

phate dehydrogenase can be used as a marker for small soluble enzymes.

With this set of marker enzymes, we think it is possible to answer the question whether nitrogenase in *Azotobacter* is soluble or particulate and whether its distribution in these fractions depends on the method used to rupture the cells.

Fig. 1 clearly shows that the nitrogenase sedimentation behaviour is independent of the cell rupture method and that all nitrogenase activity occurs in the pellet after centrifugation of the cell extracts 4 hours 200,000 xg. It is also clear that when the cells are ruptured by a mechanical method the cytoplasmic membrane desintegrates, and fragments sediment together with the nitrogenase, producing the so-called particulate nitrogenase. Some association with membrane fragments may occur but if so it is only a weak interaction. This conclusion is supported by experiments which confirm those of Reed et al. (12) who showed that when a nitrogenase-containing sediment, in our case P_4, is brought on a linear sucrose density gradient, nitrogenase activity separates from fragments of the cytoplasmic membrane. Nitrogenase activity is found near the top of the gradient, (0.6 M sucrose) and the cytochromes in the middle of the gradient, (around 1.6 M sucrose). Another indication that in the so-called particulate nitrogenase, there is no interaction between nitrogenase and the cytoplasmic membrane is the observation that nitrogenase activity is only found in the P_4 sediments. The activity found in the other sediments can be removed by washing the sediments, and further cytochrome analysis indicates that all sediments are parts of the same cytoplasmic membrane.

Some important aspects have to be mentioned in order to come to a proper interpretation of the sedimentation behaviour of the nitrogenase.

1. The nitrogenase concentration of the extracts is of great importance, especially when the cells are ruptured by osmotic shock. Low protein and high salt concentrations cause dissociation of the nitrogenase complex. The separated components are unstable, more oxygen sensitive and the rate of sedimentation is lower than that of the complexed components (30). To compare the rate of sedimentation of the nitrogenase in extracts prepared with different cell breakage methods, the concentration of nitrogenase in the extract must be of the same order. Therefore we used the osmotic shock method as described by Shah et al. (17) and not the osmotic shock method as described by Oppenheim and Marcus (31) which introduces a high salt concentration and extreme dilution.

2. Cell rupture by lysozyme or by osmotic shock is not complete and a large quantity of un-lysed cells occurs in the first pellet. Sonification of these pellets indicates that the amount might vary between 10-30%. Therefore the enzyme activities which can be detected in the washings from even extensively washed pellets, may come from residual un-lysed cells.

3. During our studies we found that the pH also determines the rate of sedimentation of nitrogenase. At low pH (pH=6.0) all nitrogenase activity is sedimented after 120 min. 200,000 xg. Since rupturing the cells, especially with a French pressure cell, lowers the pH of the extract, it is necessary to readjust the pH to 7.4. This pH effect might explain differences in the rate of sedimentation of the nitrogenase which have been reported in other publications (9,10).

When artifacts due to pH, high concentrations of salts or extreme dilution are avoided, we can conclude from Fig. 1, that tightly membrane-bound enzymes such as ATP-ase remain bound to the cytoplasmic membrane; transhydrogenase can be considered as a loosely-bound membrane-enzyme in *Azotobacter*; the rate of sedimentation of nitrogenase is independent of the method used to rupture the cells and similar to that of the pyruvate dehydrogenase complex. No direct association with the cytoplasmic membrane can be observed, but our experiments cannot exclude the possibility that *in vivo* either nitrogenase or the pyruvate dehydrogenase complex are weakly associated with the cytoplasmic membrane; it is likely that nitrogenase sediments as a complex that is larger than a simple 1:1 complex of component I plus component II (mol. weight ≈ 300,000). The rate of sedimentation is much faster than that of glucose-6-phosphate dehydrogenase (mol. weight ≈ 100,000) and about the same as the pyruvate dehydrogenase complex (mol. weight 1.10^6). Our experiments confirm the idea that nitrogenase in *Azotobacter* is present as a complex (18, 32) which as will be shown below exists of at least three proteins (component I and II plus an Fe-S protein), which perhaps is present in vesicular membranes (12).

OXYGEN STABILIZING FACTOR

Oppenheim et al. (11) suggested that the method of cell rupture is important in determing whether or not nitrogenase in crude extracts is sensitive to oxygen. Based on these experiments, Drozd and Postgate (2) speculated that the cytoplasmic membranes might be involved in the protection of nitrogenase against oxygen. In a previous paper (20), we showed that the method of cell

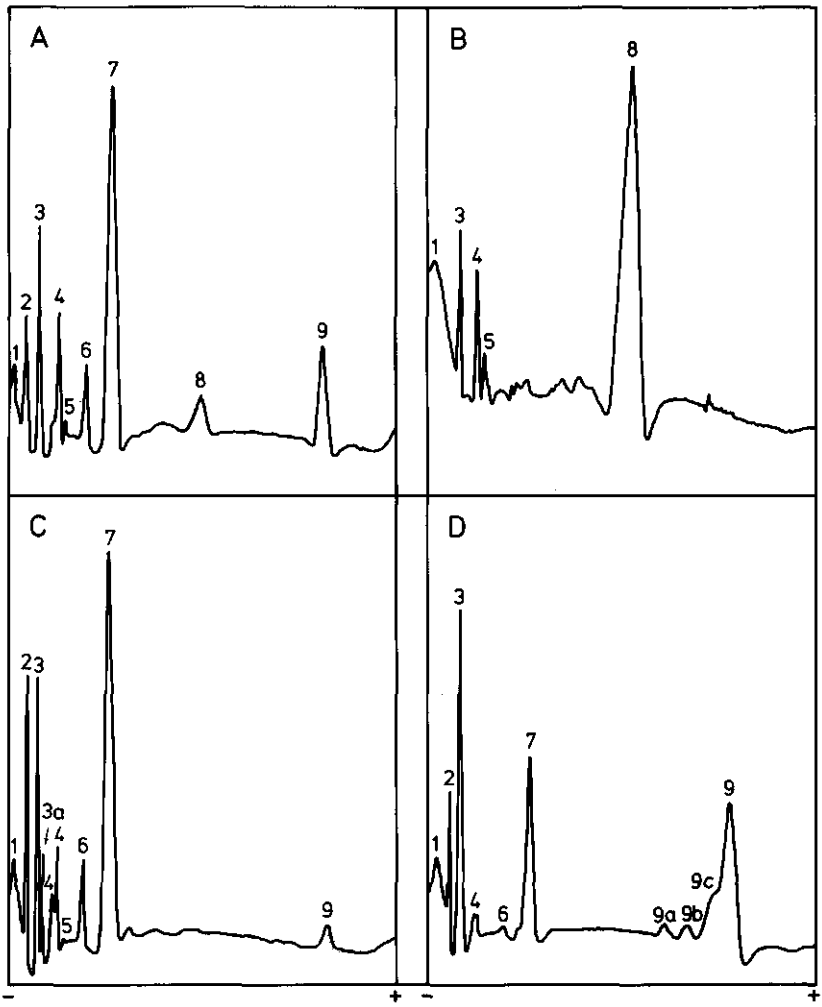

Fig. 2. Anaerobic non-denaturing polyacrylamide gel electrophoresis of nitrogenase complex before and after treatment on DEAE cellulose. Gels are prepared as described in Methods. Patterns are from the following preparations. A, nitrogenase complex (C 42-I); B, 0.15 M NaCl fraction; C, 0.27 M NaCl fraction; D, 0.40 M NaCl fraction.

