576.851.151.095.312

MEDEDELINGEN LANDBOUWHOGESCHOOL
WAGENINGEN • NEDERLAND • 74-10 (1974)

NITROGEN FIXATION AND NITROGENASE ACTIVITY OF *AZOTOBACTER CHROOCOCCUM*

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(Received 20-2-1974)

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Mededelingen Landbouwhogeschool
Wageningen 74-10 (1974)
(Communications Agricultural University)
is also published as a thesis

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1. INTRODUCTION

The biological fixation of atmospheric nitrogen is, next to the photosynthetic assimilation of CO_2, one of the most fundamental processes in nature as it maintains the balance in the nitrogen economy of the biosphere. It is known since 1838 when Boussingault proved that legumes are able to utilize atmospheric nitrogen (for historical notes on this subject see the reviews of WILSON, 1958; JENSEN, 1965; STEWART, 1966).

Although the ability to fix nitrogen is restricted to few species of microorganisms, all of them probably of the procaryotic type, these species are widely distributed in the kingdom of protists. The nitrogen-fixing microorganisms are broadly divided into two groups, the free-living ones and those living in symbiotic association with plants. However, as pointed out by several workers (cf. MULDER, 1966; DELWICHE, 1970), this distinction is not as sharp as it was once thought to be, because of the varying degrees of interdependence existing in the association between plants and microorganisms (root nodules, phyllosphere and rhizosphere associations). With the exception of nitrogen-fixing blue-green algae and photosynthetic bacteria, the free-living nitrogen fixers are indirectly dependent on plants for their carbon and energy supply.

Most of the biological nitrogen fixation in nature is brought about by symbiotic systems and to a lesser extent by nitrogen-fixing algae. The contribution of the free-living bacteria (e.g. *Azotobacter* and *Clostridium* species) to the nitrogen economy of the biosphere is generally believed to be small. This belief is based on the following facts:

(i) With very few exceptions, azotobacters and other free-living N_2-fixing bacteria are present in relatively low numbers in soil and water.

(ii) Under laboratory conditions (usually with a relatively high concentration of carbon compounds), the efficiency of nitrogen fixation (in terms of nitrogen fixed per unit weight of carbon compound consumed) is usually low.

(iii) The amount of carbon compounds available as source of carbon and energy for these bacteria in soil is limited.

However, some recent work (DALTON and POSTGATE, 1969; BECKING, 1971) suggests, that under certain conditions (low pO_2 and relatively low concentration of carbon compounds) the efficiency of nitrogen fixation of azotobacters may be several times greater. Therefore, analysis of some factors which might lead to high efficiency of nitrogen fixation by azotobacters was thought to be worthwhile.

1.1. REQUIREMENTS OF NITROGEN FIXATION

Nitrogenase, the enzyme system responsible for N_2 fixation, is a complex of an Fe protein (azoferredoxin) and a Mo-Fe protein (molybdoferredoxin) which are functioning only when occurring in combination. Using cell-free nitrogen-

fixing systems from different sources, it was found that in addition to nitrogenase, nitrogen fixation requires an electron donor, one or more electron carriers to transfer the electron from the donor to nitrogenase, ATP, and a divalent metal ion (e.g. Mg^{2+}) (cf. BURRIS, 1971).

In extracts of *Clostridium pasteurianum*, reductant and high energy phosphate for N_2 fixation can be furnished by the phosphoroclastic reaction of pyruvate. In normal cells of this bacterium, ferredoxin functions as an electron carrier between pyruvate and nitrogenase; in iron-deficient cells, however, flavodoxin substitutes for ferredoxin as an electron-transfer protein.

In extracts of *Azotobacter vinelandii*, reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$), but not reduced nicotinamide adenine dinucleotide ($NADH_2$), can serve as the source of electrons for N_2 fixation in the presence of four compounds: azotobacter ferredoxin, azotoflavine and two heat-labile unidentified components (BENEMANN et al., 1971). These compounds may function as electron-transport chain from $NADPH_2$ to nitrogenase.

BULEN et al. (1965) discovered that $Na_2S_2O_4$ functions effectively as a non-biological electron donor for nitrogenase in cell-free systems. This compound is apparently a universal reductant for the nitrogenase reaction and circumvents the usual electron carriers; it interacts directly with nitrogenase. The application of $Na_2S_2O_4$ simplifies the spectrum of components required to achieve the nitrogenase reaction in cell-free systems.

In spite of an early suggestion to the contrary (BAYLISS, 1956),it is now established that nitrogen fixation requires ATP. In the assay system for estimation of nitrogenase activity in cell-free extracts (see 2.4.1.), ATP is supplied by an ATP-generating system such as acetokinase and acetylphosphate or phosphocreatine kinase and creatine phosphate. ATP is hydrolysed to ADP and inorganic phosphate in the nitrogenase reaction. When the ATP-precursor is exhausted and the ATP/ADP ratio reaches a value of 0.5, further ATP utilization by nitrogenase and hence nitrogenase activity is completely inhibited (DALTON and MORTENSON, 1972). The number of molecules of ATP, hydrolysed per pair of electrons transferred to nitrogen, is not exactly known, although a number around 4 is reasonable (HARDY and KNIGHT, 1966). Thus, about 12 molecules of ATP are required to reduce 1 molecule of nitrogen to 2 molecules of ammonia. The stoichiometry of the reaction seems to be a sensitive function of a number of variables (KELLY, 1969).

Magnesium ions satisfy the divalent-metal-ion requirements of nitrogenase. Mn^{2+}, Fe^{2+}, Co^{2+}, and Ni^{2+} are less effective but do support the reaction. It is likely that the metal ions function as complexing agent for ATP. MORTENSON et al. (1973) published data which suggest that the reduction of N_2 (or other substrates) by nitrogenase requires the formation of the Mg-ATP-azoferredoxin complex which subsequently reacts with molybdoferredoxin to allow electron flow.

1.2. The relation between nitrogen fixation and other metabolic reactions of *Azotobacter* spp.

In whole cells nitrogen fixation is integrated with the energy-yielding metabolism, electron-transport system and ammonia-assimilation pathways of the cells. Therefore, it would be useful to discuss this related metabolism of azotobacters briefly (see Fig. 1.1.).

1.2.1. *Energy-yielding metabolism of* Azotobacter *spp.*

Glucose catabolism in *Azotobacter* spp. has been investigated both by assaying for key enzymes of the major energy-producing pathways (Mortenson and Wilson, 1954, 1955; Johnson and Johnson, 1961; Senior and Dawes, 1971) and by a radiorespirometric method (Johnson et al., 1958; Still and Wang, 1964). The results obtained suggest that glucose is metabolized mainly according to the Entner-Doudoroff pathway and to a minor degree according to the oxidative pentose-phosphate pathway. Both pathways are subjected to feed-back inhibition by the products of glucose oxidation (Senior and Dawes, 1971).

The functioning of the tricarboxylic-acid (TCA) cycle in *Azotobacter* spp. was doubted at first, because when *Azotobacter* cells were grown in the presence of lower TCA-cycle compounds (e.g. acetate), they did not promote the oxidation of higher TCA-cycle compounds (e.g. α-ketoglutarate) (Karlson and Barker, 1948). However, subsequent investigations with cell-free extracts indicated the presence of TCA-cycle enzymes in this organism (Stone and Wilson, 1952a, b; Alexander and Wilson, 1956).

Much of the reduced pyridine nucleotide, formed by the oxidation of organic acids in the TCA-cycle, is used to generate ATP via oxidative phophorylation. Ackrell and Jones (1971a, b) reported the presence of three phosphorylating sites in respiratory membranes prepared from cells of *A. vinelandii*. Phosphorylation efficiencies, particularly at site I, are very low at high ambient oxygen concentrations but increase as the dissolved oxygen concentrations become small. The low efficiency of energy coupling at high dissolved oxygen concentrations might be expected to produce a greatly decreased [ATP]/[ADP] [Pi] ratio, and thus to cause a loss of respiratory control in whole cells of this organism incubated at a high pO_2 tension. This finding supports the hypothesis proposed by Postgate and his associates (see 4.1.) that respiration in azotobacters, in addition to functioning in generating ATP, protects nitrogenase from the inhibition by oxygen.

Bresters et al. (1972) reported the presence of transacetylase activity and acetate kinase in the crude extract of *A. vinelandii*. This system permitted anaerobic ATP synthesis from pyruvate (Haaker et al., 1972).

1.2.2. *Electron-donor and electron-transport systems*

As has been mentioned briefly in 1.1, $NADPH_2$ but not $NADH_2$, has been found by Benemann et al. (1971) to function as the electron donor for nitrogen fixation in cell-free extracts of *A. vinelandii*. At first observation it is difficult to

understand how $NADPH_2$ could function in the reduction of ferredoxin, because the reaction from $NADPH_2$ to ferredoxin is thermodynamically uphill. According to BENEMANN and VALENTINE (1971) this energy barrier could be overcome by the cells in two ways. First, the $NADPH_2$-ferredoxin reaction is coupled with the ATP-driven nitrogenase reaction which is highly irreversible, thus pulling the $NADPH_2$-ferredoxin reaction to completion. Secondly, by coupling the $NADPH_2$-ferredoxin reaction with the extremely active isocitrate-dehydrogenase system, thus maintaining a high $NADPH_2$ concentration in the cels and thereby helping to push the reaction.

The isocitrate dehydrogenase of *A. vinelandii* has been purified by CHUNG and FRANZEN (1969), who found that *Azotobacter* spp. are a rich source of NADP-linked isocitrate dehydrogenase, which makes up about 1 % of the total soluble protein of the organism. KURZ and LA RUE (1973) recently provided indirect evidence that isocitrate dehydrogenase is the source of reductant for nitrogen fixation by azotobacters. When *A. chroococcum* was grown on glycolic acid as the sole source of carbon, the organism was unable to utilize N_2 and must be provided with reduced nitrogen. Glycolic acid is metabolized via Kornberg's dicarboxylic-acid cycle. In such cells the TCA-cycle enzymes are low in activity, and isocitrate dehydrogenase is practically absent. When glucose was the carbon source, N_2 fixation proceeded normally.

Other cellular NADP-specific dehydrogenases such as glucose-6-phosphate dehydrogenase and malate dehydrogenase, can, of course, also provide $NADPH_2$ for the electron-transport system connected to nitrogenase.

Azotobacters possess an electron-transport system which brings electrons from electron donors to nitrogenase, and which is different from the respiratory electron-transport system which transfers electrons to oxygen. BENEMANN et al. (1969) and YOCH et al. (1970) have isolated four compounds from *A. vinelandii* which could function as an electron-transport chain from $NADPH_2$ to nitrogenase. These four compounds are azotobacter ferredoxin, azotoflavin and two unidentified heat-labile components.

1.2.3. *The assimilation of ammonia*

Ammonia assimilation is the enzymatic conversion of ammonium ions to organic compounds of the cell. In many species of bacteria, the formation of glutamate, catalysed by glutamate dehydrogenase, is assumed to be the primary, if not the sole, route for the incorporation of ammonia into amino acids. But in most bacilli, glutamate dehydrogenase is absent and in this group of bacteria alanine dehydrogenase has been isolated from all the species studied (WIAME and PIERARD, 1955). According to SHEN et al. (1959) this enzyme is responsible for the assimilation of ammonia in most bacilli.

At least four different routes for the incorporation of ammonium ions into amino acids of microbial cells can be distinguished (ELMERICH, 1972). These include the formation of: (1) L-glutamate from 2-oxoglutarate and NH_4^+, catalysed by glutamate dehydrogenase (GDH); (2) L-glutamine from L-glutamate and NH_4^+, catalysed by glutamine synthetase (GS); (3) L-alanine from

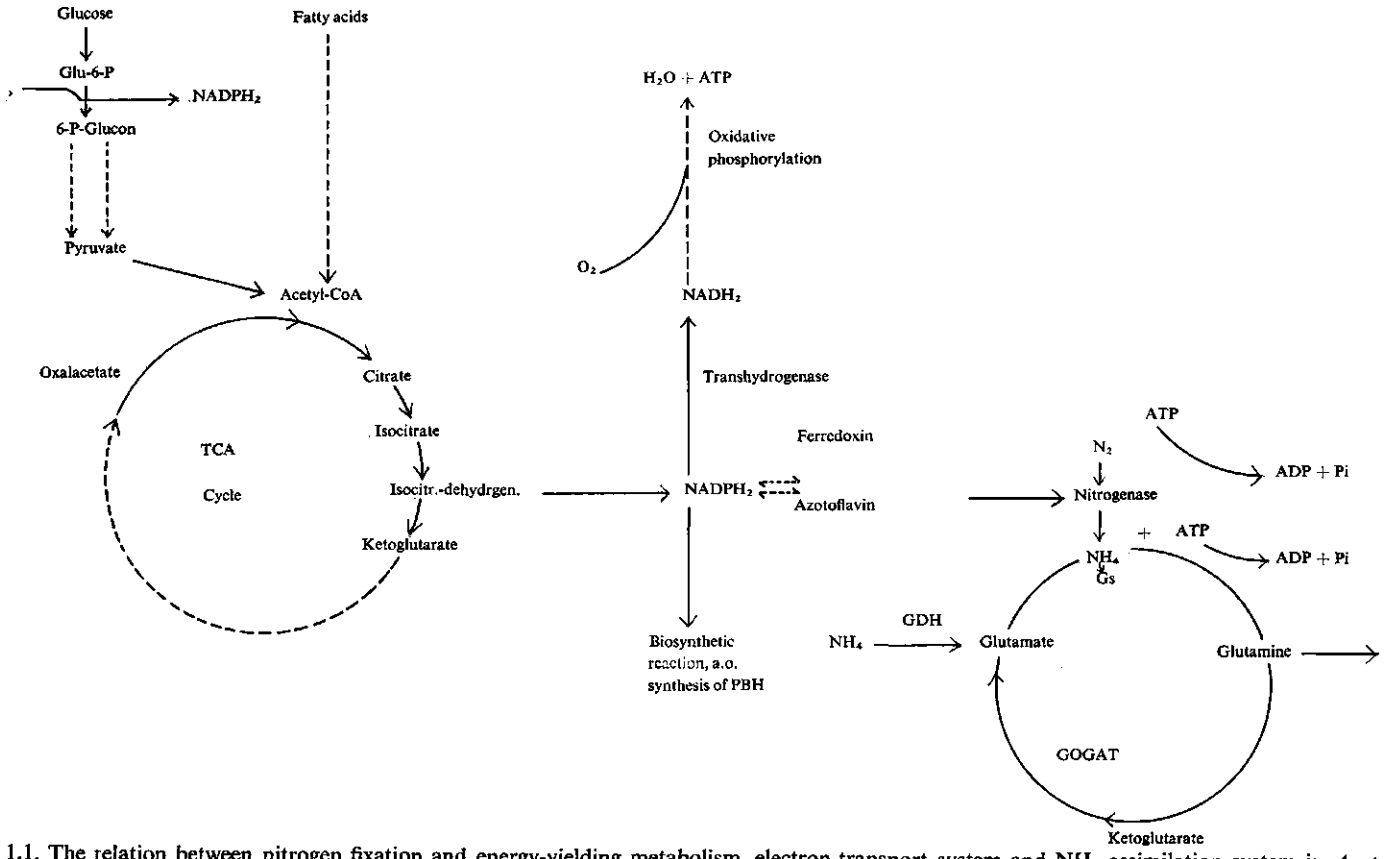


FIG. 1.1. The relation between nitrogen fixation and energy-yielding metabolism, electron-transport system and NH_4-assimilation system in *Azotobacter sp.*. (modified from BENEMANN and VALENTINE, 1972). Further explanation see text.

pyruvate and NH_4^+, catalysed by alanine dehydrogenase; (4) L-aspartate from fumarate and NH_4^+, catalysed by aspartase.

TEMPEST et al. (1970) have recently discovered a new pathway for the assimilation of ammonia to glutamate in ammonia-limited *Aerobacter aerogenes*. This organism synthesizes glutamate by a two-step process that involves the amidation of endogenous glutamate to glutamine and subsequently the reductive transfer of the glutamine-amide nitrogen to the 2-position of 2-oxoglutarate, producing a net synthesis of two molecules of glutamate (reaction 1). The latter step involves a novel enzyme, glutamate synthetase (Glutamine (amide): 2-oxoglutarate amino transferase oxido-reductase (NADP), which for convenience is designated as GOGAT).

$$\text{L-Glutamate} \xrightarrow[\text{GS}]{\text{ATP, } NH_4^+} \text{L-glutamine} \xrightarrow[\text{GOGAT}]{NADPH_2,\ \text{2-oxoglutarate}} \text{2 L-glutamate} \qquad (1)$$

It is now evident that GOGAT is not peculiar to ammonia-limited *A. aerogenes*. The presence of GOGAT and functioning of reaction (1) have been reported in *Erwinia carotovora*, *Pseudomonas fluorescens*, *Bacillus subtilis* (MEERS et al., 1970), *B. megaterium* (MEERS et al., 1970; ELMERICH and AUBERT, 1971) and in several nitrogen-fixing bacteria (NAGATANI et al., 1971). The finding of NAGATANI et al. (1971), that mutants of nitrogen-fixing *Klebsiella pneumoniae*, missing the new ammonia assimilatory route, are unable to fix nitrogen, suggests the possibility that the new pathway plays an important role in nitrogen-fixing microorganisms. However, data recently reported by DHARMAWARDENE et al. (1973) concerning the assimilation of ammonia, formed during nitrogen fixation by *Anabaena cylindrica*, do not support the suggestions of NAGATANI et al. (1971). Although the alga was found to have a very high concentration of glutamine synthetase, especially in the heterocysts, its GOGAT activity was very low.

