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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

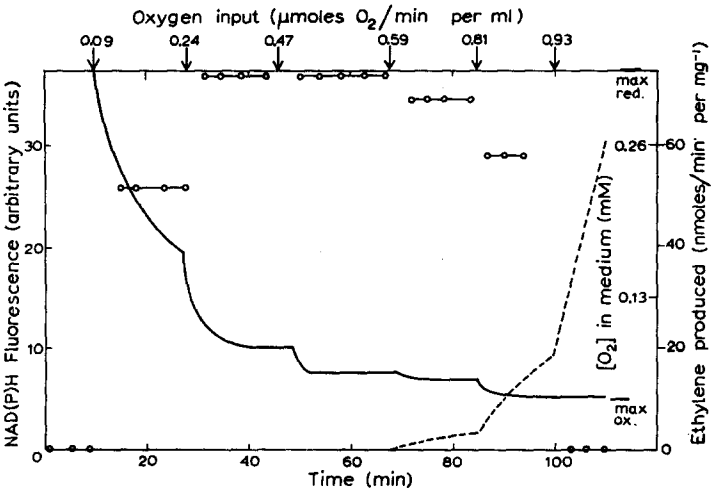

Fig. 1. The effect of increasing oxygen input on the NAD(P)H level and nitrogenase of whole *A. vinelandii* cells. "Oxygen-adapted" cells (0.42 mg/ml; sucrose oxidation, 1.3 μmoles oxygen · min^{-1} · mg^{-1}) were suspended in 7 ml standard incubation mixture with sucrose (20 mM) as substrate. 10 min 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

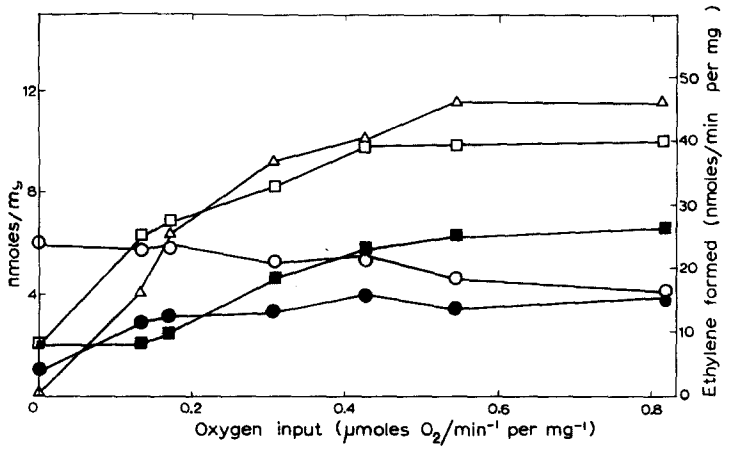

Fig. 2. Levels of ATP, ADP, NAD^+, $NADP^+$ and nitrogenase activity in intact cells of *A. vinelandii* with different oxygen supply. "Oxygen-adapted" cells (0.8 mg/ml; sucrose oxidation; 1.1 μmoles oxygen · min^{-1} · mg^{-1}) were suspended in 6 ml standard incubation mixture with sucrose (20 mM) as 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^-

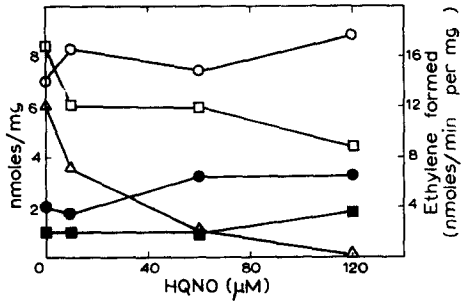

Fig. 3. The effect of HQNO on ATP, ADP, NAD^+, $NADP^+$ levels and nitrogenase activity of intact *A. vinelandii* cells under constant oxygen supply. "Oxygen-limited" cells (1.4 mg/ml; sucrose oxidation, 0.19 μmoles oxygen · min^{-1} · mg^{-1}) were suspended in 10 ml standard incubation mixture with 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