rupture is not important, but that the salt concentration and the concentration of nitrogenase determine the oxygen lability of preparations. Yates(30) showed that a fraction with membrane-bound NADH dehydrogenase activity protected a partially purified preparation of nitrogenase against oxygen inactivation. We have re-examined this question using as starting material an oxygen-stable nitrogenase complex (C 42-I), prepared according to Bulen and Le comte (18) from extracts made with a French pressure cell or by an osmotic shock. This

complex shows at least 9 protein bands after electrophoresis in a polyacrylamide gel, indicating that such a nitrogenase complex is far from pure. Several enzyme activities are detectable e.g. NADH oxidase, 0.21 μmoles NADH. min^{-1}, mg^{-1}; NADPH oxidase, 0.16 μmoles NADPH min^{-1}. mg^{-1}; transhydrogenase, 3 nmoles s-NADH. min^{-1}. mg^{-1}; NADH dehydrogenase, 0.19 μmoles benzylviologen reduced min^{-1}. mg^{-1}; NADPH dehydrogenase, 16 nmoles benzylviologen reduced min^{-1}. mg^{-1}; NADH-flavodoxin oxidoreductase, 10 nmoles flavodoxin semiquinone min^{-1}. mg^{-1}; NADPH-flavodoxin oxidoreductase, 0.9 nmoles flavodoxin semiquinone min^{-1}. mg^{-1} and nitrogenase activity, 125 nmoles ethylene min^{-1}. mg^{-1}.

When the complex is adsorbed on a DEAE cellulose column and eluted with Tris-HCl buffer containing successively 0.15 M, 0.27 M and 0.4 M NaCl, the elution pattern observed is similar to that described by Bulen and Le Comte (18). Analysis of each fraction by polyacrylamide gel electrophoresis show that all of the components in the initial complex can be accounted for (Fig. 2 b,c,d), but that a few new bands appear (band 3a, 9a, b,c). It must be noted that crude nitrogenase component II (Fig. 2D) contains large amounts of component I, even after extensive washing of the DEAE cellulose column with 0.27 M NaCl; evidently this concentration of NaCl does not completely dissociate the nitrogenase complex. Recombination of crude component I with crude component II gives good nitrogenase activity (0.132 mg crude I + 0.07 mg crude II per ml gave an activity of 35.8 nmoles C_2H_4 formed per min). As known from the literature and shown in Fig. 3, such a reformed complex is extremely oxygen labile. However, it can be stabilized by addition of the fraction eluted from the DEAE-cellulose column with 0.15 M NaCl (Fig. 3). There is an optimal concentration of this fraction that gives maximum protection against oxygen. The largest protein component in this fraction is that corresponding with component 8 in the original complex (compare Fig. 2a and 2b); component 8 is unique to the 0.15 M NaCl fraction and therefore it seems likely that this component is responsible for the stabilizing effect. Further evidence for this is obtained by testing a further fraction in which the ratio of component 8 to components 1, 3, 4 and 5 was different from that in the fraction used in the experiment of Fig. 3. The stabilizing effect is related to the concentration of component 8 and not to the other components in the fraction. In addition to its effect on the oxygen stabilization of nitrogenase, the 0.15 M NaCl fraction also protects the enzyme against inactivation that occurs at low enzyme concentrations. The amount of this fraction that gave maximum stabilization is the same as that required for

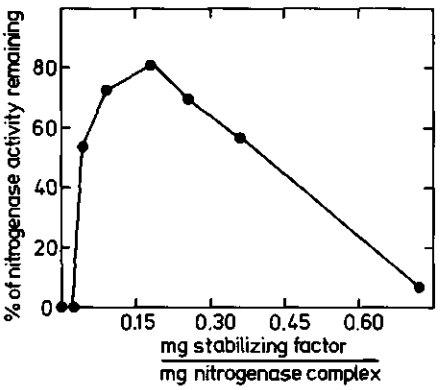

Fig. 3. Influence of stabilizing factor on the oxygen stability of a nitrogenase complex reconstituted from crude components, Oxygen inactivation was performed as described in Methods. 0.120 mg crude component I plus 0.03 mg crude component II were mixed with variable amounts of the 0.15 M NaCl fraction; in a volume of 1 ml.

stabilization against oxygen. These results and our earlier results (20) strongly suggest that protection of the nitrogenase against oxygen in *Azotobacter* is not caused by the presence of cytoplasmic membrane fragments but by a complexation of the nitrogenase proteins I + II with component 8, producing the so-called conformational-protected nitrogenase (1). We have also shown that nitrogenase in *A.vinelandii* is found to be oxygen tolerant (20) independent on the cell rupture method. The oxygen stable complex has a high activity with flavodoxin hydroquinone. Thus we propose that *in vivo* nitrogenase is protected against oxygen inactivation by complexation with component 8 (Fig. 2). Since flavodoxin hydroquinone is highly oxidizable by O_2, we propose that the switch-off state in *Azotobacter* is caused by the oxydation of flavodoxin hydroquinone rather than by a reversible inactivation of the nitrogenase as proposed by Dalton and Postgate (1).

We have tried to characterize component 8. When the 0.15 M NaCl eluate of the DEAE cellulose column is applied on a Sephadex G75 column and eluted with 25 mM Tris-HCl, pH 7.5 several fractions are obtained. The main fraction was analysed. Its E.P.R. spectrum, optical absorption spectrum and circular dichroism spectrum, show it is an Fe-S protein (33). Its mol.weight as determined by sodium dodecylsulphate gel electrophoresis and with a Sephadex G100

column is 24,000. Those characteristics indicate that component 8 is *Azotobacter* Fe-S protein II as isolated by Shethna et al. (34).
G. Scherings in our laboratorium (33) has shown that in the nitrogenase complex, C 42-I, the Fe-S protein (component 8, Fig. 2A) may have functions in addition to its role in oxygen protection of nitrogenase. It seems to regulate the nitrogenase activity.