Glutamine synthetase, as the first enzyme in the new pathway of ammonia assimilation, has some characters which make its presence in the nitrogen-fixing microorganisms highly significant. The low Km (the high affinity) of this enzyme for ammonium ions is not only providing an efficient assimilatory mechanism for concentrations of NH_4^+ which are too low for other assimilation pathways, but it is also preventing repression of nitrogenase synthesis by NH_4^+. Furthermore, the low energy requirement of the ammonia-assimilation reaction catalysed by glutamine synthetase, as compared to the reductive amination of 2-oxoglutarate, may provide a means whereby more energy can be directed into the highly endergonic process of nitrogen fixation.

1.3. Scope of this study

In the present investigation, the effect of some chemical, physical and biological factors on nitrogenase activity and growth of *A. chroococcum* has been studied. These factors are: (1) source of carbon and energy, (2) oxygen supply, (3) non-growing or slow-growing existence, (4) removal of nitrogenous compounds excreted by this organism, and (5) combined nitrogen supplied to the medium.

2. MATERIALS AND METHODS

2.1. Microorganisms and media

The *Azotobacter* species and strains used in this study, *A. chroococcum*, strains A39 and A40, *A. agile*, strain A27, and *A. vinelandii*, strain A66, were obtained from the culture collection of the Laboratory of Microbiology at Wageningen. In the earlier stage of this study, *A. chroococcum*, strains R25 and R35, isolated by Dr. J. Ruinen from the phyllosphere of cacao were included.

All of the strains grew readily in modified Burk's medium which contains: K_2HPO_4, 0.64, KH_2PO_4, 0.16, $MgSO_4.7H_2O$, 0.2, NaCl, 0.2, $CaCl_2.2H_2O$, 0.09, $FeSO_4.7H_2O$, 0.01, and $Na_2MoO_4.2H_2O$, 0.005 g per liter. The mixture was sterilized by autoclaving at 120 °C for 20 min at least one day before being used; this measure was suggested by Dalton and Postgate (1969) to disperse the colloidal precipitate formed during sterilization. Just prior to utilization, glucose or some other carbon compound was added aseptically as a sterile solution. The pH of the complete medium was adjusted aseptically to 7.2.

Solid medium was prepared by supplying 1 l of the above-mentioned liquid medium, including the carbon compound, with 12 g of agar and sterilizing at 120 °C for 20 min.

2.2. Measurement of bacterial growth

One or more of the following methods have been used for measuring bacterial growth.

2.2.1. *Total viable count*

Several dilutions of the sample were made to obtain a suspension containing approximately 2000 bacteria per ml. Modified Burk's medium (see 2.1) without carbon compound was used as the diluent, which was prepared and sterilized in bulk and distributed in 9-ml lots aseptically into test tubes. Ten 0.02 ml spots were pipetted on an agar plate with the aid of a microscrew attachment. The agar plates had been dried by storage at 25 °C for 3 days prior to use. The spots were allowed to be absorbed by the agar before the plates were placed in the incubator. Counts were made under a Wild-M5 stereomicroscope on those spots which had populations of 20–100 viable organisms. The total viable count of the sample was deduced from these data and from the number of dilutions.

2.2.2. *Determination of the density of cell suspensions by optical measurement*

The cultures were acidified to an approximate pH of 3 with 0.1 N acetic acid (to dissolve the precipitated salts) and suitably diluted before measurement, in

order to fall within the range of 0.02–0.4 optical density units. Turbidity was measured at 420 nm by using a Kipp nephelometer. Alternatively, turbidity was measured in an EEL nephelometer, using a perspex tube (supplied by the manufacturer) as a standard.

2.2.3. *Determination of cell yields by measuring total dry weight*

The cultures were acidified to a pH of approximately 3 with 0.1 N acetic acid and the cells harvested by centrifugation. The cells were washed by resuspending in distilled water and re-centrifuging. After one to three washings, depending on the relative volume of the cell suspension, the whole cell suspension was transferred to small weighing bottles and dried at 85 °C overnight.

2.2.4. *Determination of cell yields by measuring protein contents*

Portions of 0.5 ml of washed cell suspensions prepared as described in 2.2.3 (adjusted to contain ca. 200 μg dry weight of cells per ml) were transferred to test tubes. After being supplied with 0.5 ml of 0.1 N NaOH each, these tubes were placed in a boiling water bath for 5 min and subsequently cooled in cold tap water. Each tube was then provided with 2.5 ml of Na_2CO_3-$CuSO_4$-tartrate reagent (freshly prepared by mixing 50 ml of a 5% Na_2CO_3 solution with 2 ml of 0.5% $CuSO_4.5H_2O$ in a 1% aqueous solution of potassium tartrate). The mixture was allowed to stand for 10 min and rapidly supplied with 0.5 ml of diluted Folin-Ciocalteu reagent. After standing for 30 min to allow full colour development, the optical density was measured against a reagent blank in a spectrophotometer, using a wavelength of 750 nm.

A calibration curve, prepared with bovine serum albumin as standard (25–200 μg protein per ml), was used to convert the spectrophotometer reading into μg protein per sample.

2.3. Preparation of cell-free extracts

The cultures were harvested at 5–10 °C in an MSE high speed centrifuge at 23,000 × *g* for 15 min. The cells were washed twice with cold 0.025 M phosphate buffer, pH 7.0, and again centrifuged. After being weighed, the pellets were resuspended in the same buffer solution at a ratio of 3 ml per g of cell paste. The cells in this suspension were broken in a precooled (0–5 °C) French pressure cell (American Instrument Co., Inc.) at ca. 16,000 psi. The resulting extracts were centrifuged at 38,000 × *g* for 30 min and the supernatant stored under Argon at –20 °C until just before being used for assay. In later experiments these extracts were further purified by anaerobic heating at 60 °C for 10 min. Centrifugation at 38,000 × *g* for 15 min sedimented the inactive protein fraction (Hwang and Burris, 1972).

2.4. The assay of nitrogenase activity

2.4.1. *Nitrogenase activity* in vitro

Assays were carried out in 7.4-ml bottles fitted with metal caps and silicone-rubber septa. Each cap was provided with two small holes, one for injecting a gas and the other for releasing overpressure.

One ml reaction mixture was used containing 10.5 mg creatine phosphate (ca. 30 μ moles), 0.2 mg phosphocreatine kinase, 5 μ moles ATP, 5 μ moles $MgCl_2.6H_2O$, and 20 μ moles sodium hydrosulfite (dithionite). The creatine phosphate, phosphocreatine kinase, and ATP were prepared in 44 mM sodium cacodylate buffer, pH 7.0. Three tenths of a milliliter of this solution was added to each flask, along with 0.05 ml of a $MgCl_2.6H_2O$ (100 μ moles/ml) solution and 0.25 ml sodium cacodylate buffer (0.2 M, pH 7.0). The contents of the flasks were flushed with Argon gas for 3 min. Two tenths of a milliliter of a dithionite solution containing 100 μ moles/ml (prepared anaerobically with 0.2 M sodium cacodylate buffer), was added to the reaction mixture with a syringe.

The enzyme activity was assayed by the acetylene-reduction technique. To start the reaction, 0.2 ml of the extract was added to the flask. At the same time acetylene was injected into the flask to make a final concentration of 10% (v/v) of acetylene in the gas phase. The rate of acetylene reduction was measured by periodically taking a gas sample (100 μl) from the flask and estimating the ethylene formed by a Unigraph-F-analytical gas chromatograph, type 407 (Becker, Delft, The Netherlands), equipped with a flame-ionization detector and a matched column of Porapak R, at 45°C. The ethylene content was calibrated with a standard mixture consisting of 100 ppm C_2H_4 in nitrogen gas. Under constant conditions the height of the peak was linearly related to the concentration of C_2H_4 (Hardy et al., 1968).

2.4.2. *Nitrogenase activity* in vivo

The nitrogenase activity of living cells was assayed either by measuring the increase in total nitrogen of the whole culture or by employing the acetylene-reduction technique.

The determination of total nitrogen was carried out by acid digestion of an aliquot of the whole culture, followed by colorimetric measurement of the ammonia after the addition of Nessler's reagent (see 2.8.1).

The rate of acetylene reduction was estimated by incubating 10 ml of a culture in air containing 10% C_2H_2. The affinity of nitrogenase for C_2H_2 is much higher than that for N_2 so that in such a gas mixture N_2 reduction is effectively inhibited and reduction of C_2H_2 is virtually the same as in the absence of N_2 (Akkermans, 1971). The ethylene formed was measured gas-chromatographically as described in 2.4.1.

2.5. Measurement of hydrogen

The hydrogen content of a gas mixture was measured with a Multigraph, type 409, gas chromatograph (Becker, Delft, The Netherlands), equipped with a thermal conductivity detector and a 2-m length, 4-mm I.D. column packed with 60–80 mesh of molecular sieve 13X, operating at 45 °C with argon as carrier gas at a flow rate of 40 ml/min.

2.6. Determination of glucose in the culture medium

After separating the cells from the culture by centrifugation, glucose in the culture filtrate was estimated by the method of Somogyi-Nelson (Somogyi, 1952).

Two ml of copper reagent and 2 ml of culture filtrate (if necessary diluted), containing 5–50 μg of glucose per ml, were mixed in a tube and heated for 15 min in a boiling water bath. After cooling, 2 ml of Nelson's reagent was added. The absorbancy of the blue colour was measured against a reagent blank in a Vitatron colorimeter supplied with a filter to give light with a wavelength of 520 nm. A standard solution containing 50 μg of glucose per ml was used for calibration.

The copper reagent of Somogyi was prepared by dissolving copper sulphate (4 g), anhydric sodium carbonate (24 g), sodium bicarbonate (16 g), potassium, sodium tartrate (12 g), and anhydric sodium sulphate (180 g) in water and making up this solution to 1 l.

The reagent of Nelson was prepared by dissolving ammonium molybdate (25 g) in 450 ml of water; this solution was supplied with 21 ml of concentrated sulphuric acid and subsequently a solution of 3 g sodium arsenate ($Na_2HAsO_4.7H_2O$) dissolved in 25 ml of water. This reagent must be incubated at 37 °C for 48 h prior to use.

2.7. Determination of total volatile fatty acids in the culture medium

The cells of a culture were separated by centrifugation and 5 ml of the supernatant was placed in the Markham distilling-apparatus, supplied with 5 ml of a 1 M solution of KH_2PO_4 which had been brought to pH 3.5 by the addition of 60% (w/v) aqueous H_3PO_4. The volume was made up to 100 ml by the addition of distilled water. During the distillation this volume was approximately kept constant by adding distilled water. A portion of 150 ml of distillate was collected and an aliquot titrated with a 0.01 N NaOH solution, using phenol red as the indicator.

2.8. Determination of nitrogen compounds in the culture

2.8.1. *Total nitrogen*

A portion of 10–20 ml of culture was brought into a 100-ml Kjeldahl flask and digested with 2.5 ml of concentrated H_2SO_4 and 2.5 ml of digestion solution. The composition of the digestion solution was: 2 g SeO_2, 1 g $CaSO_4$, 500 g $KHSO_4$ and 1000 ml of distilled water. In the presence of a high concentration of glucose (or another carbon compound), a few drops of a 30% H_2O_2 solution were added when the digest began foaming. The digestion was carried out until the content turned clear green and was continued for a further 30 min. When the digestion was complete, the contents of the flask were provided with distilled water, cooled, and further diluted to obtain ca. 10–15 μg of ammonia nitrogen per ml. A portion of 2 ml of the diluted digest was mixed with 2 ml of Nessler's reagent (containing per 1: 4 g HgJ_2, 4 g KJ, and 1.75 g of gum ghatti) and 3 ml of 2 N NaOH. After standing for 15 min at room temperature, the optical density was measured aginst a reagent blank in a Vitatron colorimeter supplied with a filter to give light with a wavelength of 540 nm.

Alternatively, the ammonium present in the digest was distilled with a Markham distilling-apparatus into a 4% H_3BO_3 solution containing methyl-red bromocresol-green indicator, and titrated with 0.01 N HCl.

When chloramphenicol was present in the culture, the digestion was modified according to the method described by Steyermark et al. (1958).

2.8.2. *Estimation of free amino acids of bacterial cells*

The total free amino acid content of bacterial cells was estimated by extraction with hot water, followed by analysis of the extract with ninhydrin reagent according to the method described by Rosen (1957). Leucine was used as reference standard. Ammonia and amines give also a colour with ninhydrin reagent. Therefore, when these compounds were supposed to be present in a relatively high concentration, the results were reported as the ninhydrin-positive compounds (n.p.c.) of the pool (see 7.4).

3. GROWTH AND EFFICIENCY OF NITROGEN FIXATION BY *AZOTOBACTER CHROOCOCCUM* SUPPLIED WITH DIFFERENT SOURCES OF CARBON AND ENERGY

3.1. INTRODUCTION

As mentioned in 1.1, the fixation of atmospheric nitrogen by nitrogen-fixing bacteria can occur only when these bacteria have an accessible source of energy at their disposal. The determination of the assimilability of different carbon compounds by *Azotobacter* spp. and the efficiency of nitrogen fixation by these bacteria when these compounds are utilized is of great interest. The results obtained in such investigations permit a more precise evaluation of the role of nitrogen fixation by *Azotobacter* spp. under natural conditions.

WINOGRADSKY (1935) pointed out that the use of glucose or mannitol as nutrients for *Azotobacter* spp. is not appropriate from an ecological point of view. There is no doubt that in their natural habitat these organisms never have the opportunity to feed on glucose or mannitol; most certainly they derive their energy from the decomposition products of glucose (or other sugars), such as ethanol, acetic acid, propanol, butanol, butyric acid etc. JENSEN (1965), however, raised the question whether such decomposition products of sugars could always be used as sources of energy by *Azotobacter*.

In the present study, some *Azotobacter* strains, especially those of *A. chroococcum*, have been grown in liquid media supplied with hexoses, hexitols, fatty acids (as the sodium and calcium salts) and primary alcohols, to see whether the degree of oxidation and the molecular size (chain length) of the carbon compound have any effect on the efficiency of nitrogen fixation, defined as the amount of nitrogen fixed per gram of carbon compound consumed by these organisms.

3.2. HEXOSES AND HEXITOLS

Table 3.1 shows the observations on the growth of two strains of *A. chroococcum* and one strain of *A. vinelandii* in media supplied with three different hexoses and their corresponding heixtols. From these semi-quantitative data it can be seen that one strain of *A. chroococcum* (A40) and one of *A. vinelandii* (A66) grew with galactose, mannitol and sorbitol but failed to grow with galactitol (dulcitol), mannose and sorbose. The second strain of *A. chroococcum* (R25) used in this experiment grew poorly with galactose and sorbose, and failed to grow with the rest of the hexoses and hexitols mentioned above. JENSEN (1954) inserted sorbose into the list of carbon compounds assimilable for azotobacters. But only one strain of the organisms tested here was able to

TABLE 3.1. Growth of *A. chroococcum*, strains R25 and A40, and *A. vinelandii*, strain A66, after being cultivated for 48 hr at 30 °C in a medium supplied with hexoses and hexitols[a]

Organism	Hexoses			Hexitols		
	Galactose	Mannose	Sorbose	Galactitol	Mannitol	Sorbitol
A. chroococcum, strain R25	+[b]	−	+	−	−	−
A. chroococcum, strain A40	+++	−	−	−	+++	+++
A. vinelandii, strain A66	+++	−	−	−	+++	+++

[a] 0.01 M concentrations of hexoses or hexitols have been used. [b] — indicates no detectable growth, + poor growth, +++ good growth.

grow, be it very poorly, with sorbose. For the purpose of this study, hexoses and hexitols listed above could not be further used.

3.3. SODIUM SALTS OF FATTY ACIDS AND CORRESPONDING ALCOHOLS

A. chroococcum, strain A40, was precultivated in modified Burk's mineral salts medium, containing 0.1 % (w/v) of glucose, at 30 °C for 20 hr. The cells were harvested by centrifugation, washed twice with saline phosphate buffer, and resuspended in mineral salts medium without carbon compound. The nitrogen content of this suspension, was 17 μg/ml. Portions of 2 ml of this suspension were inoculated into 18 ml of modified Burk's mineral salts medium containing the sodium salt of different fatty acids and the corresponding alcohol, in 100-ml flasks. The final concentration of carbon compounds in the cultures was 6.10^{-2}M. These cultures were then incubated on a shaker at 30 °C. At different periods of incubation (50, 70 and 90 hr) two cultures of each carbon-compound series were analysed for total nitrogen. In calculating the efficiency values of nitrogen fixation it was assumed that the carbon compound supplied to the media had been entirely consumed when no further increase of the total nitrogen content occurred (for comparison see 5.2).