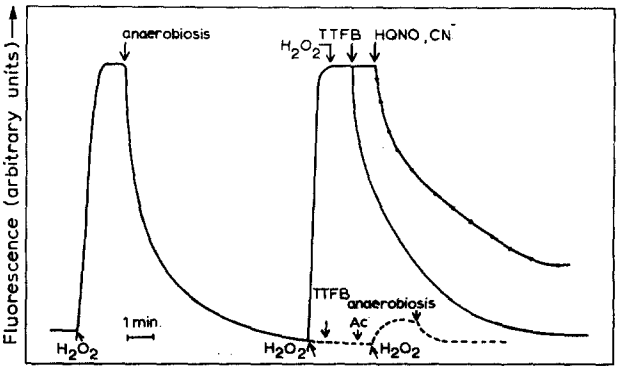

Fig. 4. Enhancement of 9-aminoacridine fluorescence by whole *A. vinelandii* cells upon energization. "Oxygen-limited" cells (0.33 mg/ml; sucrose oxidation, 0.35 μmoles oxygen · min^{-1} · mg^{-1}) were suspended in 2 ml standard incubation mixture with sucrose (20 mM), 9-aminoacridine (2 μM), catalase (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

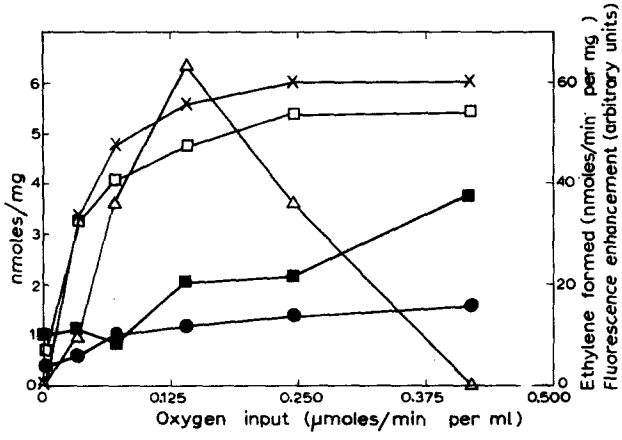

Fig. 5. Enhancement of 9-aminoacridine fluorescence, ATP, NAD^+, $NADP^+$ levels and nitrogenase activity of whole *A. vinelandii* cells on increasing oxygen input. "Oxygen-limited" cells (0.8 mg/ml; sucrose oxidation, 0.46 μmoles oxygen · min^{-1} · mg^{-1}) were suspended in 8 ml standard incubation 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

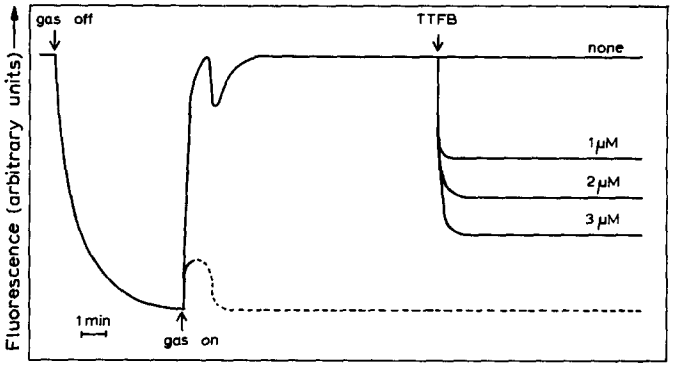

Fig. 6. The effect of TTFB on the fluorescence emission of 9-aminoacridine. "Oxygen-limited" cells (0.38 mg/ml; sucrose oxidation, 0.25 μmoles oxygen $\cdot$ min^{-1} $\cdot$ mg^{-1}) were suspended in 4 ml standard incubation mixture with acetate (20 mM) and 9-aminoacridine (2 μM). 10 min after a constant 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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