Table I. Distribution of NADH-flavodoxin oxidoreductase, transhydrogenase and NADH dehydrogenase over cytoplasmic membrane preparations and soluble cell fraction after cell rupture. *Azotobacter* cells, containing 2.7 g of protein were disrupted with a Manton-Gaulin homogenizer and centrifuged as described in Methods. The preparations were dialysed against 25 mM TES-KOH, 1 mM $MgCl_2$, pH 7.4. The enzyme activities were measured as described in Methods.

Centrifugation	NADH-flavodoxin oxidoreductase ($\mu moles.min^{-1}$)	Transhydrogenase ($\mu moles.min^{-1}$)	NADH dehydrogenase ($\mu moles.min^{-1}$)
30 min, 100,000 x g pellet	3.25	10.1	598
120 min, 200,000 x g pellet	0.47	138.5	1587
120 min, 200,000 x g supernatant	0	151.3	224

ROLE OF CYTOPLASMIC MEMBRANE IN ELECTRON DONATION FOR NITROGENASE

The physiological electron donating system to nitrogenase in obligate aerobes such as *Azotobacter*, *Mycobacterium flavum* or *Rhizobium*, is still unknown. There are reports in which low nitrogenase activity is demonstrated in membrane preparations with NADH as electron donor and without artificial electron carriers (13,14). We showed low activities with pyruvate as substrate (20), but a few intact cells remaining in the membrane preparations could have been responsible for the activity. We have previously obtained evidence that in intact cells of *Azotobacter* the cytoplasmic membrane, especially its energized state,is important for electron transport to nitrogenase (15). Benemann et al. (35) showed that with the electron carriers, ferredoxin and flavodoxin and a crude enzyme preparation, low nitrogenase activity could be obtained with NADPH as electron donor. Since their preparation corresponds to our P_4 fraction by French pressure rupture, it seems highly likely to us that cytoplasmic membranes were present which could have played

a role in their observed activity. By using the reducing power of illuminated spinach chloroplasts, Arnon and co-workers showed that ferredoxin and flavodoxin can couple between chloroplasts and nitrogenase (36,37). Further, Yates (38) demonstrated that substrate amounts of dithionite-reduced flavodoxin acts as an electron donor for nitrogenase. These latter experiments can be critisized from the fact that flavodoxin-hydroquinone can be oxidized by SO_3^{2-} forming SO_2^- radicals (39) which in turn can be responsible for reduction of the nitrogenase. In order to avoid this complication we reduced substrate amounts of flavodoxin photochemically with deazaflavin (20) and showed that even higher activities than with dithionite can be obtained. Substrate levels of photochemically reduced *Azotobacter* ferredoxin (c.f. ref. 19) are not able to act as a reducing agent for nitrogenase.

Table I shows that a NADH-flavodoxin oxidoreductase is localized mainly in the phosphorylating membranes. The activity is low in smaller membrane vesicles and not detectable in the soluble cell-fraction. The NADH-flavodoxin reductase activity may be a side reaction of other enzymes. Two possible candidates, transhydrogenase and NADH-benzylviologen oxidoreductase were tested and can be excluded (Table I).

Tabel II shows the influence of the electron donating system on the activity of NADH-flavodoxin oxidoreductase and on the ratio of flavodoxin semiquinone to flavodoxin oxidized after reaching equilibrium. NADH is a better electron donor than NADPH for NAD(P)H-flavodoxin oxidoreductase. By using the purified transhydrogenase from *Azotobacter*, coupled with glucose-6-phosphate and glucose-6-phosphate dehydrogenase a very efficient NADPH and NADH regenerating system can be obtained. This system does not increase the initial rate and does not reduce flavodoxin beyond its semiquinone state. G. Scherings has shown (33) that nitrogenase activity is observed only when the ratio of flavodoxin hydroquinone to flavodoxin semiquinone is high. It is the therefore clear that the reduced pyridine nucleotides via the membrane-bound NADH-flavodoxin oxidoreductase are not capable to give any significant nitrogenase activity. We have tried to energize cytoplasmic membranes of *Azotobacter* with ATP, using the fluorescence of acridine as a probe for energization, but we did not succeed. The coupling between ATP hydrolysis and energization is probably too loose in isolated cytoplasmic membranes. The cytoplasmic membrane can only be energized with high rates of electron transport through the respiratory chain.

Table II. Influence of the electron donating system on the NAD(P)H-flavodoxin oxidoreductase activity and the amount of flavodoxin semiquinone formed. A membrane preparation P_2 was prepared after cell rupture with a French pressure cell as described in Methods. The membrane preparation was dialysed against 2 x 1 liter 25 mM TES-KOH, 1 mM $MgCl_2$, pH 7.4. 0.49 mg membrane protein was incubated as described in Methods, a spectrum was scanned and the reaction was started by adding flavodoxin to the sample cuvet. When the absorbance at 580 was constant, a further spectrum was scanned and the amount of flavodoxin semiquinone was calculated. The NAD(P)H regenerating system is described in Methods.

Electron donating system	Initial velocity, nmoles flavodoxin semiquinone formed.min^{-1}. mg^{-1}	Ratio flavodoxin semiquinone/oxidized after reaching an equilibrium
NADH	4.9	0.57
NADPH	1.8	0.52
NAD(P)H regenerating system	5.8	0.72

Toluene treatment causes bacterial cells to become permeable for small molecules (40), and enzyme systems, such as the DNA replicase system of *E.coli* (41), where labile interactions with the cytoplasmic membrane are necessary for maintenance of activity, remain active after a toluene treatment. We used a toluene treatment to investigate if labile interactions exist between cytoplasmic membranes, flavodoxin and nitrogenase, which may be lost during cell breakage.

Fig. 4a shows that in toluene-treated *Azotobacter* cells good nitrogenase activities with dithionite and ATP can be obtained. The photochemical reducing system deazaflavin with methylviologen as electron carrier also supports high nitrogenase activity. These activities indicate that nitrogenase is fully active in toluene-treated *Azotobacter* cells. Very low activities are observed with the photochemical reducing system alone and in the presence of added flavodoxin (Fig. 4A). Since photochemically reduced deazaflavin by itself is a poor electron donor for purified nitrogenase, hardly any activity in toluene-treated cells can be expected, by direct reduction of the nitrogenase within the cell. However in the case of transferring its electrons via endogenous carriers nitrogenase activity can be expected. Added flavodoxin does not stimulated the activity presumable because it cannot enter the cell.