The results obtained (Table 3.2) show that ethanol gave the highest efficiency of nitrogen fixation and glucose the lowest. Acetate gave much lower values than did ethanol. The results suggests that the more reduced carbon compound is a more favourable source of energy and reducing power for nitrogen fixation. This assumption could not be confirmed for the couples propanol-propionate and butanol-butyrate as in both instances the alcohol did not support growth of *A. chroococcum*. No much difference was observed between the nitrogen-fixing efficiencies of *A. chroococcum* supplied with the sodium salts of different fatty acids. There was a slight tendency that fatty acids with longer carbon chains gave a somewhat higher efficiency value.

To see whether the harmful effect of propanol and butanol on growth and nitrogen fixation of *A. chroococcum* could be alleviated by lowering their con-

TABLE 3.2. Efficiency of nitrogen fixation by *A. chroococcum*, strain A40, grown with the sodium salts of fatty acids and the corresponding alcohols[a].

C compound[b]	Nitrogen fixed (μg N/10 ml)	Efficiency of nitrogen fixation (mg N/g substrate)
Ethanol	383	13.9
Propanol	—	—
Butanol	—	—
Na-acetate	235	6.6
Na-propionate	322	7.3
Na-butyrate	392	7.4
Glucose	529	4.9

[a] Values are averages of 2 replicates. [b] Final concentration 6.10^{-2}M.

centration, an experiment was carried out in which the concentration of the carbon compounds was reduced to 10^{-2}M. The inoculum used in this experiment contained 79 μg N/ml.

The results of this experiment (Table 3.3) show that even at a concentration of 10^{-2}M, propanol and butanol did not support growth and nitrogen fixation of *A. chroococcum*, strain A40, as effectively as it was the case with propionate and butyrate. The other carbon compounds tested gave similar results as in the previous experiment, except that the efficiency values of nitrogen fixation were generally higher. It is undecided, whether these differences were caused by the decreased concentration of the carbon compounds or were due to the increased size of the inoculum used in this experiment. In both experiments the cultures had been aerated at a low rate of shaking. BECKING (1971) reported an increased efficiency of nitrogen fixation by *Azotobacter* and *Beijerinckia* spp. by lowering the carbon-compound concentration of the medium.

TABLE 3.3. Efficiency of nitrogen fixation by *A. chroococcum*, strain A40, grown with the sodium salts of fatty acids and the corresponding alcohols[a].

C compound[b]	Nitrogen fixed (μg N/10 ml)	Nitrogen-fixing efficiency (mg N/g substrate)
Ethanol	73	15.9
Propanol	14	2.3
Butanol	31	4.2
Na-acetate	66	11.0
Na-propionate	92	12.4
Na-butyrate	110	12.5
Glucose	183	10.2

[a] Values are averages of 3 replicates. [b]Final concentration 10^{-2}M.

3.4. Calcium salts of fatty acids

The medium used in the present experiments was somewhat different from that described in 2.1, and consisted of K_2HPO_4, 2.50 g; $MgSO_4.7H_2O$, 0.25 g; NaCl, 0.25 g; $CaCl_2.2H_2O$, 0.25 g; $FeSO_4.7H_2O$, 10 mg; $Na_2MoO_4.2H_2O$, 5 mg, dissolved in 900 ml of distilled water. Portions of 22.5 ml of this medium were transferred to 100-ml flasks and supplied with 2.5 ml of solutions of calcium salts of different fatty acids to make a final concentration of 0.1 % (w/v). These media were inoculated with 0.5 ml of an *Azotobacter* culture, precultivated for 48 hr in a similar medium with 0.5 % glucose. After being incubated at 30 °C for 96 hr, the total nitrogen of the cultures was determined according to the method described in 2.8.1 (ammonia was estimated by distillation and titration).

Table 3.4 shows the composite results of 4 of such experiments carried out at different times and using different *Azotobacter* strains as test organisms. These results show that up to four carbon atoms, with calcium propionate as the exception, the amount of nitrogen fixed increased with increased length of the carbon chain. The low values obtained with calcium valerate and calcium caproate were presumably caused by the poor solubility of these compounds in water. The amount of nitrogen fixed with calcium butyrate as the carbon compound was higher than that obtained with glucose. In these experiments the amount of substrate consumed was not determined so that the efficiency of nitrogen fixation could not be computed.

In a subsequent experiment with different fatty acids, the amounts of carbon compounds consumed during the experimental period were estimated according to the methods described in 2.6 and 2.7. Ca-valerate and Ca-caproate, because of their poor solubility in water, and Ca-formate, because of the poor growth of azotobacters obtained with this compound, were omitted from the experi-

Table 3.4. Nitrogen fixation by *Azotobacter* spp. grown with the calcium salts of different fatty acids and glucose, respectively, for 96 hr at 30 °C[a].

C compound[b]	Nitrogen fixed (µg/25 ml of culture)			
	A. chroococcum strain R25	*A. chroococcum* strain R35	*A. agile* strain A27	*A. vinelandii* strain A66
Ca-formate	[50	76	53	60
Ca-acetate	241	392	258	270
Ca-propionate	241	384	60	386
Ca-butyrate	421	610	473	470
Ca-valerate	43	74	43	50
Ca-caproate	52	49	71	62
Glucose	328	475	332	340

[a]All the values reported are averages of 3 replicates. [b]The concentration of the carbon compounds was 0.1 % (w/v).

TABLE 3.5. Growth and nitrogen fixation of *A. chroococcum*, strain R35, cultivated in media with the calcium salts of different fatty acids and glucose for 96 hr at 26 °C[a].

C compound[b]	pH	O.D.	Dry weight (mg/25 ml)	Substrate consumed (mg/25 ml)	Nitrogen fixed (μg N/25 ml)	Efficiency of nitrogen fixation (mg N/g C compound)
Ca-acetate	8.6	0.056	1.2	17.80	280	16.39
Ca-propionate	8.4	0.086	1.7	17.55	302	17.21
Ca-butyrate	8.6	0.142	2.4	16.78	412	24.55
Glucose	6.8	0.113	2.4	25.00	389	15.56

[a]Values reported are averages of 3 replicates. [b]The final concentration of the carbon compounds was 0.1 % (w/v).

ment. From the results of this experiment, with *A. chroococcum*, strain R35, as test organism (Table 3.5), it can be seen that at the end of the experimental period, the pH of the cultures supplied with the calcium salts of fatty acids had become alkaline. This was caused by the assimilation of the fatty acids, thus leaving free Ca^{2+} in the medium. It is possible that the high pH of the medium has adversely affected the rate of consumption of the added fatty acids. At the end of the experimental period residual fatty acids were found in the cultures supplied with these compounds, whereas no residual substrate was found in the culture supplied with glucose. In the cultures provided with the calcium salts of fatty acids, the efficiency of nitrogen fixation by *A. chroococcum*, strain R35, increased with the increase of the molecular weight of the acid. The efficiency of nitrogen fixation with butyrate was approximately 60 % higher than that with glucose. This higher efficiency could be due to one or more of the reasons discussed in 3.5.

3.5. OXYGEN UPTAKE BY *A. CHROOCOCCUM* SUPPLIED WITH CALCIUM BUTYRATE OR GLUCOSE

A. chroococcum, strain R35, was cultivated in modified Burk's medium with calcium butyrate as the source of carbon and energy at 30 °C for 20 hr. The cells were harvested by centrifugation, washed twice with distilled water, and resuspended in a phosphate-buffer solution (0.1 M, pH 7.0). Portions of 1 ml were transferred to Warburg vessels and after being supplied with 10 μ moles of calcium butyrate, 10 μ moles of glucose, and distilled water (endogenous substrate), respectively, the oxygen-uptake values were measured. The results of this experiment (Fig. 3.1) show that ca. 725 μl of O_2 was taken up by the cells for the consumption of 10 μ moles of butyrate and ca. 930 μl for the consumption of 10 μ moles of glucose. From these values it can be calculated that ca. 65 % of the butyrate and ca. 70 % of the glucose given as substrate had been respired. From the same data it can be calculated that for the consumption of

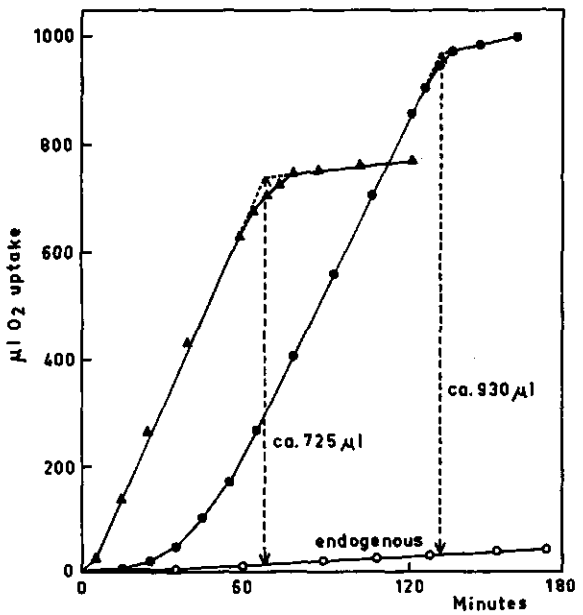


FIG. 3.1. Oxygen uptake of a washed suspension of *A. chroococcum*, strain R35, previously grown on butyrate, after the addition of 10 µ moles of butyrate (▲), and glucose (●).

1 g of butyrate the cells took up 824 ml of O_2 and for the consumption of 1 g glucose, 517 ml. Using a theoretical P/O (ATP formed/O consumed) ratio of 3, these values would correspond with a production of 220 m moles of ATP per g of butyrate consumed and of 138 m moles per g of glucose consumed.

From these calculations it might be concluded that the higher efficiency of nitrogen fixation by *A. chroococcum* supplied with butyrate as compared to that with glucose is due either to the greater reducing capacity (higher equivalent of 'available electrons' according to MAYBERRY, PROCHAZKA and PAYNE, 1967) of the former compound (as shown by a greater O_2 uptake) or by the production of more ATP per weight unit of butyrate. Since azotobacters have very high Q_{O_2}-values (oxygen uptake, µl O_2/mg dry cells/hr), it may be expected that these organisms consistently overproduce ATP and suffer from insufficient reducing capacity and carbon skeletons for cell synthesis (cf. GUNSALUS and SHUSTER, 1961; MULDER and BROTONEGORO, 1974). Therefore, of the two possible explanations given above, the greater reducing capacity per weight unit of butyrate is obviously the cause of the higher efficiency of N_2 fixation by azotobacters supplied with this compound. In chapter 4 it will be shown that excess oxygen is harmful to nitrogenase. Thus it seems logical to expect that substrates possessing greater reducing capacities would be more efficient in protecting nitrogenase against oxygen toxicity.

3.6. Growth in a semi-solid medium supplied with calcium butyrate or glucose

That the higher efficiency of nitrogen fixation by *A. chroococcum* supplied with butyrate as compared to that with glucose is due to the removal of larger amounts of oxygen per weight unit of butyrate, is confirmed by the results of an experiment reported below. This experiment is based on observations of Tschapek and his associates (e.g. Tschapek and Gaborsky, 1953; Tschapek and Giambiagi, 1954).

These investigators found that azotobacters growing in nutrient solutions or in semi-solid media containing small amounts (<0.01 g/l) of carbon compounds have a tendency to thrive and to form layers of bacterial colonies at some distance from the surface of the media where the pO_2 is optimal for N_2 fixation and growth. This distance was found to depend on two factors: (a) concentration of the carbon compound, and (b) the pO_2 at which the culture was incubated.

Under normal conditions, i.e., at a pO_2 of 0.2 atm., the *Azotobacter* colonies develop on the surface of the nutrient solution only if the concentration of carbon compounds is above 0.5 g/l. When the concentration of carbon compounds is below this value, layers of bacterial colonies will be formed in the nutrient solution at distances from the surface inversely proportional to the concentration of the carbon compound.

If the same low concentration of carbon compound is applied, but the pO_2 is varied, the localization of the colonies varies in proportion to the pO_2 level: the higher the pO_2, the greater is the distance from the layer of bacterial colonies to the surface of the media.

In the present investigation *A. chroococcum*, strain R35, was grown in a semi-solid Burk's medium (containing 0.3 % agar) supplied with 10 and 30 ppm of glucose and of calcium butyrate, respectively. The results of this experiment (Table 3.6) show that cells with butyrate developed layers of bacterial colonies at shallower locations than cells supplied with the same concentrations of glucose. This was obviously due to the ability of *Azotobacter* cells supplied

Table 3.6. Effect of different carbon compounds on the depth of the layer of colonies of *A. chroococcum*, strain R35, grown in a semi-solid agar medium.

C compound	Concentration (ppm)	Depth of the layer of bacterial colonies* (mm)
Glucose	10	26
	30	12
Ca-butyrate	10	10
	30	5

* Measured from the surface of the medium. Values reported are averages of 3 replicates.

with butyrate to remove larger amounts of oxygen, and consequently to tolerate higher pO_2 values in the medium, than do cells supplied with the same concentration of glucose.

3.7. Discussion

From the results of the experiments described in this chapter it may be assumed that more-reduced compounds give higher efficiency values of nitrogen fixation by *A. chroococcum*. This assumption was not confirmed for the couples propanol-propionate and butanol-butyrate, presumably owing to the inhibitory effect of these alcohols on cell growth at the concentrations used.

Throughout the investigation, *Azotobacter* cells grown with butyrate gave higher efficiency values than those with glucose. These higher efficiency values might have been due either to the higher reducing capacity or to the higher energy yield of butyrate. Since azotobacters have very high Q_{O2}-values, it may be expected that these organisms consistently overproduce ATP and suffer from shortage of reducing capacity and carbon skeletons for cell synthesis. Obviously the greater reducing capacity per weight unit of butyrate has been the primary cause of the higher efficiency of N_2 fixation with butyrate. If the efficiency values of nitrogen fixation with assimilable carbon compounds obtained in the preceding experiments are compared with the equivalents of available electrons (cf. Mayberry et al., 1967) per gram of carbon compounds, calculated for aerobic cultures (Table 3.7), a good correlation is found to exist between these two values.

Table 3.7. Correlation between efficiency values of nitrogen fixation by *A. chroococcum* and the equivalents of available electrons per g of carbon compound in aerobic cultures (e^-/g).

C compound	Efficiency values adopted from Table			e^-/g
	3.2	3.3	3.5	
Ethanol	13.9	15.9		0.26
Acetic acid	6.6	11.0	16.39	0.13
Propionic acid	7.3	12.4	17.21	0.19
Butyric acid	7.4	12.5	24.55	0.23
Glucose	4.9	10.2	15.56	0.13

4. THE EFFECT OF OXYGEN ON GROWTH AND NITROGENASE ACTIVITY

4.1. INTRODUCTION

Oxygen has a remarkably complicated effect on the growth of *Azotobacter* spp. because these bacteria are only capable of conducting nitrogen fixation, an anaerobic reductive process, in the presence of oxygen. Thus, they are faced with the problem of maintaining their internal concentration of oxygen at a level low enough to enable nitrogenase to function effectively, yet high enough to allow efficient formation of ATP via oxidative phosphorylation. Furthermore, the growth of azotobacters, similar to that of other aerobic bacteria, is delayed or inhibited at very high oxygen tensions. Therefore, it is not surprising to find many contradictory results and different explanations on this subject in the literature (cf. RUBENCHICK, 1960).

MEYERHOF and BURK (1928) originally regarded oxygen toxicity in azotobacters to be due to inhibition of nitrogen fixation, but it was later considered by BURK (1930) to be a general inhibition of growth, since he observed a similar effect of oxygen when the organisms were supplied with combined nitrogen. PARKER (1954), and TSCHAPEK and GIAMBIAGI (1955) also found inhibition of nitrogen fixation by oxygen but they did not determine whether the effect was specific or due to a general reduction of growth caused by inhibition of some other metabolic system. DILWORTH (1962), for example, observed inhibition of pyruvate dehydrogenase by excess oxygen in *A. vinelandii*. PARKER and SCUTT (1960) suggested that oxygen is a competitive inhibitor of nitrogen fixation because it competes for electrons required for nitrogen fixation. This competitive relationship may be masked when the oxygen tension is raised to a very high level where inhibition of growth of the organism limits the rate of nitrogen fixation. On the other hand, DALTON and POSTGATE (1969) assumed that the oxygen sensitivity of azotobacters is related to the known oxygen sensitivity of components of nitrogenase (BULEN and LECOMTE, 1966; KELLY, 1969) and proposed the hypothesis that: (a) in resting bacteria, the nitrogenase is 'conformationally protected', i.e. it acquires a conformation in which the oxygen-sensitive sites are inaccessible to O_2; (b) in growing bacteria the organism adjusts its respiration rate in such a manner that oxygen is prevented from reaching the sensitive site of nitrogenase ('respiratory protection'). Later, Postgate and his associates (DROZD and POSTGATE, 1970a, b; YATES, 1970; HILL, DROZD and POSTGATE, 1972) mentioned the possibility of 'conformational protection' in growing cells when 'respiratory protection' is for some reason (e.g. carbon-limited) inadequate ('switch off' state). Recently WONG and BURRIS (1972), working with subcellular particles of *A. vinelandii*, found that oxygen inhibits nitrogen fixation uncompetitively by affecting nitrogenase-dependent ATP hydrolysis.