When the cytoplasmic membrane is ruptured by sonication or by a lysozyme treatment there is an increase in nitrogenase activity with dithionite as

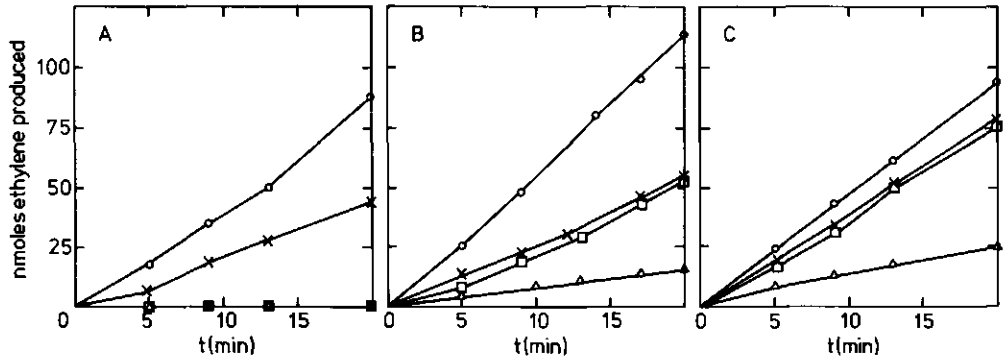

Fig. 4. Effects of different electron donors on the nitrogenase activity in toluene-treated *A.vinelandii* cells, sonicated-toluene-treated *A.vinelandii* cells and lysozyme-toluene-treated *A.vinelandii* cells. Preparations were made and activities measured as described in Methods. A, 1.1 mg toluene-treated *A.vinelandii* cells, were suspended in 0.5 ml of the acetylene assay mixture, electron donors: o-o, dithionite; x-x, light, 40 µM deazaflavin, 40 µM methylviologen; □-□, light, 40 µM deazaflavin, 3.8 nmoles *A.vinelandii* flavodoxin, Δ-Δ, light, 40 µM deazaflavin. B, 1.3 mg sonicated toluene-treated *A.vinelandii* cells were suspended in 0.5 ml acetylene assay mixture. C, 1.2 mg lysozyme-toluene-treated *A.vinelandii* cells were suspended in 0.5 ml acetylene assay mixture. B and C, same additions and symbols as A.

reductant (Fig. 4B). Several explanations are possible. In toluene-treated cells the diffusion of the strong inhibitor ADP out of the vesicle may be rate limiting or the diffusion of dithionite, but not diffusion of the more lipid soluble methylviologen, to the reducing site of nitrogenase may be hindered. The nitrogenase activity with endogenous electron carriers reduced photochemically, increases dramatically after rupturing (Fig. 4B,C) the cytoplasmic membrane. Fig. 4B,C also shows that low amounts of flavodoxin added to the sonicated toluene-treated *Azotobacter* cells increase the rate of nitrogenase activity considerably. The results presented in Fig. 4 show that in the presence of deazaflavin alone, before rupture of the cytoplasmic membrane, the low potential electron carriers cannot be reduced and thus mediate reducing equivalents to nitrogenase, indicating that they are presumably burried in the cytoplasmic membrane. This view is supported by the observation that the active sites of the nitrogenase are constantly attainable as the activity with dithionite and with reduced methylviologen shows.

A combination of our earlier work in which we showed that an energized cytoplasmic membrane is necessary for electron transport to nitrogenase, and the observations presented in this paper, that *Azotobacter* cells contains a membrane-bound NADH-flavodoxin oxidoreductase and that the low potential electron carriers may be burried in the membrane, allow us to propose the scheme shown in Fig. 5. The electron donating system consists of three components. A membrane bound NADH-flavodoxin oxidoreductase; a binding site for flavodoxin on the NADH-flavodoxin oxidoreductase; flavodoxin. In addition we did not get evidence for the presence of a NAD(P)H-ferredoxin reductase within the cells.

It is generally accepted that during oxidative phosphorylation a pH gradient is formed over the cytoplasmic membrane; a difference of two pH units over an energized cytoplasmic membrane is not uncommon. The pH on the inside of the cytoplasmic membrane could be 9 while the pH in the medium is 7. At pH 9.0 the redox couple $NADH/NAD^+$ has a midpoint potential of -380 mV (Fig. 5) NADH-flavodoxin oxidoreductase is reduced and electrons are transferred to the flavodoxin-reducing site, where flavodoxin semiquinone is bound. Proton transfer through the membrane coupled with electron transport through the respiratory chain, lowers the pH at this site. At pH=5 the midpoint potential of the redox couple flavodoxin hydroquinone/flavodoxin semi-

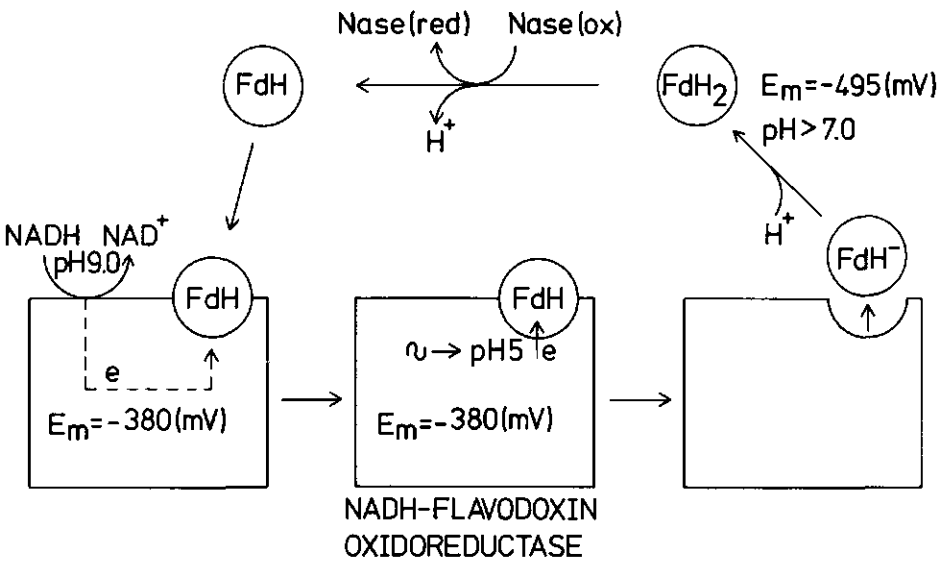

Fig. 5. Proposal for electron transport to nitrogenase in *Azotobacter vinelandii*.

quinone is -380 mV (22,42), and flavodoxin semiquinone can be reduced by NADH-flavodoxin oxidoreductase. The fully reduced flavodoxin does not bind and diffuses into the cytosol where the pH is higher than 7.0. At a pH > 7.0 the midpoint potential of the couple flavodoxin hydroquinone/flavodoxin semiquinone is -490 mV (c.f. ref. 42) low enough to reduce nitrogenase. Essential to this hypothesis is the idea that the cytoplasmic membrane creates a difference in pH at two sites of NADH-flavodoxin oxidoreductase e.q. pH=9 at the input site (NADH), pH=5 at the output site (flavodoxin). If this is possible, then NADH can act as an electron donor for nitrogenase in *Azotobacter*.

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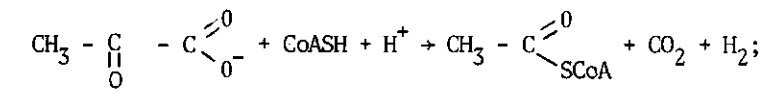
Discussion

ELECTRON TRANSPORT TO NITROGENASE

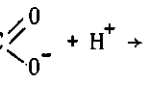
A. ELECTRON DONORS FOR NITROGENASE

Isolated nitrogenase has full activity at pH 8.0 at a redox potential of -500 mV but has no activity when this potential becomes higher than -420 mV (1,2). Therefore an efficient electron donor for nitrogenase must have a midpoint potential of at least -420 mV at pH 8.0. In anaerobic bacteria several enzyme systems are known to catalyse reactions that produce reducing equivalents at a sufficiently low potential:

1. The so-called phosphoroclastic reaction is catalysed by pyruvate dehydrogenase, ferredoxin and hydrogenase:



E_m = -510 mV at pH 7.0 (3).

2. The formate decarboxylation reaction is catalysed by formate dehydrogenase, ferredoxin and hydrogenase:  E_m = -420 mV at pH 7.0 (3).

   In these systems a ferredoxin or flavodoxin functions as the direct electron acceptor and can subsequently donate electrons to hydrogenase for the reduction of protons, or to nitrogenase. The reduction of protons is reversible so that molecular hydrogen can also be used to reduce ferredoxin and ultimately nitrogen.

3. H_2 + 2 ferredoxin(ox) → $2H^+$ + 2 ferredoxin(red); E_m = -420 mV at pH 7.0.

In photosynthetic organisms, ferredoxin has a similar nodal function in transferring reducing equivalents at low potential, but in such organisms the reducing equivalents originate in photosystem I with a redox potential lower than -700 mV (4). No similar ferredoxin-linked oxidative enzyme systems with a sufficiently low redox potential are known in aerobic or symbiotic

nitrogen-fixing organisms; the pyruvate oxidizing system in *A.vinelandii* for example is coupled to the reduction of NAD^+ (E_m(pH 7.0) = -320 mV) and not to the reduction of ferredoxin.

Thermodynamically it is possible to reduce nitrogenase with the reduced pyridine nucleotides, by coupling the pyridine nucleotides to a more or less irreversible reaction. For example: when 5 mM glucose-6-phosphate and 0.2 mM $NADP^+$ is brought in equilibrium at pH 8.0 with 6-phosphogluconate and NADPH by adding glucose-6-phosphate dehydrogenase, and taking into account the measured equilibrium constant, for this reaction (K=60 c.f.ref.5), at t = 25°C a ratio for NADPH/$NADP^+$ of 999.2 can be calculated. From this ratio and the Nernst equation the theoretical redox potential is -0.44 mV, low enough to give a low nitrogenase activity. Indeed with glucose-6-phosphate, $NADP^+$, glucose-6-phosphate dehydrogenase, and $NADP^+$-ferredoxine oxidoreductase plus ferredoxin to couple between NADPH and nitrogenase, a low nitrogenase activity can be obtained (6-8). But it is doubtful whether such high ratios of reduced to oxidized pyridine nucleotides ever exist in living organisms. For *Azotobacter* we have shown that under conditions of maximum nitrogenase activity, the ratio of NAD(P)H/$NAD(P)^+$ varies between 0.25-0.75 (paper II,III) and thus equivalent with redoxpotentials of around -310 mV.

A more definite proof that the reducing power necessary for nitrogen fixation is generated in a process other than that via a high ratio of reduced to oxidized pyridine nucleotides, is given in paper I. A possible energy source which could generate low potential reducing equivalents for nitrogen fixation, is NADH oxidation via the cytoplasmic membrane coupled to membrane energisation.

In the experiments to investigate the role of the energized cytoplasmic membrane in electron donation to nitrogenase, acetate was used as substrate. Acetate was used because, in contrast with substrates like sucrose or succinate, energy is not requires for its uptake into *Azotobacter vinelandii* (9). When *Azotobacter* cells are respiring acetate, low concentrations of uncoupler do not influence the oxygen uptake, indicating that the rates of the main metabolic processes are constant. This facilitates interpretation of experimental results considerably. As shown in paper I, low concentrations uncoupler do not influence the intracellular ratio of ATP, a substrate for nitrogenase, and ADP, an inhibitor of nitrogenase, but the same concentration of uncoupler inhibits the nitrogenase activity completely. Assuming that the acridine fluorescence can be used as a marker for the energized state, we have shown that a high energy level of the cytoplasmic membrane in *Azotobacter*

is necessary to generate reducing equivalents for the nitrogenase. We tried to prove this hypothesis with isolated cytoplasmic membranes and nitrogenase, but no nitrogenase activity could be observed. An explanation for this failure could be the fact that the cytoplasmic membranes of *Azotobacter* are not energizable with ATP, only with an oxidizable substrate and oxygen. Another indication that the energized cytoplasmic membrane is necessary for electron transport to the nitrogenase is given in paper III, in which it is shown that a toluene treatment makes *Azotobacter* cells permeable for small molecules. Normally *Azotobacter* has good coupling between pyruvate oxidation and nitrogenase activity (paper II). After a toluene treatment no pyruvate oxidation is observed. The pyruvate oxidation can be restored by adding the appropriate cofactors, but no associated nitrogenase activity is found. Since we have no evidence that enzymes leak out of the cell envelope under these conditions, a membrane which is energizable may be the missing link between pyruvate oxidation and nitrogenase activity.