One of the very distinct physiological characteristics of azotobacters is their exceptionally high rate of respiration. Q_{O2}-values of 4000 to 5000 have been reported in the literature (cf. WILLIAMS and WILSON, 1954), No other living organism has been found to have a higher Q_{O2}-value than that of azotobacters. PHILLIPS and JOHNSON (1961), and particularly DALTON and POSTGATE (1969), suggested that this high rate of respiration functions as an 'oxygen-wasting system' which maintains a low Eh value within the cell, presumed to be necessary for nitrogen fixation.

Several investigators (MEYERHOF and BURK, 1928; PARKER, 1954; SCHMIDT-LORENZ and RIPPEL-BALDES, 1957; PARKER and SCUTT, 1960; DALTON and POSTGATE, 1969) have shown that the efficiency of nitrogen fixation decreases with increasing pO_2 (from 0.04 atm to 0.2 atm) of cultures of azotobacters. According to DALTON and POSTGATE (1969) the decreased efficiency of nitrogen fixation with increased pO_2 is due to increased respiration for protecting nitrogenase as mentioned above.

Other physiological and physical (structural) characteristics of azotobacters which are presumably functioning in the protection of nitrogenase from the harmful effect of excess oxygen were recently discussed by MULDER and BROTONEGORO (1974).

In an attempt to clarify the inconsistency mentioned above, in the present study some experiments were carried out dealing with certain aspects of the effect of oxygen on growth, respiration and nitrogenase activity of *A. chroococcum*.

4.2. THE EFFECT OF pO_2 ON GROWTH AND NITROGEN FIXATION

A. chroococcum, strain A39, was inoculated into modified Burk's medium containing 0.1 % (w/v) calcium butyrate as the source of carbon and energy, and urea or N_2 as the source of nitrogen. This culture was subsequently incubated at 30 °C for 14 hr. Portions of 25 ml of this culture were transferred to Erlenmeyer flasks of 100 ml capacity, closed with cotton stoppers, and put in desiccators. The air inside the desiccators was replaced by gas mixtures of different pO_2. These gas mixtures were prepared by the displacement method as described by BURRIS and UMBREIT (1957). The replacement of air from the desiccators by the gas mixture was performed according to the evacuation method as described by BURRIS (1957). The desiccators with their contents were then put in an incubation room at 30 °C. During the first 4 hours precautions were taken to release gas overpressure every 15 min.

In agreement with the results reported in the literature (cf. DALTON and POSTGATE, 1969), the data of the present study (Table 4.1) show that *A. chroococcum*, when depending on N_2 for the source of nitrogen, grew better at lower pO_2. Such an effect was not observed when urea had been supplied to the medium. These results suggest that up to a pO_2 of 0.4 atm. the reduction of the growth of azotobacters (growing on N_2) by oxygen was due to increased consumption of the carbon compound for respiratory protection of nitrogenase.

TABLE 4.1. The effect of different partial pressures of oxygen on cell yield[a] and cell nitrogen of *A. chroococcum* grown without or with combined nitrogen.

Source of nitrogen	pO_2 [b]	O.D.	Cell nitrogen (μg N/25 ml.)
N_2	0.4	0.155	241
	0.2	0.174	330
	0.04	0.215	469
	0.02	0.252	506
Urea	0.4	0.244	305
	0.2	0.208	316
	0.04	0.222	319
	0.02	0.222	294

[a] Measured as optical density (O.D.). [b] Approximate pO_2 at the beginning of the experiment. Values given are averages of 3 replicates.

The lower cell yield obtained in the media supplied with urea as compared to that supplied with N_2 at low pO_2, may be explained by an insufficient supply of combined nitrogen owing to the loss of ammonia derived from catabolism of urea. This loss was due to the alkaline reaction of the culture solution resulting from the utilization of butyric acid (see results reported in Table 3.5).

4.3. THE EFFECT OF AERATION ON RESPIRATION AND NITROGENASE ACTIVITY

A. chroococcum, strain A39, was grown in modified Burk's medium with 0.1 % (w/v) calcium butyrate as the source of carbon end energy. Portions of 3 ml of this culture were put in Warburg flasks of ca. 16.5 ml capacity. In one series of these flasks, the glass stoppers of the side arms were replaced by suitable Suba seal caps; this series was used for assaying nitrogenase activity with the acetylene-reduction technique. The other series was used for estimating the rate of oxygen consumption. Both series of flasks were then put in a Warburg apparatus, operated at 30 °C, and after the addition of acetylene to one series, all of the flasks were incubated at a certain rate of shaking. The amplitude of shaking had previously been adjusted to 2.5 cm. This test was repeated at other rates of shaking.

The results of the experiment show that at a rate of aeration corresponding with 80 and 120 strokes per min, nitrogenase activity had reached a maximum value. When the rate of shaking was increased to 160 strokes per min, nitrogenase activity was much lower (Fig. 4.1 B). Such an effect of aeration was not observed in the case of respiration (Fig. 4.1 A). Here it is seen that up to 160 strokes per min, the rate of oxygen uptake increased with increased rate of aeration. Thus shaking at 160 strokes per min, which inhibited nitrogenase activity, did not inhibit the respiration of the organism.

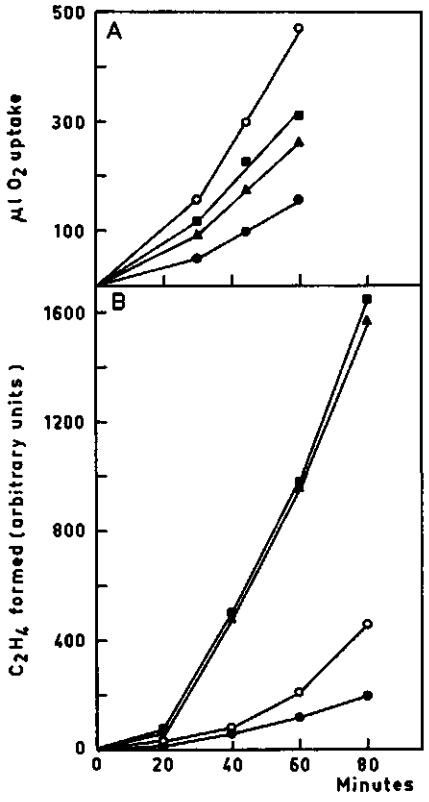


FIG. 4.1. Effect of aeration on respiration (A) and nitrogenase activity (B) of *A. chroococcum*, strain A39.
Rate of shaking: 40 (●), 80 (▲), 120 (■) and 160 strokes per min (○). Amplitude: 2.5 cm.

4.4. THE EFFECT OF CELL DENSITY ON THE OPTIMUM AERATION RATE FOR NITROGENASE ACTIVITY

In subsequent experiments, the optimum aeration rate for nitrogenase activity was found to vary from that corresponding with 80 strokes per min to that with 160 strokes per min. This variation was presumably due to the different cell densities of the *Azotobacter* cultures used for conducting the experiments. It might be anticipated that cultures of higher cell densities could remove larger amounts of oxygen, thus giving a better protection to nitrogenase at a high aeration rate than could be expected from cultures of lower cell densities.

To test this assumption, a similar experiment to that reported in 4.3 has been carried out with *Azotobacter* cultures of two different cell densities. A washed cell suspension, harvested from a 16-hr old culture of *A. chroococcum*, strain A39, was resuspended in two batches of fresh Burk's medium to make two suspensions with cell densities of 43 and 116 µg N/ml, respectively. From each suspension, portions of 3 ml were transferred to Warburg flasks which had been fitted with Suba seal caps. A number of these flasks were then put in a Warburg apparatus at 30 °C and, after the addition of acetylene up to 10 %, were

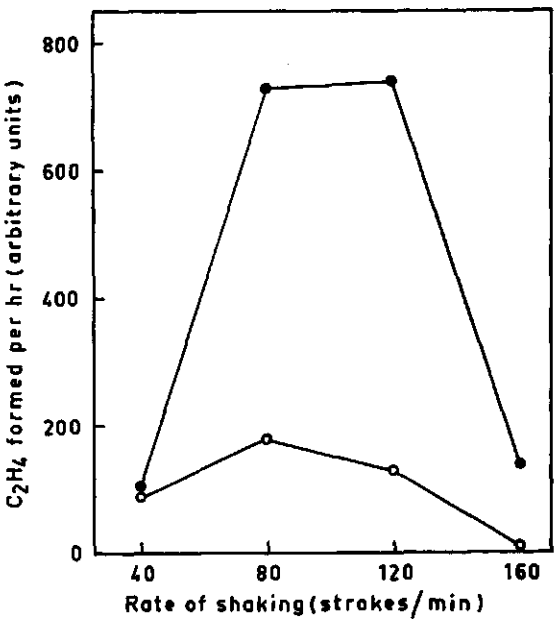


FIG. 4.2. Effect of aeration on nitrogenase activity of cultures of *A. chroococcum*, strain A39, with cell densities corresponding to 43 (○) and 116 (●) μg N per ml, respectively.

shaken at 40 strokes per min with an amplitude of 2.5 cm. The ethylene produced during 24 min in each flask was measured. This performance was repeated at aeration rates corresponding to 80, 120, and 160 strokes per min, respectively.

The results of this experiment (Fig. 4.2) show that the optimum aeration rate for nitrogenase activity depends upon the density of the cell suspensions used. At a low cell density (corresponding to 43 μg N/ml) nitrogenase activity reached a maximum value at the rate of shaking of 80 strokes per min and already showed a decrease at 120 strokes per min. At the higher cell density (corresponding to 116 μg N/ml) the nitrogenase activity assayed at 120 strokes per min was similar to, or even somewhat higher than that assayed at 80 strokes per min. These results suggest that *Azotobacter* cultures with high cell densities can grow at aeration rates which inhibit growth of cultures with low cell densities owing to a more efficient removal of excess oxygen.

4.5. DISCUSSION

The results reported in this chapter show that increased O_2 supply of *A. chroococcum* up to a certain level favoured nitrogenase activity of the cells (Figs. 4.1B and 4.2). This was presumably due to the improved production of energy (ATP) and reductant ($NADPH_2$) resulting from an increased catabolism of the carbon compound (increased respiration). The optimum level of O_2 supply for nitrogenase activity was influenced by the density of the cell suspension (Fig. 4.2). Upon further increase of the O_2 supply of the cells, the nitrogen-

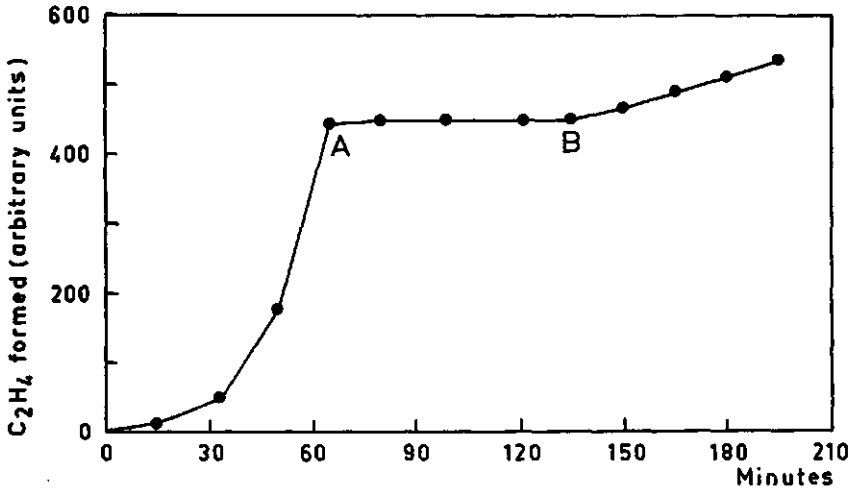


FIG. 4.3. Partially reversible inhibition of nitrogenase activity by O_2. Cells of *A. chroococcum*, strain A39, in Burk's medium with glucose as carbon source, were shaken at 80 strokes per min, amplitude 2.5 cm. at 30°C, under air containing 0.1 atm of C_2H_2. The rate of shaking was changed to 160 strokes per min at A and returned to 80 strokes per min at B.

ase activity decreased, possibly as a result of competition between O_2 and nitrogenase for reductant. At this level of O_2 supply, the rate of cell respiration still increased (Fig. 4.1A). Although the increased respiration (i.e. oxidation of cellular substrate) may have partly protected nitrogenase from being damaged by excess oxygen (Postgate's 'respiratory protection'), it could not prevent a serious drop in nitrogenase activity. In addition, it resulted in a lower efficiency of nitrogen fixation by *A. chroococcum*.

YATES (1970) observed that when the aeration rate was changed from a high, inhibitory value, at which the nitrogenase activity was low or absent, to a medium one, nitrogenase resumed its high activity. DROZD and POSTGATE (1970) found that in some of their experiments the resumption of nitrogenase activity was preceded by a short lag period. This phenomenon could be explained by assuming that $NADPH_2$ should accumulate first before nitrogenase could resume its activity. In the present investigation it was observed that when a culture of *A. chroococcum* was exposed to a high aeration rate for a relatively long time (1 hr) and then was returned to a medium one, the recovery of nitrogenase activity was low (Fig. 4.3). This low recovery was probably due to the inactivation of the oxygen-sensitive component of the nitrogenase.

5. NITROGENASE ACTIVITY OF NON-GROWING OR SLOW-GROWING CELLS OF *A. CHROOCOCCUM*

5.1. INTRODUCTION

Although the biochemistry of nitrogen fixation in nodules of leguminous plants is essentially similar to that of free-living nitrogen-fixing bacteria (KOCH, EVANS and RUSSEL, 1967; KLUCAS, KOCH, RUSSEL and EVANS, 1968; BERGERSEN and TURNER, 1968), a pronounced difference between the two systems exists as to the amount of nitrogen fixed per unit of nitrogen-fixing material. In symbiotic nitrogen fixation, a relatively small amount of nodule tissue is able to supply a large amount of plant tissue with fixed nitrogen. The free-living bacteria, on the contrary, generally fix nitrogen in amounts no more than 10–12% of their cell weight. MULDER (1966) suggested that one of the reasons of this difference should be sought in the fact that the free-living nitrogen fixers only fix nitrogen when they are growing, whereas in the symbiotic system nitrogen fixation takes place in non-growing bacteroids (KENNEDY, 1966; BERGERSEN and TURNER, 1967; KOCH et al., 1967) for several weeks.

There are, however, several reports indicating the possibility of nitrogen fixation in non-growing cells of *Azotobacter* spp. (WINOGRADSKY, 1941; NEWTON, WILSON and BURRIS, 1953; BRUEMMER and RINFRET, 1960; KALININSKAYA and GOLOVACKEVA, 1969). No detailed quantitative data are given in these papers. In his comprehensive review of nonsymbiotic nitrogen fixation JENSEN (1965) concluded, that 'it seems necessary to consider the possibility that nitrogen fixation *in terra* may under some conditions cease to be strictly tied to cell proliferation and to be accompanied by copious excretion products'.

Inspired by these reports and suggestions, in the present study several methods have been tried to obtain nitrogen fixation by non-growing (but still living) cells of *A. chroococcum*. The results of these experiments will be reported in this chapter.

5.2. GROWTH AND NITROGEN FIXATION OF CULTURES TREATED WITH CHLORAMPHENICOL

Chloramphenicol is known to inhibit the protein synthesis, and thus the growth, of procaryotic microorganisms in much lower concentrations than those required for inhibiting the processes of fermentation, respiration, accumulation of free amino acids and nucleic-acid synthesis (GALE and FOLKES, 1953; VAZQUEZ, 1966). Therefore, the application of chloramphenicol for inhibiting the growth of *Azotobacter* cells might be one of the best methods to achieve the aim of the present study.

By using chloramphenicol at a concentration of 100 μg per ml of culture,

BRUEMMER and RINFRET (1960) stopped the growth of *A. vinelandii*. At this concentration of the antibiotic, O_2 uptake was inhibited for about 40%, but the rate of $N_2{}^{15}$ fixation was reduced only 20% as compared to that of control cultures. This would indicate that nitrogen fixation of *A. vinelandii* does not depend upon proliferating cells.

In the present investigation, several experiments have been carried out to test the effect of chloramphenicol on the growth and nitrogen fixation of *A. chroococcum*. The following three representative experiments will be recorded.

5.2.1. *The effect of different concentrations of chloramphenicol on growth and nitrogen fixation at 30°C*

Different amounts of chloramphenicol, dissolved in distilled water and sterilized by filtration through a bacterial filter, were added to 8-hr old cultures of *A. chroococcum*, strain A39, to obtain final concentrations of 20, 60 and 100 μg of the antibiotic per ml of culture. These cultures were continued to be incubated at 30°C. Four and eight hours later, three flasks of each treatment were used for determining cell yields, cellular nitrogen and total nitrogen of the cultures.

The results of this experiment (Table 5.1) show that 4 hr after the addition of chloramphenicol at a concentration of 100 μg per ml of culture, growth of *A. chroococcum* had nearly completely come to a standstill, apparently owing to inhibition of protein synthesis. Increase in yield of *Azotobacter* cells during this period was only 2% when calculated as dry weight but 13% when calculated as cell nitrogen. The difference between these two values can not be explained exactly, but it was most probably due to (1) the abnormally high yield value of 8-hr old cultures (before the addition of cloramphenicol) and (2) the loss of some cellular substance during the washing procedure preceding the estimation

TABLE 5.1. The effect of different concentrations of chloramphenicol on cell yield and nitrogen fixation by cultures of *A. chroococcum*, strain A39, incubated at 30°C.

Time (hr)	CAM (a)	Cell yield (b)	Cell N (c)	Total N (c)	Increase of:[(d)]		
					Cell yield	Cell N	Total N
8	0	532	48	56			
12	0	712	72	84	33	50	50
	20	688	70	83	29	45	48
	60	589	63	77	11	31	37
	100	544	54	70	2	13	25
16	0	1040	108	122	95	125	118
	20	876	101	118	65	110	111
	60	720	81	99	35	69	77
	100	660	67	89	24	40	60

(a) μg chloramphenicol per ml; (b) dry weight, μg per ml; (c) μg per ml of culture; (d) calculated as % of values obtained at 8 hr.

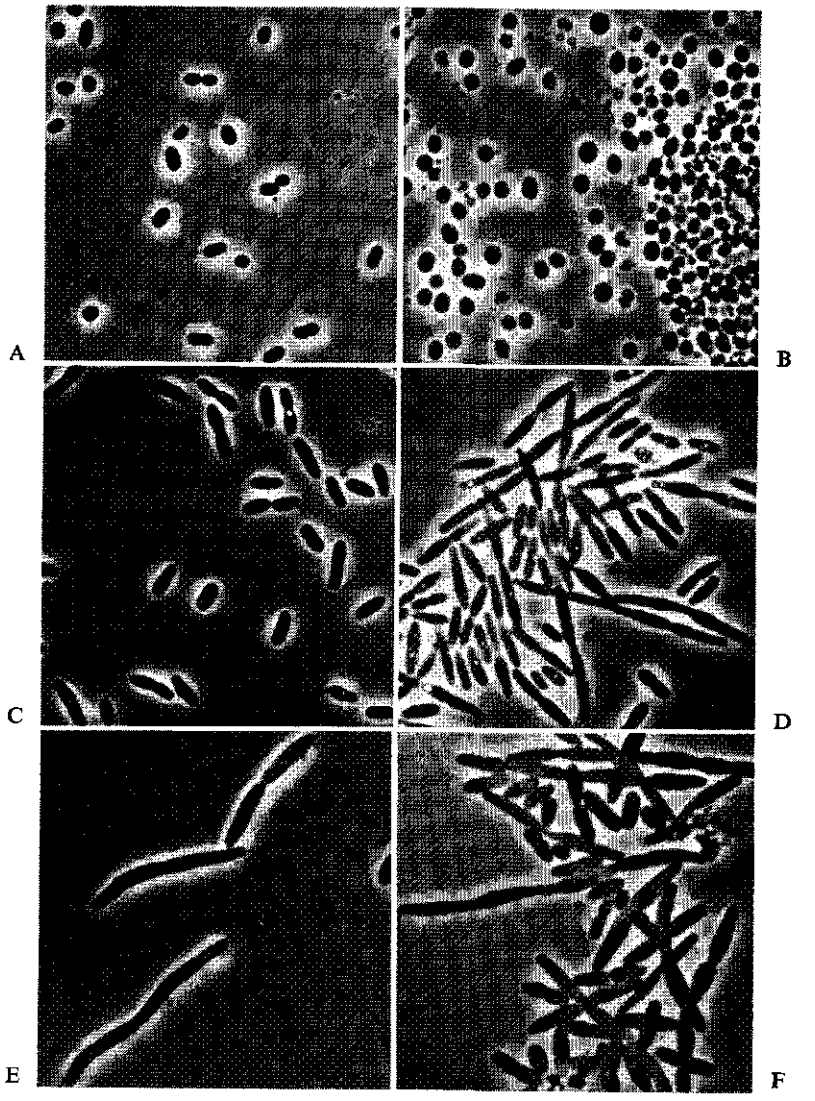


Plate 1
The effect of chloramphenicol (40 μg/ml) on the morphology of *Azotobacter chroococcum*, strain R25, (C and D), and strain A39, (E and F), as compared to that of control cells of strain R25, (A and B). Photos A, C and E were taken 12 hr after the addition of chloramphenicol to cultures C and E, photos B, D and F, 48 hr after the addition of chloramphenicol to cultures D and F.

of cell dry weight. Such a procedure was not applied in the determination of cell nitrogen. Under the microscope, chloramphenicol-treated cells were observed as long, swollen cells which seemed easily to break (see Plate I). In spite of the strong inhibition of cell growth, total nitrogen content of the culture had increased with 25%, showing that nitrogen fixation was less severely affected by chloramphenicol than cell growth.

When these cultures were incubated for a further 4-hr period, cell growth recovered slightly, presumably due to degradation, thus inactivation, of chloramphenicol. During the 8-hr treatment, the increase in yield of cells treated with 100 μg of chloramphenicol per ml of culture was 24% when calculated as cell dry weight or 40% when calculated as cell nitrogen. During the same period, total nitrogen content had increased with 60%. These results show again that the inhibition of growth by chloramphenicol was more pronounced than the inhibition of nitrogen fixation, which confirms the finding of BRUEMMER and RINFRET (1960) cited above.

In subsequent experiments, carried out to confirm the conclusion of this experiment, erratic results were obtained which indicated that at the temperature used in this experiment (30°C), chloramphenicol was to a certain extent degraded and inactivated by *A. chroococcum*. Therefore, another series of experiments were conducted at lower temperatures of incubation, to see whether under these conditions chloramphenicol was more stable.

5.2.2. *The effect of a low concentration of chloramphenicol on growth and nitrogen fixation at 22°C*

Sterile aqueous solutions of chloramphenicol were added to 6-hr old cultures of *A. chroococcum*, strain A39, to obtain a final concentration of 20 μg of the antibiotic per ml of culture. These cultures were then incubated in a shaker at a moderate rate of shaking, at 22°C. After different periods of incubation, a number of cultures were analysed for glucose consumption, cell yield, cell nitrogen and total nitrogen.

The results of this experiment (Table 5.2) show that glucose consumption and

TABLE 5.2. Glucose consumption, cell yield and nitrogen fixation of cultures of *A. chroococcum*, strain A39, incubated in the absence or presence of chloramphenicol (20 μg/ml) at 22°C.

Time (hr)	Glucose consumed[a]		Cell yield[b]		Cell N[c]		Total N[c]	
	Control	+CAM	Control	+CAM	Control	+CAM	Control	+CAM
0			270	310	26	26	29	31
24	9.9	9.2	1160	710	137	83	171	108
32	10	9.9	1130	750	130	92	159	123
48	10	10	1000	630	127	80	162	117
72	10	10	960	520	129	73	165	116
96	10	10	1000	390	130	40	168	116

[a]mg glucose per ml; [b]μg cell dry weight per ml; [c]μg N per ml of culture.

growth, as measured by increase in cell yield or in cell nitrogen, of the control cultures were somewhat faster than those of the chloramphenicol-treated cultures. The maximum yield of the control cultures (1.16 mg dry cells per ml of culture) was obtained 8 hr earlier than that of chloramphenicol-treated cultures (0.75 mg dry cells per ml of culture). The maximum values for growth and nitrogen fixation of chloramphenicol-treated cultures were 50 and 65%, respectively, of those of the control cultures. It can be concluded that chloramphenicol had inhibited the growth of *A. chroococcum* to a slightly higher extent than it had inhibited nitrogen fixation of this organism.

The results of this experiment also show that after having reached a maximum level of growth, cells were undergoing lysis. This was more pronounced in the treated cultures than in the controls.

5.2.3. *The effect of a moderate concentration of chloramphenicol on growth and nitrogen fixation at 20°C*

In this experiment, the final concentration of chloramphenicol was 40 μg per ml of culture. The cultures were then incubated in a shaker at a moderate rate of shaking, at 20°C.

The results (Table 5.3) show that the addition of chloramphenicol at a concentration of 40 μg per ml of culture caused an almost complete inhibition of growth of *A. chroococcum*, strain A39, incubated at 20°C. It can be seen that at 20°C, chloramphenicol was more stable than at 30°C, as a recovery of cell growth in treated cultures took place only after 48 hr of incubation. The increase in cell yield of treated cultures during this period was less than 5% of that of control cultures. Although the consumption of glucose by the former cultures continued, nitrogen fixation of these cultures was completely eliminated. This might indicate that nitrogen fixation does not take place in non-growing cells of *A. chroococcum*, or that chloramphenicol is an inhibitor of nitrogen fixation.

TABLE 5.3. The effect of chloramphenicol (40 μg/ml) on glucose consumption, cell yield and nitrogen fixation, as compared to that of control, of cultures of *A. chroococcum*, strain A39, incubated at 20°C.

Time (hr)	Glucose consumed[a]		Cell yield[b]		Cell N[c]		Total N[c]	
	Control	+CAM	Control	+CAM	Control	+CAM	Control	+CAM
0			170	170	12	12	25	29
24	9.0	7.1	620	170	72	10	103	29
30	9.9	7.6	860	170	104	10	133	29
48	10.0	9.2	790	200	102	14	137	31

[a]mg glucose per ml; [b]μg cell dry weight per ml; [c]μg N per ml of culture.

5.3. The effect of chloramphenicol on nitrogenase activity

In an attempt to find the nature of the depression of nitrogen fixation in chloramphenicol-treated *Azotobacter* cultures, some experiments have been carried out to study the effect of this antibiotic on the nitrogenase activity of intact cells (*in vivo*) as well as of cell-free extracts (*in vitro*) of *A. chroococcum*. The acetylene-reduction technique was used for the assay of the nitrogenase activity.

5.3.1. *The effect of chloramphenicol on nitrogenase activity* in vivo

Portions of 2.7 ml of a cell suspension obtained from a 12-hr old culture of *A. chroococcum*, strain R25, were transferred to 25-ml flasks. Aliquots of 0.3 ml of sterile aqueous solutions of chloramphenicol were added to these flasks to obtain final concentrations of 20, 40, 60 and 80 μg of the antibiotic per ml of culture. These flasks were then closed with suitable Suba seal caps and after the contents were flushed with argon for 3 min, portions of 4 ml of O_2 and 2 ml of C_2H_2 were injected into each flask.

From the rate of acetylene reduction obtained (Fig. 5.1) it appears that the nitrogenase activity of living cells of *A. chroococcum*, strain R25, was adversely affected by the addition of chloramphenicol. The maximum depression, which was not complete, was obtained at the concentration of 40 μg of the antibiotic per ml of culture. Higher concentrations of chloramphenicol hardly increased the level of depression of nitrogenase activity in this organism.

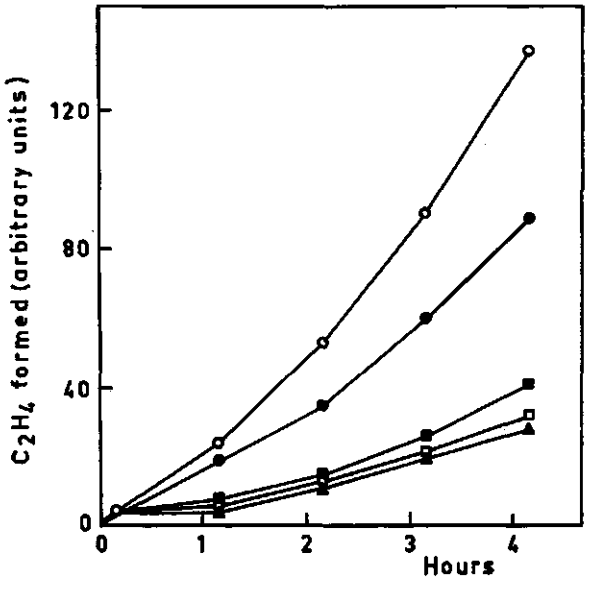


FIG. 5.1. Effect of chloramphenicol on nitrogenase activity of *A. chroococcum*, strain R25. Acetylene reduction in the absence (○) or presence of 20 (●), 40 (■), 60 (□) and 80 μg (▲) of chloramphenicol per ml of culture.

In a subsequent experiment a denser cell suspension of the same organism was used. Sterile aqueous solutions of chloramphenicol were added 96 min after the cell suspensions had been incubated under acetylene. The purpose of this delayed application of chloramphenicol was to eliminate the small amount of soluble nitrogen, including ammonia, before adding the antibiotic. As is shown in Fig. 5.2, addition of chloramphenicol to *Azotobacter* cells which were reducing acetylene, adversely affected further reduction of this compound. The depression reached the maximum level at the concentration of 40 μg of chloramphenicol per ml of culture. This maximum level of depression was somewhat lower than that obtained in the preceding experiment, possibly due to the elimination of ammonia.

The erratic results obtained in some of the preceding experiments with chloramphenicol were supposed to have partially been due to different rates of aeration. To test this assumption a culture of *A. chroococcum*, strain R25, was supplied with chloramphenicol to obtain a final concentration of 20 μg of the antibiotic per ml of culture. Portions of 3 ml of this culture were transferred to

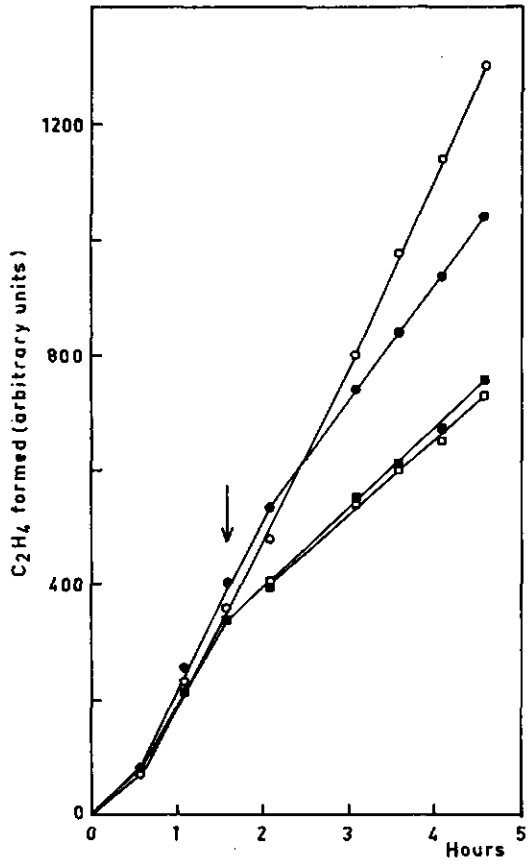


FIG. 5.2. Effect of chloramphenicol, added to cultures of *A. chroococcum*, strain R25, which were reducing acetylene. Arrow indicates the time at which chloramphenicol was added. For the explanation of symbols, see FIG. 5.1.

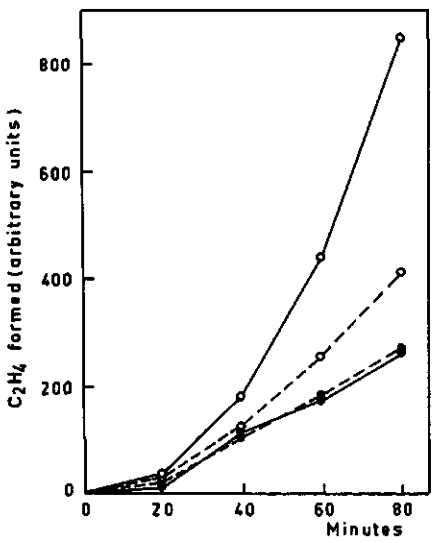


FIG. 5.3. Nitrogenase activity of cultures of *A. chroococcum*, strain R25, incubated in the absence (solid lines) or presence of 20 μg of chloramphenicol per ml of culture (broken lines) at aeration rates corresponding to 80 (closed symbols) and 120 strokes per min (open symbols), respectively.

Warburg flasks which had been fitted with Suba seal caps. A number of these flasks with their contents were then put in a Warburg apparatus and, after the addition of C_2H_2, shaken at 80 strokes per min. After completion of the incubation period of 80 min, the remaining cultures were shaken at 120 strokes per min.

The results of this experiment (Fig. 5.3) show that the decline of the nitrogenase activity of chloramphenicol-treated cultures depended upon the aeration rate to which the cultures were submitted. At the concentration of 20 μg per ml of culture, the antibiotic depressed the nitrogenase activity of cultures incubated at an aeration rate corresponding with 120 strokes per min. However, the same amount of chloramphenicol added to cultures shaken at a rate of 80 strokes per min did not affect nitrogenase activity of the *Azotobacter* cultures.