B. ROLE OF FERREDOXIN AND FLAVODOXIN IN NITROGEN FIXATION

Four small and soluble non-heam iron electron transfer proteins with "labile sulfur" have been isolated from *Azotobacter* (10,11). The ferredoxins isolated by Shetna et al. (10) cannot either replace spinach ferredoxin in the photoreduction of $NADP^+$ by illuminated chloroplasts or substitute clostridial ferredoxin in nitrogen fixation in a ferredoxin-free extract of *C.pasteurianum*. In contrast however, the two ferredoxins isolated by Yoch and Arnon (11,12) are biologically active in the above mentioned tests. These ferredoxins can also transfer the reducing power of illuminated spinach chloroplasts to nitrogenase of *Azotobacter*. This property led to the postulate that ferredoxin is a physiological reductant for nitrogenase in *Azotobacter* (2). In our view however it is unlikely that the ferredoxin as isolated by Yoch (11) from *Azotobacter* plays a role in nitrogen fixation. Firstly, substrate levels of reduced ferredoxin are not capable of transferring electrons to nitrogenase (G.S. Scherings to be published). Secondly the amount of ferredoxin extractable from *Azotobacter* grown under nitrogen-fixing conditions is very low compared with another electron carrier - flavodoxin - isolated by the same method (20 mg ferredoxin and 500 mg flavodoxin from 1 kg cell paste).

Shethna et al. (13) isolated a flavodoxin from *Azotobacter*, which differs from flavodoxins from other organisms. Firstly it is not inducible at low concentrations of Fe in the culture medium. Secondly, it is a poor replacent

for ferredoxin in the pyruvate phosphoroclastic reaction and in the photochemical reduction of $NADP^+$ by spinach chloroplasts (14). Thirdly the coupling capacity of flavodoxin between illuminated chloroplasts and nitrogenase is poor (12). Yates (15) and we have shown (paper III) that substrate amounts of flavodoxin hydroquinone act as a very efficient electron donor for *Azotobacter* nitrogenase. In paper III we have shown that there are several interactions between *Azotobacter* flavodoxin and nitrogenase.

1. High activity with substrate amounts of flavodoxin from *Azotobacter* compared with flavodoxins from *P.elsdenii* and *D.vulgaris* and also compared with the activities of dithionite and reduced methylviologen.
2. Nitrogenase activity is independent of the redox potential of *Azotobacter* flavodoxin over a long range.
3. *Azotobacter* flavodoxin prevents dissociation of the nitrogenase complex at low protein concentration, which was also shown by Yates (22).

These observations together with the inability of reduced ferredoxin without a reducing system to react with the nitrogenase and the low amount of ferredoxin extractable from *Azotobacter* suggest that flavodoxin, and not ferredoxin, is the physiological electron donor for nitrogenase in *Azotobacter*.

C. ELECTRON TRANSPORT CHAIN TO NITROGENASE OR THE FLAVODOXIN REDUCING SYSTEM

As described in part A of this discussion, we think that the reducing power for nitrogenase in *Azotobacter* is generated in the cytoplasmic membrane. We have tried to isolate the electron donation system to nitrogenase, but we did not succeed with any of the cell-rupture methods that were used. An illustration of the lability of this system is given in paper III. Under anaerobic conditions, *Azotobacter* cells have low nitrogenase activity coupled with pyruvate oxidation. *Azotobacter* cells lyse spontaneously after a lysozyme treatment. During this autolysis a decrease in nitrogenase activity coupled with loss of pyruvate oxidation activity and an increase in dithionite -mediated nitrogenase activity has been observed. The pyruvate driven nitrogenase activity is not restored by adding the appropriate cofactors for pyruvate oxidation and an ATP regenerating system.

Despite our failure to isolate or reconstitute the physiological electron donating system to nitrogenase in *Azotobacter*, we have found two indications that the cytoplasmic membrane and flavodoxin are involved.

1. Flavodoxin seems to be burried within the membrane of toluene-treated cells (paper III).
2. A NAD(P)H-flavodoxin oxidoreductase is present in the cytoplasmic membrane (Paper IV).

Based on these observations, we give in paper IV a proposal how flavodoxin can be reduced to the hydroquinone form by the cytoplasmic membrane. In this hypothesis differences in pH created energy-linked, are the driving force for the reduction of flavodoxin by NADH.

Possibly a similar phenomenon, an energy-linked electron transport from NADH to ferredoxin occurs in *Clostridium pasteurianum*. Jungermann et al. (16) have shown that Acetyl CoA is necessary for electron transport from NADH to ferredoxin. It is possible that the energy present in Acetyl CoA is used to transport electrons from NADH to ferredoxin, a carrier with a lower redox potential. No further information about this clostridial system is available.

OXYGEN PROTECTION OF NITROGENASE

A Respiration protection

It was long known that oxidation by *Azotobacter* cells depends on the oxygen input during growth. Dalton and Postgate (17) suggested that augmented respiration in *Azotobacter* keeps the environment of the nitrogenase oxygen-free, and protects the oxygen-sensitive nitrogenase. They called this phenomenon the respiration protection. Jones and co-workers have described the changes which occur within the respiratory system of *A.vinelandii* upon an increase in oxygen input during growth.

1. Increased NAD(P)H dehydrogenase activity.
2. Increased concentration of cytochrome d.
3. Loss of energy conservation at site I (18,19).

In our opinion the experiments of Ackrell and Jones (18,19) are questionable. Firstly, they grow the bacteria in a batch culture, which in generally yields cells of a non-defined physiological state. Secondly, they do not take into account mutual spectral overlap of cytochromes c_4 + c_5 and cytochromes b + 0 and furthermore they do not discriminate between cytochrome b and 0.

We used cells grown under specified conditions in a chemostat and used the method of Sinclear and White (20) to calculate the amounts of cytochromes present in a membrane preparation. The following observations have been made.

When *A.vinelandii* cells are grown under oxygen-limited conditions mainly cytochromes $c_4 + c_5$ and o are detectable. Coupling sites are found between NADH and CoQ (site I), between malate and oxygen and between cytochrome $c_4 + c_5$ and oxygen. When the cells are grown with an excess of oxygen, beside a decrease in cytochrome $c_4 + c_5$, an increase in cytochromes b and d is observed. Only between CoQ and oxygen phosphorylation is detectable. Our results support the suggestion of Jones and coworkers (18,19) that cells grown oxygen-limited, have cytoplasmic membranes with a high oxidative phosphorylation capacity, while cells grown in excess oxygen have membranes with a low oxidative phosphorylation capacity. But in contrast to their suggestions our results in Paper II clearly show that the changes within the respiratory system are not responsible for the observed phenomenon of enhanced whole cell respiration (see discussion Paper III). It turned out that the uptake of sugar into *Azotobacter* is the rate-limiting step in sugar oxidation. We have also shown that the changes in the respiratory system are not related to the phenomenon of respiration protection of the nitrogenase, by using substrates like pyruvate or acetate. Pyruvate and acetate are taken up into *Azotobacter* without energy in contrast with sugars and the tricarboxylic acid cycle intermediates which uptake is energy-dependent (9). It may be possible that only during growth on substrates which are taken up energy-dependent, changes in the respiratory system occur as a regulating mechanism for substrate uptake. More experiments are necessary to give a physiological explanation for the changes observed in the respiratory system and the regulation of these changes in *Azotobacter*.