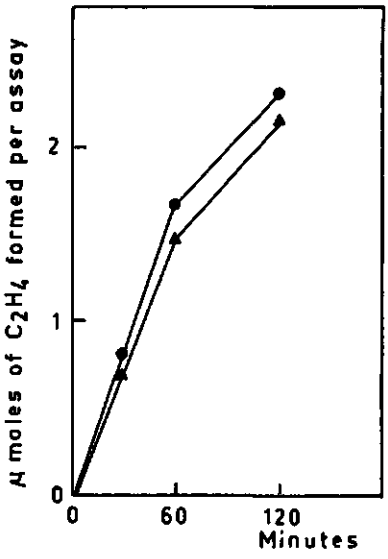
5.3.2. *The effect of chloramphenicol on nitrogenase activity* in vitro

A washed cell suspension, harvested from a 16-hr old culture of *A. chroococcum*, strain R25, was extracted as described in 2.3 and used for measuring nitrogenase activity *in vitro* as described in 2.4.1. To part of the assay mixture, an aqueous solution of chloramphenicol was added to make a final concentration of 100 μg of the antibiotic per ml of mixture. A control-assay mixture was provided with distilled water only. As can be seen from the results of this experiment (Fig. 5.4), chloramphenicol did not inhibit the *in vitro* nitrogenase acitivity of this organism.

5.3.3. *The stability and functioning of nitrogenase in chloramphenicol-treated cells*

The experiments with intact cells of *A. chroococcum* reported in 5.3.1 gave the impression that chloramphenicol inhibited nitrogenase activity rather than

FIG. 5.4. Nitrogenase activity of cell-free extract of *A. chroococcum*, strain R25, in the absence (●) or presence of 100 μg of chloramphenicol per ml of assay mixture (▲).



repressed the synthesis of the enzyme. In the experiment reported in 5.3.2, however, it was shown that chloramphenicol did not inhibit nitrogenase activity *in vitro*. To clarify this apparent discrepancy, some additional experiments have been carried out.

A sterile aqueous solution of chloramphenicol was added to a culture (5l) of *A. chroococcum*, strain A40, to give a final concentration of 100 μg of the antibiotic per ml of culture. After 0.5, 4 and 12 hr, the cells from part of this culture were harvested and washed. The greater portion of the cell suspension was extracted as described in 2.3 and used for measuring its *in vitro* nitrogenase activity as described in 2.4.1. The remaining portion of the washed cell suspension was used for estimating its *in vivo* nitrogenase activity. The acetylene-reduction technique has been used for both assays.

The results of these measurements as compared to those of control cells (Table 5.4) show that the concentration of nitrogenase in chloramphenicol-treated cells of *A. chroococcum*, estimated as specific activity in cell-free extracts of these cells, was decreasing at a rate of approximately 3% per hr. In contrast to the nitrogenase activity of these extracts (measured in the presence of an added ATP-generating system and reductants), the activity of intact cells of the same culture (estimated with endogenous sources of ATP and reductants) decreased much more strongly. Therefore, it can be concluded that although nitrogenase in chloramphenicol-treated cells was present in relatively large concentrations, it was not functioning properly in intact cells. This low *in vivo* activity of nitrogenase may have been due to competition for reductants between nitrogenase and chloramphenicol. In intact cells of *A. chroococcum*, the antibiotic is presumably serving as an alternative and preferred acceptor for electrons originating from reduced ferredoxin. This is concluded from the ob-

TABLE 5.4. Nitrogenase activity of living cells of *A. chroococcum*, strain A40, measured at different periods of incubation after the addition of chloramphenicol (100 μg/ml) to the culture, as compared to that of cell-free extracts prepared from the same culture at the same time[a].

Time of incubation (hr)	Living cells		Cell-free extracts	
	Control	+CAM	Control	+CAM
0.5	109	65	10.2	10.2
4	180	46	10.0	7.7
12	137	4.1	15.2	6.7

[a]All the values given are mean specific activities of three replicates (in nmoles C_2H_4 formed/mg protein/min), calculated from the linear portions of the acetylene-reduction curves.

servation of O'Brien and Morris (1971) concerning a ferredoxin-dependent reduction of chloramphenicol in *Clostridium butylicum*.

An additional explanation of the chloramphenicol-dependent depression of nitrogenase in non-growing azotobacters in air could be that the slowly continued nitrogen fixation caused an accumulation of soluble nitrogen (NH_4^+) in the non-growing cells which may have adversely affected the nitrogenase activity (see 7.3.1).

5.4. GROWTH AND NITROGEN FIXATION OF CULTURES DEPRIVED OF POTASSIUM IONS

ENNIS and LUBIN (1961, 1965) suggested that potassium-depleted conditions in cultures of *Escherichia coli* produced a specific impairment of protein synthesis similar to that imparted by chloramphenicol. HAROLD and BAARDA (1968) confirmed this finding by showing that replacement of potassium in *Streptococcus faecalis* by sodium ions caused the inhibition of protein synthesis.

The purpose of the present experiment was to study the effect of potassium deficiency on cell growth and metabolism, including nitrogen fixation, of *A. chroococcum*.

Cells of *A. chroococcum*, strain A40, harvested from a 14-hr old culture, were washed once with Na-phosphate buffer and transferred to a medium deficient in potassium and calcium ions. After an incubation for 6 hr, the cells were collected and transferred to a fresh deficient medium and after the same incubation period this procedure was repeated once more. The purpose of these transfers was to remove (by simple dilution and by cell growth) traces of K^+ and Ca^{2+} adhering to, or remaining within the cells. At the beginning of the experiment, the cells were centrifuged and washed aseptically with sterile Na-phosphate buffer. Part of the washed cells were inoculated into a 'normal' medium, and another part were used for inoculating a medium in which potassium had been replaced by sodium ions. At several intervals aliquots of the

TABLE 5.5. Glucose consumption, viable counts, cell protein and nitrogen fixation of cultures of *A. chroococcum*, strain A40, deprived of potassium or calcium ions, as compared to that incubated in complete medium.

Time (hr)	Media	Glucose consumed (a)	Viable count (b)	Cell protein (c)	Total N (d)	Increase of Cell protein (c)	Increase of Total N (d)
0			45	26	80		
4	Complete	0.7	58	47	112	21	32
	Minus K^+	0.4	74	40	103	14	23
	Minus Ca^{2+}	0.3	44	26	80	0	0
12	Complete	3.0	313	180	463	154	383
	Minus K^+	1.8	137	49	150	23	70
	Minus Ca^{2+}	1.4	58	32	80	6	0
20	Complete	4.8	658	410	980	384	900
	Minus K^+	2.1	83	50	160	24	80
	Minus Ca^{2+}	1.7	74	34	70	8	−10

(a) mg glucose per ml of culture; (b) viable count per 10^{-5} ml of culture; (c) μg protein per ml; (d) μg N per 10 ml.

cultures were taken for the estimation of glucose consumption, viable cells, protein nitrogen and total nitrogen.

From the results obtained (Table 5.5), it can be seen that during the first four hours, the potassium-deprived cells synthesized less protein than the cells of the control cultures. Hereafter the growth of potassium-deprived cells ceased almost completely. The number of viable cells increased until 12 hr of incubation. At 20 hr of incubation, the number of viable cells was lower. The reduction of nitrogen fixation (calculated from data on total nitrogen) of cultures deprived of potassium ions was less pronounced than the reduction of growth (calculated from data on protein nitrogen). This resulted in an accumulation of non-protein nitrogen in K-deficient cells.

As a consequence the ratio of soluble nitrogen to protein nitrogen in such cells was higher than in control cells, an observation which is in agreement with the effect of potassium deficiency in higher plants.

5.5. GROWTH AND NITROGEN FIXATION OF CULTURES DEPRIVED OF CALCIUM IONS

Calcium has been reported to play an important part in the metabolism of azotobacters. Decrease of the calcium concentration of the nutrient medium prolonged the lag phase of *Azotobacter* cultures growing at the expense of atmospheric nitrogen (ESPOSITO and WILSON, 1956). Therefore, it was thought to be interesting to study nitrogen fixation of *A. chroococcum* under such conditions.

Potassium- and calcium-deficient cells of *A. chroococcum*, strain A40, obtained in the pre-treatment of the previous experiment (5.4), were inoculated into a medium deficient in calcium ions. At different periods of incubation, aliquots of this culture were analysed for glucose consumption, viable cells, protein nitrogen and total nitrogen.

From the results obtained (Table 5.5) it can be seen that, although the consumption of glucose continued, both growth (calculated from data on protein nitrogen) and nitrogen fixation (calculated from data on total nitrogen) of calcium-deprived cultures had ceased. If it is assumed that the removal of potassium from *Azotobacter* cells in the pre-treatment was as effective as that of calcium ions, then it can be concluded that calcium deficiency affects growth and nitrogen fixation of *A. chroococcum* more seriously than potassium deficiency does.

5.6. Growth and nitrogenase activity of cultures deprived of nitrogen

In most bacteria, non-growing cultures can be obtained by suspending cells, previously washed with a sterile phosphate buffer, in a medium devoid of assimilable nitrogenous compounds. This method can not be applied to bacteria that use atmospheric nitrogen as the source of nitrogen. However, nitrogenase which normally catalyses nitrogen fixation, is also able to reduce non-physiological substrates such as acetylene. Since the estimated Km value of nitrogenase for C_2H_2, based on partial pressure, is only about 5% of that for N_2 (Hardy, Holstein, Jackson and Burns, 1968), nitrogen fixation, and thus the growth of *A. chroococcum*, can be stopped by incubating this organism in air containing acetylene.

In the present study, *A. chroococcum*, strain A40, was incubated in air containing 10% acetylene. At different periods of incubation, growth and nitrogenase activity of such cultures were determined. In the first experiment the number of viable cells, used as a measurement of cell growth, increased very slightly during an incubation period of 8 hr (Fig. 5.5-IA). In the second experiment cell growth was measured as increase of protein content; here practically no growth of *Azotobacter* cells occurred when the organism was incubated under acetylene-containing air. In spite of the nearly complete standstill of growth in both experiments, the nitrogenase continued to reduce acetylene. The specific activity of nitrogenase of cells incubated under acetylene-containing air was greater than that of normally growing cells incubated under air. This can be seen from Fig. 5.5-IC, where the specific activity of nitrogenase is expressed as ethylene produced per 10^8 cells per min, and from Fig. 5.5-IIC, where it is expressed as ethylene produced per mg of protein per min. These differences in specific activity of nitrogenase between growing cells incubated under air and non-growing cells incubated under acetylene-containing air may have been due to one or more of the factors discussed below.

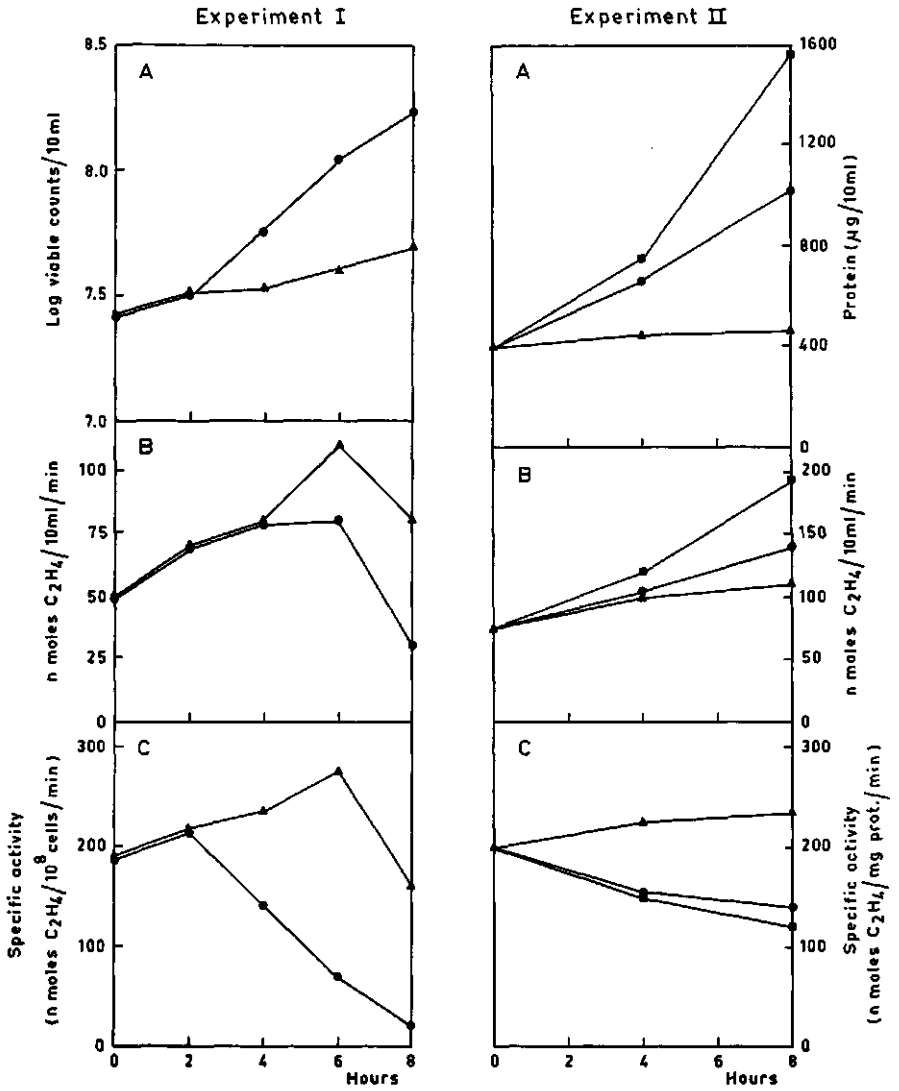


FIG. 5.5. Growth (A), nitrogenase activity (B) and specific activity of nitrogenase (C) of *A. chroococcum*, strain A40, incubated under acetylene (▲), air with rubber caps (●) and air with cotton stoppers (■).

In the closed systems used in these experiments, lack of oxygen might have been the limiting factor for the nitrogenase activity. Although excess oxygen is harmful to nitrogenase (see chapter 4), O_2 is required by *Azotobacter* cells for the synthesis of ATP. In closed flasks under air, oxygen was depleted by *Azotobacter* cells sooner than in closed flasks under acetylene-containing air, because in the former case the cells grew vigorously as contrasted to the cultures under

acetylene-containing air which did not grow owing to nitrogen deficiency. Thus lack of oxygen, with the consequence of a diminishing supply of ATP, might have been the cause of the decline of nitrogenase activity of cultures incubated for 4 hr under air in closed vessels (Fig. 5.5-IB). Oxygen deficiency has been reported by DART and DAY (1971) to be the limiting factor for the nitrogenase activity of root nodules from leguminous plants incubated in closed vessels.

The assumption that lack of oxygen was the limiting factor of the nitrogenase activity of growing cells incubated under air in closed vessels was not supported by results obtained in the second experiment, in which *Azotobacter* cells were incubated in vessels closed with rubber caps as well as with cotton stoppers. The cotton stoppers, which prevented lack of O_2 to be the limiting factor of nitrogenase activity, were replaced by rubber caps only during the assay of the nitrogenase activity. Although the cells in flasks with cotton stoppers grew better than those in flasks with rubber caps, the specific activity of nitrogenase of the former cells was similar to or even somewhat lower than that of the latter.

Another reason for the reduced specific activity of nitrogenase in growing *Azotobacter* cultures incubated under air as compared to that of non-growing cultures incubated under acetylene-containing air might be found in the different reaction products of nitrogenase with N_2 and acetylene, respectively. Under air, nitrogenase catalyses the conversion of N_2 into ammonia which is further assimilated to amino acids. For the assimilation of ammonia, ATP and $NADPH_2$ are required (see chapter 1, section 2.3). These two compounds are also two of the most important co-factors for nitrogenase activity. Thus, in nitrogen-fixing *Azotobacter* cells there may be a competition between nitrogenase activity and ammonia assimilation for these two co-factors. Such a competition does not exist when *Azotobacter* cells are incubated under air containing acetylene at a concentration that competitively inhibits nitrogen fixation. Under such conditions nitrogenase catalyses the reduction of acetylene and the ethylene produced is not further assimilated, and as a consequence is not affecting the nitrogenase activity.

A third possible reason for the reduced specific activity of nitrogenase of cells incubated under air is the repressing effect of NH_3 formed during nitrogen fixation on the synthesis of nitrogenase.

The results of these experiments clearly show the important phenomenon that non-growing cells of *A. chroococcum* are able to maintain a highly active nitrogenase system for at least 8 hr if the formation of ammonia, the product of nitrogen fixation, can be prevented. This can be achieved by using acetylene as substrate for the enzyme. If N_2 is the only substrate present, nitrogenase activity in living cells of azotobacters can only be preserved if the ammonia produced is constantly removed and utilized for the synthesis of amino acids and protein of readily growing cells (for more details on the effect of ammonia on nitrogenase see chapter 7).

5.7. Hydrogen evolution by living cells of *A. chroococcum*

Most of the known nitrogen-fixing microorganisms are capable of producing H_2. Although cell-free extracts of azotobacters are also capable of producing this gas (Table 5.6), the evolution of H_2 by living cells of these organisms has never been observed. Some theories have been suggested to explain this seeming discrepancy.

Table 5.6. H_2 evolved by cell-free extracts of *A. chroococcum* grown on N_2*.

Atmosphere	H_2 evolved (nmoles/0.2 ml/30 min.)
Argon + 10% (v/v) C_2H_2	76
N_2	227

* For assay mixture see 2.4.1.

The absence of a detectable H_2 evolution by living cells of azotobacters might be the result of the action of an unusual hydrogenase present in these organisms which would readily utilize H_2 but could not evolve it. This hydrogenase would reoxidize any H_2 evolved by the nitrogenase of these organisms (personal communication of Dr. Bulen, cited by Postgate, 1971). According to Postgate (1971), this theory is not supported by experimental results. Carbon monoxide (CO), which inhibits conventional hydrogenase, does not cause azotobacters to evolve H_2. Postgate (1971) subsequently gave an alternative theory which suggested that in living azotobacters, the site of nitrogen fixation is effectively anhydrous; the hydrogen ion may be unable to reach this site.

On comparing the consumption of oxygen by *Azotobacter* cells incubated under air to that by cells incubated under air containing approximately 0.1atm C_2H_2, it was found in the present investigation that in the latter case the cells evolved H_2. Subsequently, some experiments were carried out to find the nature of the H_2 production by *A. chroococcum*.

Portions of 10 ml of a culture of *A. chroococcum*, strain A40, were transferred to 124-ml flaks which were closed with suitable Suba seal caps. After subjecting the atmosphere of the flasks to different treatments to obtain 7 variables shown in Table 5.7, these flasks with their contents were incubated in a shaker equipped with a waterbath which had been adjusted to 30°C. After an incubation period of 1 hr, aliquots of 100 µl of gas were removed from the flask for measuring H_2 evolved, using a method described in 2.5.

The results of this experiment (Table 5.7) show, that no H_2 evolution was detected in the cultures which had been incubated under air, either in the absence or presence of carbon monoxide. However, the injection of acetylene at a concentration of approximately 0.1 atm which inhibits N_2 fixation competitively, did cause the production of H_2. Injection of 1% (v/v) CO to such cul-

TABLE 5.7. Hydrogen evolution by living cells of *A. chroococcum*, strain A40, exposed to different atmospheres.

Atmosphere	H_2 evolved (μmoles/10ml/h)
1. Air	0
2. Air + 1% (v/v) CO	0
3. Air + 10% (v/v) C_2H_2	1.139
4. (3) + 1% (v/v) CO	4.439
5. Argon + 20% (v/v) O_2	0
6. (5) + 1% (v/v) CO	0
7. (5) + 3% (v/v) CO	0.100

tures inhibited acetylene reduction but stimulated H_2 evolution. To see if removal of N_2 from the atmosphere would cause H_2 evolution, the air of some flasks was replaced by argon which contained 20% (v/v) oxygen. Under these conditions, as well as in the presence of 1% (v/v) CO, no evolution of H_2 was detected. As small amounts of N_2 were present, either as impurity of argon and O_2 used or as leftover from the air, this N_2 may have prevented H_2 evolution. When 3% (v/v) CO had been injected into the argon-O_2 mixture, some H_2 was evolved. In a further experiment which is not shown here, it was found that the addition of ammonium acetate to a culture of *A. chroococcum*, which was subsequently incubated under acetylene-containing air, prevented the production of H_2. The addition of CO to obtain approximately 10% (v/v) CO in acetylene-containing air inhibited respiration, acetylene reduction as well as H_2 evolution by a culture of this organism. From these results it is concluded that at least part of the H_2 evolved by living cells of *A. chroococcum*, strain A40, reported here, was the product of 'ATP-dependent, CO-insensitive, nitrogenase-catalysed H_2 evolution'.

Although the evolution of H_2 by azotobacters is a complex process, depending on the activities of both hydrogenase and nitrogenase, the lack of H_2 evolution by *Azotobacter* cells incubated under air might be explained as follows. As has been discussed in 5.6, *Azotobacter* cells incubated under air reduce N_2 to ammonia which is further assimilated to amides and amino acids. For the assimilation of ammonia, ATP and/or $NADPH_2$ are required (see 1.2.3). These two compounds are also the most important co-factors required for nitrogen fixation. Therefore, it could be anticipated that in a system with N_2-fixing cells the donation of ATP and electrons to protons for the evolution of H_2 is prevented. Such a prevention would not occur when *Azotobacter* cells are incubated under acetylene containing air (with a final concentration of acetylene of 10%) or in a system with cell-free extracts of azotobacters where the assimilation of ammonia is practically absent (MORTENSON, 1962). GEST (1972) came to the same conclusion in his explanation of the lack of H_2 evolution by *Rhodospirillum rubrum* (a photosynthetic nitrogen-fixing bacterium) growing on N_2 or NH_4. This organism produces H_2 when it is growing on the expense of an amino acid and a carbon source.

5.8. Discussion

In the present study several methods were applied to stop the growth of azotobacters, viz. addition of chloramphenicol (which prevents protein synthesis), depriving the cultures of K^+ or Ca^{2+}, and by incubating the cells in air containing 10% C_2H_2 (which prevents N_2 assimilation). Although the elimination of growth of *Azotobacter* cells by chloramphenicol in some cases was found to be attended with a complete elimination of nitrogen fixation, the latter process was mostly less seriously depressed by the antibiotic than it was the case with cell proliferation.

To explain the suppression of N_2 fixation in non or very poorly growing cells of chloramphenicol-treated azotobacters, the following possibilities should be considered.

(a) Addition of chloramphenicol to growing cultures prevents protein synthesis and as a consequence prevents the synthesis of nitrogenase. If the activity of nitrogenase would not be affected by the antibiotic, nitrogen fixation by cultures incubated under air would continue and would result in accumulation of soluble nitrogen, including NH_4^+, in the non-growing, chloramphenicol-treated cells. This would cause a decline of nitrogen fixation as NH_4^+ depress nitrogenase activity in living cells (see 7.3.1).

(b) Chloramphenicol, in addition to preventing protein synthesis and growth, adversely affects the nitrogenase activity of living cells by: (i) inhibiting the activity of the enzyme, (ii) favouring the degradation of nitrogenase, or (iii) competing with nitrogenase activity for reductants. To determine which of these three possibilities takes part in the depression of nitrogenase activity of chloramphenicol-treated *Azotobacter* cultures, they will be discussed in connection with the observations of the present study.

(i) Addition of chloramphenicol to cell-free extracts did not inhibit the *in vitro* nitrogenase activity of azotobacters (Fig. 5.4).

(ii) Estimation of the *in vitro* nitrogenase activity at different periods of time after the addition of the antibiotic to cultures incubated under air, showed that the degradation of the enzyme in these (non-growing) cells proceeded quite slowly (Table 5.4). Consequently, neither chloramphenicol nor the possibly accumulated soluble nitrogenous compounds (including NH_4^+) have promoted the degradation of nitrogenase.

(iii) The *in vivo* nitrogenase activity of *Azotobacter* cells incubated under air upon the addition of chloramphenicol declines much more readily than the *in vitro* activity of the same cells (Table 5.4). This is thought to be due to competition for reductants between nitrogenase activity on the one hand and chloramphenicol and/or accumulated nitrogenous compounds (including NH_4^+) on the other hand in living cells treated with the antibiotic (Tables 5.1, 5.2 and 5.3).

Submitting a culture of *Azotobacter* cells to potassium deficiency slackens and ultimately entirely stops the growth of the cells. Nitrogen fixation follows this trend, although, similar to chloramphenicol-treated cells, it is less severely affected by K deficiency than growth. This indicates that the effect of K defi-

ciency on nitrogenase activity is indirect, for instance by the accumulation of soluble nitrogenous compounds including NH_4^+. Decreased metabolic activity of the cells, leading to shortage of reductants and (or) ATP, may also be involved.

Depriving growing *Azotobacter* cultures of calcium almost entirely stops cell growth, while N_2 fixation completely ceases. This is in contrast to K-deficient cells which continue to fix some nitrogen, even when cell growth has almost completely come to a standstill.

The most convincing way of showing that non-growing cells of azotobacters may be able to preserve a high nitrogenase activity for a prolonged period is by incubating a culture of growing nitrogen-fixing cells under air containing 10% acetylene (Fig. 5.5). Under such conditons nitrogenase reduces C_2H_2 to C_2H_4 and fixation of N_2 is completely suppressed. As a consequence, cell growth ceases owing to nitrogen deficiency. In such cells the accumulation of soluble nitrogen, in particular NH_4^+, does not take place so that the depressing effect of NH_4^+ on nitrogenase activity can be prevented.

In the absence of acetylene, preservation of an active nitrogen-fixing system is only possible in growing cells in which the ammonia produced is constantly removed and utilized for protein synthesis.

Azotobacter cultures kept under air containing 10% C_2H_2 were found to evolve small amounts of H_2. This evolution was enhanced by adding 1% CO. No H_2 production was obtained when the cells were incubated under air. The explanation of this difference was thought to depend on the fact that part of the reductants and ATP which in growing N_2-fixing cultures were used for the assimilation of fixed N_2, were available for H_2 production when the cells were incubated under air + 10% acetylene.

6. THE EFFECT OF REMOVING NITROGENOUS COMPOUNDS EXCRETED BY *AZOTOBACTER* CELLS ON NITROGENASE ACTIVITY

6.1. INTRODUCTION

Azotobacter cells are known to excrete some nitrogenous compounds into the medium. This excretion depends upon the strain and the species of *Azotobacter*, and on the growing conditions (carbon source, alkalinity of the medium, etc.). For review of earlier work, see e.g., RUBENCHICK (1960).

In a preliminary experiment, using five different strains of *Azotobacter*, it was found that 7–13% of the total nitrogen fixed by these bacteria was excreted by the cells (see Table 6.1). Only a small portion of these excreted nitrogenous compounds was ammonia. Whether this ammonia was a direct product of nitrogen fixation or a product of deamination of amino acids or amides is not known.

In the present investigation some efforts have been made to remove the excreted nitrogenous compounds from cultures of *A. chroococcum*, strain A40, and to study the effect of this removal on the nitrogenase activity of the cultures. It was expected that the removal of the excreted nitrogenous compounds from the cultures would enhance the rate of outward flow of ammonia from the pool and thus might alleviate the repressing effect of ammonia on nitrogenase synthesis.

TABLE 6.1. Growth and nitrogen fixation of five *Azotobacter* strains observed at two periods of incubation.

Species and strain	Incubated for 14 hours				Incubated for 22 hours			
	Total N (a)	Cellular N (b)	Extra-cel.-N (a-b)	Glucose consumed (c)	Total N (a)	Cellular N (b)	Extra-cel.-N (a-b)	Glucose consumed (c)
A. chroococ. A25	98	91	7 (7)	5.60	143	132	11 (7)	11.60
A. chroococ. A27	57	51	6 (11)	2.80	121	108	13 (10)	8.76
A. chroococ. A40	71	63	8 (12)	4.24	118	104	14 (12)	10.48
A. vineland. A66	28	24	4 (13)	3.08	87	79	8 (9)	7.80
A. agile A33	65	61	4 (7)	3.88	120	106	14 (12)	9.72

Values reported here are averages of three replicates; those found in parentheses are % from total nitrogen. (a) and (b) microgram nitrogen per ml of culture; (c) mg glucose per ml of culture.

6.2. Replacement of the culture solution by fresh medium and its effect on nitrogenase activity

Modified Burk's nitrogen-free liquid medium was inoculated with *A. chroococcum*, strain A40. After having been incubated at 30°C for 12 hr, half of this culture was harvested, washed twice aseptically with sterile saline phosphate buffer, and then resuspended in a fresh medium containing the same amount of sugar as present in the discarded supernatant. This cell suspension, as well as the original culture were then divided into several aliquots of 10 ml, transferred to 124-ml flasks and used for measuring the nitrogenase activity by the acetylene-reducing technique as described in 2.4.2.

Fig. 6.1 shows the result of such an experiment. It appears that the rate of acetylene reduction of cells which had been transferred to a fresh medium was slightly higher than that of the original culture. Although the difference in nitrogenase activity was small, it always occurred in the cultures of the two species of *Azotobacter* which have been tested.

6.3. Nitrogen fixation by *A. chroococcum* grown together with a *Rhodotorula* sp.

Many investigators have reported the stimulation of nitrogen fixation in mixed cultures consisting of *Azotobacter* spp and other microorganisms as compared to pure cultures of the *Azotobacter* spp (cf. Rubenchick, 1960; Kalinins-

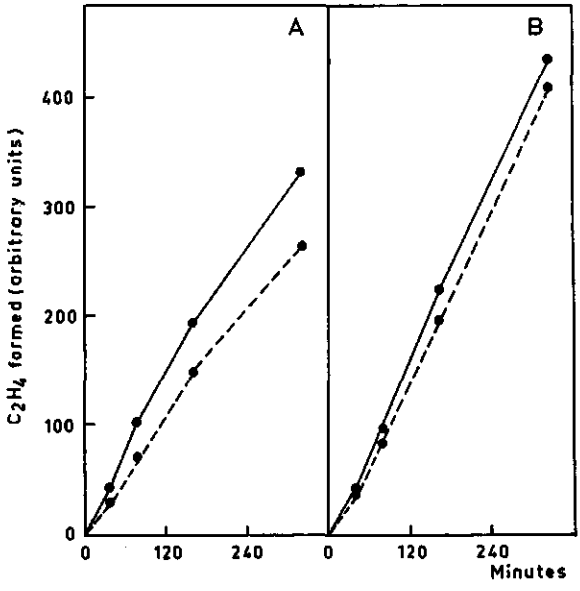


Fig. 6.1. Nitrogenase activity of *A. chroococcum*, strain A25 (A), and A. *vinelandii*, strain A66 (B), after being resuspended in fresh medium (solid lines), as compared to cultures of these bacteria remaining in the original culture media (broken lines).

KAYA, 1967). Different explanations have been put forward to account for the observed phenomena, e.g.: (a) partial decomposition of a substrate otherwise unavailable to *Azotobacter* spp, (b) removal of excess fixed nitrogen, (c) synthesis of growth factors required by *Azotobacter* spp, (d) removal of excess oxygen. In the present investigation, some experiments have been carried out to remove extracellular nitrogenous compounds from cultures of *A. chroococcum*, by growing this organism in the presence of yeast cells.

A 12-hr old culture of *A. chroococcum*, strain A40, was harvested and washed once aseptically with a sterile saline phosphate buffer. At the same time, a 12-hr old culture of a *Rhodotorula* sp., grown on yeast-extract glucose liquid medium was harvested and washed three times aseptically with a sterile saline phosphate buffer. Both *Azotobacter* and *Rhodotorula* cells were resuspended in modified Burk's liquid medium and mixed in different proportions (see Table 6.2). These cultures were then divided into three parts. One part was incubated stationary for 168 hr, a second part was subjected to a moderate rate of shaking for 66 hr and a third part to a high rate of shaking for 16 hr. All of these cultures were incubated at 30 °C. At the end of the incubation periods the cultures were analysed for total nitrogen and glucose.

A yeast, a *Rhodotorula* sp., was used as the associated organism because in a preliminary experiment this microorganism was found to be able to grow in a cell-free supernatant of a culture of *A. chroococcum*, strain A40.

The results of this experiment (Table 6.2) show that after having been incubated for 16 hr at a high rate of shaking, *Azotobacter* cells in pure culture had fixed only a small amount of nitrogen per gram of glucose consumed. *Azotobacter* cells grown with yeasts had fixed clearly larger amounts of nitrogen than those grown in pure culture. Mixed cultures subjected to a moderate rate of shaking for 66 hr had fixed much higher amounts on N_2 than the pure bacterial culture. From the low amounts of nitrogen fixed by the agitated cultures of *Azotobacter* it can be concluded that both rates of shaking had been too high

TABLE 6.2. Glucose consumed, nitrogen fixed and efficiency of nitrogen fixation by *A. chroococcum*, strain A40, incubated at three rates of aeration for different periods of incubation, either in the absence or in the presence of a yeast a *Rhodotorula* sp.

Rate of shaking	Time of incubation (hr)	Azotobacters			Azotobacters + Yeasts (A : Y = 1 : 1)[a]			Azotobacters + Yeasts (A : Y = 1 : 10)[a]		
		G.c. (b)	N.f. (c)	Eff. (d)	G.c. (b)	N.f. (c)	Eff. (d)	G.c. (b)	N.f. (c)	Eff. (d)
High	16	2.9	51	1.8	2.2	74	3.3	3.2	140	4.4
Moderate	66	6.2	75	1.2	10.7	1011	9.5	13.6	1300	9.6
Stationary	168	16.9	2273	13.5	15.8	1478	9.4	17.5	1738	9.9

(a) Ratios of azotobacters to yeast cells, based on cell dry weight of the inocula. (b) Glucose consumed, mg per ml. (c) Nitrogen fixed, µg per 10 ml. (d) Efficiency of nitrogen fixation, mg N per g glucose consumed. All of these values are averages of three replicates.

for optimum nitrogen fixation of the pure culture. The presence of *Rhodotorula* cells apparently improved the efficiency of nitrogen fixation by consuming excess oxygen. This assumption is supported by the observation that the mixed cultures of azotobacters and yeasts had consumed considerably more glucose than the pure culture of azotobacters.