CONFORMATIONAL PROTECTION

Nitrogen-fixing systems have been extracted from *Azotobacter* in a so-called particulate form which is reasonable stable in air. Dalton and Postgate (17) proposed a conformational protection of nitrogenase in which the oxygen sensitive sites of the nitrogenase are protected against oxygen. Cytoplasmic membrane fragments, present in a particulate nitrogenase preparation (22) or a crude NADH dehydrogenase preparation (22) are thought to interact with the nitrogenase to form the oxygen stable conformational-protected nitrogenase. The experiments of Oppenheim et al. (23) confirmed this view. They ruptured *Azotobacter* cells with a French pressure cell and nitrogenase was found particulate and oxygen stable. When *A.vinelandii* was ruptured with an osmotic shock, nitrogenase was found soluble and oxygen labile. However in Paper III,

we show that the nitrogenase complex isolated from *Azotobacter* after cell rupture by an osmotic shock, is oxygen stable, in spite of the fact that the nitrogenase in these extract is non-particulate. High salt concentrations and extreme dilution makes nitrogenase from *Azotobacter* oxygen labile. We also show that membrane fragments with a high NADH dehydrogenase activity which are proposed to protect oxygen sensitive sites of the nitrogenase (24) in fact do not protect. In paper IV, we demonstrate that one cannot speak of a particulate nitrogenase. Nitrogenase is found to be a soluble enzyme complex comparable with the pyruvate dehydrogenase complex. Depending on the cell-rupture method, cytoplasmic membrane fragments sediment together with the nitrogenase. We also show that the oxygen stability of nitrogenase from *Azotobacter* is due to a complexation of the nitrogenase components with a Fe-S protein. This Fe-S protein is identified as ferredoxin II, isolated from *Azotobacter* by Shethna et al. (10).

With our results it is possible to re-evaluate the mechanism of the switch-on, switch-off phenomenon of whole *Azotobacter* cells (17). Since nitrogenase is present active and oxygen-protected in *Azotobacter*, the observed inhibition of whole cell nitrogen fixation upon increased oxygen input, must be due to oxidation of electron carriers involved in electron transport to nitrogenase and not be due to a change into conformational protected nitrogenase, oxygen stabile but inactive.

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Summary

I ELECTRON DONATION TO NITROGENASE

Paper I shows that the hypothesis, that a high ratio of (NADH + NADPH)/ (NAD^+ + $NADP^+$) is the source of reducing power for nitrogenase in intact *A.vinelandii*, is invalid. On the contrary, with a decreasing ratio of reduced to oxidized pyridine nucleotides, the nitrogenase activity of whole cells increases. The experiments described in paper I, indicate that the reducing power necessary for nitrogen fixation in *A.vinelandii* is generated within the cytoplasmic membrane. It is demonstrated that transport of reducing equivalents to the nitrogenase requires a high energy level of the cytoplasmic membrane. The energy level of the cytoplasmic membrane was measured by the intracellular ATP concentration and by using 9-amino acridine as a fluorescent probe. Other regulating factors of the nitrogenase activity in *A.vinelandii* are shown to be the intracellular ATP/ ADP ratio and the presence of oxygen.

Paper III shows that toluene makes *A.vinelandii* cells permeable for small molecules but not for enzymes. In toluene-treated cells, enzyme activities can be measured by adding the appropriate cofactors and substrates. It is possible to restore the oxidation of organic substrates but no concomitant nitrogenase activity can be observed. We suggest that an observed lack of energization of the cytoplasmic membranes is the missing link between oxidation and generation of the reducing equivalents for nitrogen fixation. In paper III and IV we show that the endogenous low potential electron carriers in toluene-treated cells are not reduced. In paper IV a membrane-bound NAD(P)H-flavodoxin oxidoreductase is demonstrated and a proposal is given in which NADH is the electron donor for nitrogenase. The electron carrier flavodoxin is reduced by the membrane-bound NADH-flavodoxin oxidoreductase at a low pH, that is developed in an energy-linked process.

II OXYGEN PROTECTION OF NITROGENASE

In paper II the source of respiration protection is investigated. Experi-

ments with radioactive pyruvate and sucrose show that the rate of sucrose oxidation by *A.vinelandii* is associated with the sucrose translocator activity. We show that the respiration protection of the nitrogen-fixing system in *A.vinelandii* is dependent of the oxygen input during growth. The oxidation capacity intrinsically depends on the type of substrate and can be partly adapted.

Membranes rich in cytochromes c_4 + c_5 and o and with phosphorylation between NADH and CoQ, CoQ and oxygen and cytochrome c_4 + c_5 and oxygen, can be isolated from *A.vinelandii* grown O_2-limited. Cytochromes b and d can be detected in addition when *A.vinelandii* cells are grown N_2 limited. The activity of the NADH oxidase system is increased in such cells and phosphorylation is only observed between CoQ and oxygen. Under saturating oxygen concentrations the type of respiratory membranes was not observed to influence the intracellular energy charge.

In paper III and IV the mechanism of the conformational protection of nitrogenase was investigated. It is shown that nitrogenase can be isolated as an oxygen-stable complex form *A.vinelandii* independent of the cell rupture method. Also no influence of the cell rupture method on the rate of sedimentation of the nitrogenase can be observed. The rate of sedimentation of the nitrogenase is found to be concentration and pH dependent. At pH=7.4 the rate of sedimentation of the nitrogenase complex is comparable with that of the pyruvate dehydrogenase complex.

No evidence was found for a particulate nitrogenase, it is demonstrated that the oxygen stability of nitrogenase in crude extracts is caused by complexation of the nitrogenase components with an Fe-S protein. An alternative proposal for the switch-on switch-off phenomenon in whole *Azotobacter* cells is given. Nitrogenase is present in vivo as an active and oxygen tolerent complex but nitrogen fixation in whole cells is inhibited by the oxidation of flavodoxin hydroquinone.

Samenvatting

De experimenten welke in dit proefschrift beschreven en bediscussieerd zijn, hebben alle betrekking op de stikstoffixatie in *Azotobacter vinelandii*. Het onderzoek kan worden onderverdeeld in twee hoofdrichtingen. De bescherming van de nitrogenase tegen zuurstof en het genereren van reductie-equivalenten voor de nitrogenase.

I BESCHERMING VAN HET STIKSTOFFIXEREND SYSTEEM TEGEN ZUURSTOF

De bescherming tegen zuurstof vindt plaats op twee niveau's. Op het niveau van de hele cel (ademhalingsbescherming) en op enzymatisch niveau conformatiebescherming).

a) Ademhalingsbescherming

In de tweede publicatie is nader op de ademhalingsbescherming in gegaan. Aangetoond is dat de aanpassing van de ademhalingssnelheid van hele cellen aan het zuurstofaanbod tijdens de groei berust op een aanpassing van de opnamesnelheid van de koolstofbron. Door substraten aan te bieden (pyruvaat en acetaat), waarvan de oxidatie de snelheidsbeperkende stappen van de suikerademhaling overslaat, is aangetoond dat voor het zuurstof vrijhouden van het stikstoffixerend systeem in intacte *Azotobacter*, er geen speciale aanpassingen van het cytoplasmamembraan en het stikstoffixerend systeem, noodzakelijk zijn. Dit is tegenstrijdig met wat in de literatuur gesuggereerd is . Naast het verschil in suiker translocator activiteit, zijn afhankelijk van de hoeveelheid zuurstof tijdens de groei, de volgende fysiologische verschillen tussen twee typen cellen aangetoond. Zuurstofbeperkend gegroeide cellen bevatten een ademhalingsketen met een hoge oxidatieve fosforyleringsefficiëntie. Fosforylering is aangetoond tussen NADH en CoQ, tussen CoQ en zuurstof en tussen cytochroom c_4 + c_5 en zuurstof. De belangrijkste cytochromen zijn cytochroom c_4 + c_5 en cytochroom o. Wanneer de cellen in overmaat zuurstof gegroeid zijn, worden ademhalingsmembranen aangetroffen met

een lage oxidatieve fosorylerings-efficiëntie. Fosforylering wordt alleen waargenomen tussen CoQ en zuurstof. De ademhalingsmembranen bevatten minder cytochroom c_4 + c_5 en o en meer cytochroom b en d. Ook neemt het NADH oxidase systeem sterk toe in activiteit. De verminderde oxidatieve fosforylerings-efficiëntie van de geïsoleerde membranen, veroorzaakt geen lagere intracellulaire energielading. Geen verschillen in kinetische eigenschappen van glucose-6-fosfaat dehydrogenase, 6-fosfogluconaat hydro-lyase en 3-deoxy-2-keto-6-fosfogluconaat aldolase in ruwe extracten van de twee typen cellen zijn waargenomen.

b) Conformatiebescherming

In artikel IV wordt aangetoond dat nitrogenase uit *Azotobacter* in tegenstelling tot de literatuur, onafhankelijk van de methode waarmee de cellen open gebroken worden, sedimenteert als een complex. Gedurende de zuivering van dit complex m.b.v. selectieve protaminesulfaat-precipitatie en een $MgCl_2$-precipitatie, blijft dit complex zuurstof stabiel (artikel III). Na scheiding van dit complex in 3 fracties m.b.v. DEAE-cellulose chromatografie, is door middel van reconstitutie-experimenten aangetoond welk eiwit in het oorspronkelijke complex de nitrogenase componenten tegen zuurstofinactivatie beschermt. Dit eiwit heeft een molecuulgewicht van 24.000 en bevat als karakteristieke groep een Fe-S kluster.

Daar in artikel 3 is aangetoond dat het zuurstof stabiele nitrogenase complex met flavodoxine hydrochinon als reductie middel een hoge activiteit vertoont, is het duidelijk dat de reversibele remming van de stikstof binding bij *Azotobacter* (switch-on-switch-off) berust op een oxidatie van flavodoxine hydrochinon .

II HET GENEREREN VAN REDUCTIE-EQUIVALENTEN VOOR DE STIKSTOFFIXATIE

In aeroob stikstofbindende micro-organismen zonder foto-systeem zijn geen enzymsystemen aangetoond, waarbij redoxcarriers zoals ferredoxine of flavodoxine volledig gereduceerd kunnen worden. In artikel I hebben we aangetoond voor intacte *Azotobacter* cellen dat voor het genereren van reductie-equivalenten voor de stikstofbinding energie nodig is, verkregen door electronen transport naar zuurstof door de ademhalingsketen.

In het derde artikel wordt het terminale gedeelte van de electronentransportketen naar de nitrogenase behandeld. Hierbij is gebruik gemaakt van de

methode om m.b.v. fotoreductie van 3,10 dimethyl-5-deaza-isoalloxazine flavodoxine te reduceren. Aangetoond is: een hoge activiteit en een speciale fit tussen flavodoxine en nitrogenase uit *Azotobacter* vergeleken met flavodoxines uit *P.elsdenii* en *D.vulgaris*. Met fotochemisch gereduceerd flavodoxine uit *Azotobacter* is de nitrogenase-activiteit over een groot gebied onafhankelijk van de redoxpotentiaal in tegenstelling tot fotochemisch gereduceerd methylviologen als reductiemiddel voor de nitrogenase.

Experimenten met tolueen behandelde *Azotobacter* cellen geven aan dat flavodoxine in intacte structuren niet reduceerbaar is voor het fotochemisch reductiesysteem (artikel III en IV). In artikel IV wordt het begin van de electronentransportketen naar de nitrogenase beschreven. Enige eigenschappen van een membraan-gebonden NADH-flavodoxine oxidoreductase worden gegeven. Tevens wordt in het vierde artikel een voorstel gedaan hoe via een pH-verschil tussen de plaats waar NADH NADH-flavodoxine oxidoreductase reduceert en waar dit enzym electronen overdraagt op flavodoxine, NADH (E_m(pH 7.0 = -320 mV) in staat geacht moet worden flavodoxine semichinon te reduceren tot flavodoxine hydrochinon (E_m(pH 7.0) = -495 mV).

Curriculum vitae

De schrijver van dit proefschrift is geboren op 24 juli 1946 te Badhoevedorp. In 1965 behaalde hij het HBS-B diploma aan het Pius X Lyceum te Amsterdam. In hetzelfde jaar begon hij zijn studie chemie aan de Universiteit van Amsterdam. In september 1968 werd het kandidaatsexamen S_1 behaald en in juni 1971 het doctoraal examen, cum laude (hoofdvak biochemie, bijvak dierfysiologie en speciale richting fysische- organische chemie).

Vanaf 1 augustus 1971 tot heden is de auteur werkzaam bij het Laboratorium voor Biochemie van de Landbouwhogeschool te Wageningen. Tot 1 januari 1975 was hij in dienst bij de Stichting Scheikundig Onderzoek Nederland (S.O.N.) welke gesubsidieerd wordt door de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.).