The highest amounts of N_2 were fixed in stationary cultures of *A. chroococcum* incubated for 7 days without the *Rhodotorula* sp. In the presence of yeasts, the efficiency of nitrogen fixation of these cultures was considerably lower. This may have been due to lack of O_2.

6.4. Discussion

The failure of removing fixed N_2 (ammonia) from *Azotobacter* cells is probably due to the following factors: (a) the synthesis processes in the growing cells of *Azotobacter* spp. appear to be rapid enough to remove ammonia formed by the N_2 fixation, hence little ammonia is found as a free metabolite in the pool; (b) the excretion of amino acids, amides and ammonia is not simply effected by concentration gradients between intra and extracellular concentrations of these compounds.

Replacement of the culture solution by fresh medium gave a meagre increase in nitrogenase activity of living azotobacters. This was possibly due to the fact that ammonia made up only a small part of the extracellular nitrogenous compounds. As it will be shown in chapter 7, amino acids do not adversely affect nitrogen fixation by this organism.

The beneficial effect of growing *Azotobacter* cells in the presence of yeast cells was apparently due to the consumption of excess oxygen, thus creating better conditions for nitrogen fixation, rather than to the consumption by the yeast of excreted ammonia as was suggested when starting these experiments. Similar findings have been reported by Kalininskaya (1967) in an association of a nitrogen-fixing *Mycobacterium* sp. with a yeast.

7. THE EFFECT OF COMBINED NITROGEN ON THE SYNTHESIS AND FUNCTIONING OF NITROGENASE

7.1. INTRODUCTION

Extracellular nitrogenous compounds (mainly amino acids), excreted in small amounts by azotobacters, have only little effect on the nitrogenase activity of the bacterial cells (see Chapter 6). This conclusion raises one of the perennial questions in this field: what is the effect of supplemented combined nitrogen on nitrogenase activity. Although this question has been the subject of many investigations (see e.g. RUBENCHICK, 1960; BRADBAER and WILSON, 1963, for review of the earlier work), the mechanism by which combined nitrogen is affecting nitrogenase is not completely understood.

Because of their agronomic importance, most of the earlier studies were primarily concerned with the effect of ammonium ions and nitrate. The effect of organic nitrogenous compounds on nitrogen fixation has been studied much less frequently. BRADBAER and WILSON (1963) suggested that the inhibition of nitrogen fixation by a supplemented nitrogenous compound depended on the readiness of conversion of this compound to ammonia. SORGER (1969) showed that nitrate *per se* did not inhibit nitrogen fixation. Ammonium ions derived from the reduction of nitrate would most likely be the ultimate cause of the inhibition. THOMAS and DAVID (1969) came to the same conclusion following their work with an *Anabaena* sp, a nitrogen-fixing blue-green alga. HILL et al. (1972) on the other hand believed that nitrate *per se* did affect nitrogen fixation, because they found an immediate effect of nitrate on nitrogenase activity. Inhibition by urea is practically the same as that by ammonium ions since urea is readily metabolized to ammonia by azotobacters (owing to the presence of urease). Asparagine (BRADBAER and WILSON, 1963) and glutamine (PAREJKO and WILSON, 1970) are only partially inhibiting, whereas aspartate (PATIL, PENGRA and YOCH, 1967) and glutamate (BECKING, 1962) have been found without effect.

In an attempt to clarify some remaining contradictions found in the literature, in the present investigation the effect of ammonium ions, nitrate, some amino acids and amides on nitrogenase activity has been studied. Furthermore some experiments have been carried out on the combined effect of ammonium ions and oxygen or chloramphenicol on nitrogenase activity in living cells of *A. chroococcum*.

7.2. Effect of ammonia, nitrate, amino acids and amides on nitrogenase activity of living cells of *A. chroococcum*

Fourteen to 18-hr old cultures of *A. chroococcum*, strain A40, were divided into portions of 9 ml and transferred to 124-ml flasks. These cultures were supplied with aliquots of 1 ml of sterile solutions of NH_4Cl, KNO_3, Na-glutamate, Na-aspartate, alanine, leucine, Casamino acids, glutamine and asparagine, respectively, in concentrations giving 60 μg combined nitrogen per ml of culture. The flasks were then closed with Suba seal caps, injected with 10 ml of acetylene gas and afterwards the rate of acetylene reduction was followed by periodically sampling and analysing the ethylene produced by gas chromatography.

The results of these experiments (Figs. 7.1 and 7.2) show that: (1) ammonia immediately and completely suppressed nitrogenase activity *in vivo* of *A.chroococcum* (Fig. 7.1); (2) nitrate inhibited nitrogenase activity slightly less effectively than ammonia did; (3) glutamate had no effect on nitrogenase activity; aspartate, alanine and leucine, when tested separately, gave a result similar to glutamate; (4) Casamino acids gave a partial inhibition; (5) glutamine also partially inhibited nitrogenase activity; asparagine gave a result similar to glutamine. The inhibition of nitrogenase activity by Casamino acids or amides was possibly due to the ammonium ions present in Casamino acids or released by amides. From the estimation of growth of cultures incubated under C_2H_2, as measured by the increase in optical densities, amino acids were found to be hardly assimilated by *A. chroococcum*. Thus, the absence of inhibition of nitrogenase activity of *A. chroococcum* by amino acids so far tested in the present study, is presumably due to the slow uptake of these compounds by this organism.

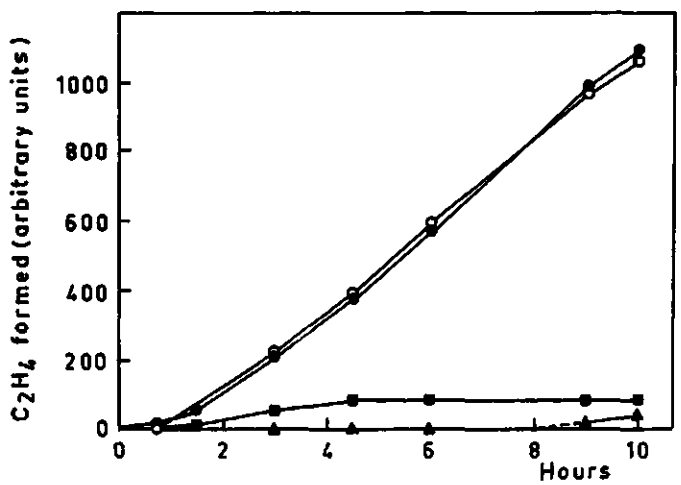


Fig. 7.1. Nitrogenase activity of cultures of *A. chroococcum*, strain A40, after being supplied with NH_4Cl (▲), KNO_3 (■), and Na glutamate (○) at concentrations giving 60 μg N per ml of culture. Control (●).

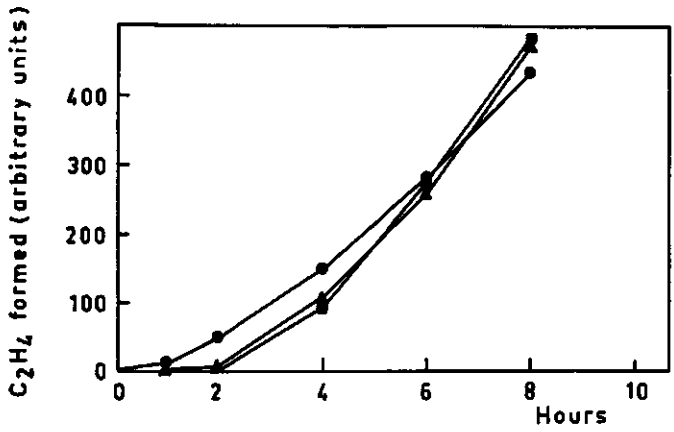


FIG. 7.2. Nitrogenase activity of cultures of *A. chroococcum*, strain A40, after being supplied with Casamino acids, 42 μg N/ml (▲) and glutamine, 60 μg N/ml (■). Control (●).

7.3. THE EFFECT OF AMMONIUM IONS ON NITROGENASE ACTIVITY

In an attempt to find the nature of the inhibition of nitrogenase activity by ammonium ions, some experiments have been carried out to study the effect of these ions on the nitrogenase activity of *A. chroococcum*, *in vivo* as well as *in vitro*. The acetylene-reducing technique was used throughout these experiments for the assay of the nitrogenase activity.

7.3.1. *Nitrogenase activity of living cells of* A. chroococcum *supplied with different amounts of ammonium acetate*

Portions of 7 ml of 16-hr old cultures of *A. chroococcum*, strain A40, were transferred to 124-ml flasks and provided with 2 ml of sterile solutions containing different amounts of ammonium acetate. The acetate concentration of these cultures was brought to the same level by adding different amounts of sodium acetate dissolved in 1 ml of distilled water. The final concentrations of ammonium acetate in the nutrient solution were 0, 1, 2 and 4 mM. The flasks were then closed with Suba seal caps, injected with 10 ml of acetylene gas whereupon the rate of acetylene reduction was estimated by periodically sampling and analysing the gas for ethylene by gas chromatography.

The results of this experiment (Fig. 7.3) show that during the first 72 min, different concentrations of ammonium acetate inhibited the *in vivo* nitrogenase activity of *A. chroococcum* to the same extent. Approximately 110 min after the addition of the highest concentration of ammonium acetate (4 mM), the nitrogenase activity of the organism was completely inhibited. This inhibition lasted ca. 330 min, whereupon the nitrogenase resumed its activity at a very slow rate. The nitrogenase activity of cultures containing ammonium acetate at a concentration of 2 mM was nearly completely suppressed. The enzyme

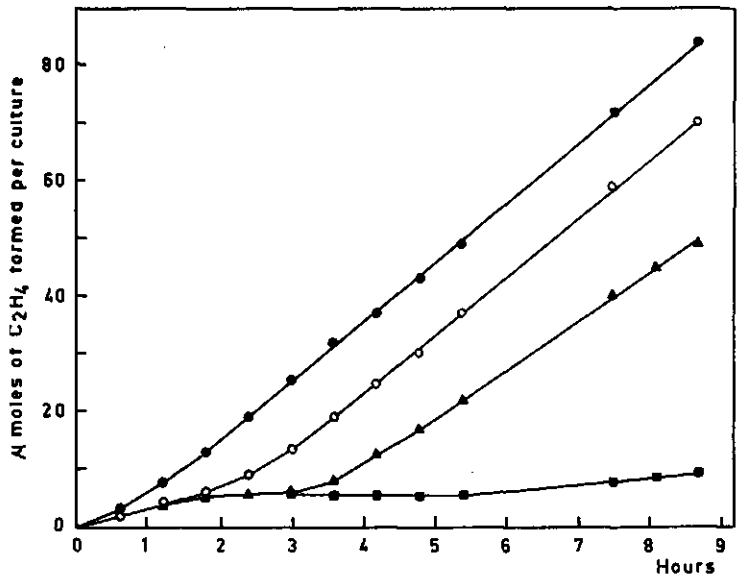


FIG. 7.3. Loss and recovery of nitrogenase activity of cultures of *A. chroococcum*, strain A40, after being provided with ammonium acetate giving final concentrations of 1 (○); 2 (▲) and 4 mM (■). Control (●).

system resumed its activity at a rate which was slightly lower than that of the control cultures approximately 210 min after the addition of the ammonium ions. Cultures containing ammonium acetate at a concentration of 1 mM showed only a partial inhibition of their nitrogenase activity and resumed the activity at a rate similar to that of the control cultures after approximately 2 hr.

These results suggest that the reduction of the nitrogenase activity *in vivo* of cultures of *A. chroococcum*, after the addition of small amounts of NH_4^+, is a combination of an immediate effect (feedback inhibition or a competition for reductants and/or ATP between nitrogenase activity and assimilation of ammonia) and a slower effect owing to repression of nitrogenase synthesis. That repression is partly responsible for the reduced activity is concluded from the fact that in spite of the increased cell density owing to the assimilation of the ammonia, the restored nitrogenase activity of the NH_4^+-treated cultures never exceeds that of the control cultures.

7.3.2. *The effect of ammonium ions on nitrogenase activity* in vitro

Added ammonium ions immediately depress the nitrogenase activity of living cells of *A. chroococcum*, suggesting either a feedback inhibition or a competition between nitrogenase activity and ammonia assimilation for reductants and/or ATP. The purpose of the following experiments was to check which part of this depression was due to feedback inhibition by investigating the effect of ammonium ions on nitrogenase activity *in vitro*.

The contents of five flasks of 5–1 capacity, each containing 1 l of an 18-hr old culture of *A. chroococcum*, strain A40, were harvested, washed and extracted in a manner described in 2.3. Part of this cell-free extract, unpurified, was used for this experiment. NH_4Cl was added to the assay mixture (see 2.4.1) to obtain a final concentration of 56 μg N per ml. The control was supplied with NaCl to obtain the same concentration of Cl ions as the test mixture.

The results of one of such experiments (Fig. 7.4) show that ammonium ions at a concentration high enough to eliminate nitrogenase activity of intact cells of *A. chroococcum* had hardly any effect on the initial rate of nitrogenase activity in cell-free extracts. This result confirms the finding of STRANDBERG and WILSON (1968). On the other hand, HARDY et al. (1968), using much higher concentrations of NH_4Cl, found an adverse effect of this compound on the nitrogenase activity of cell-free extracts of *A. vinelandii*. The same effect, however, was obtained with equimolar concentrations of NaCl.

The results obtained in the present investigation, as well as those reported in the literature (STRANDBERG and WILSON, 1968), suggest that the depression of nitrogenase activity by ammonium ions is not due to feedback inhibition.

7.3.3. *The effect of ammonium ions on the synthesis and functioning of nitrogenase*

The result of the experiment reported in 7.3.2 showed that feedback inhibition did not take part in the depression of nitrogenase activity. The following experiments have been carried out to determine which part of the depression of

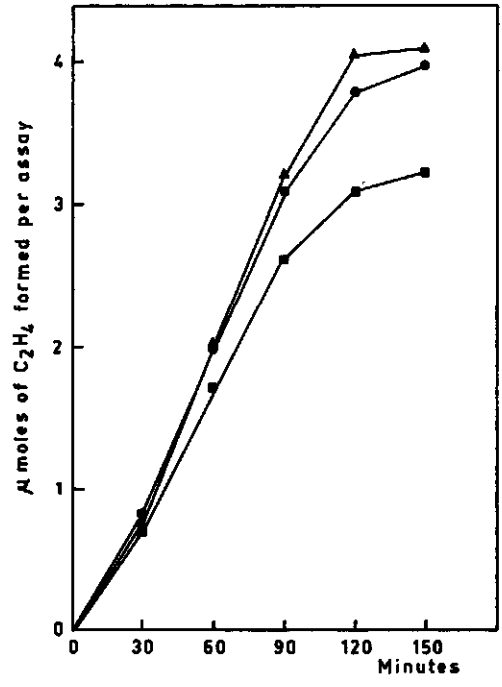


FIG. 7.4. Effect of ammonium ions on nitrogenase activity *in vitro*: acetylene reduction by a cell-free extract of *A. chroococcum*, strain A40, in the presence of 4 mM of NH_4^+ (■) or Na^+ (▲) as compared to that of control (●).

nitrogenase activity in ammonia-treated cultures of *A. chroococcum* was due to the repression of nitrogenase synthesis.

Ammonium acetate was added to cultures of *A. chroococcum*, strain A40, to obtain a final concentration of 5mM. At different time intervals, samples were removed, the bacteria were harvested by centrifugation and the greater part of the cells was extracted. The specific activity of the nitrogenase of living cells and that of cell-free extracts prepared form the same cells, at the same time, were compared. The results of this experiment (Table 7.1) show that the specific activity of nitrogenase of cell-free preparations extracted from cells harvested one hour after the addition of ammonium is slightly higher than that of cells harvested before the addition of ammonium ions. This suggests that the repression of nitrogenase synthesis after the addition of ammonium ions is not immediate. However, the loss of nitrogenase activity of intact cells supplied with ammonium ions began immediately and proceeded at a much faster rate than that of cell-free preparations of the same culture. This result suggests that although the concentration of nitrogenase in cells supplied with ammonium ions was still high, the enzyme was not functioning as it should be. As it was shown earlier (7.3.2) that feedback inhibition was not involved in the depressing effect of NH_4^+ on nitrogenase activity, it can be concluded that competition between assimilation of ammonia and nitrogenase activity for $NADPH_2$ and/or ATP was responsible for this effect. In the presence of excess ammonia, insufficient $NADPH_2$ and /or ATP are assumed to be available for the nitrogenase activity. In cell-free preparations, such a competition does not occur because assimilation of ammonia is practically absent (MORTENSON, 1962).

7.3.4. *Effect of ammonium ions in the presence of excess oxygen on the nitrogenase activity* in vivo

As has been discussed extensively in chapter 4, excess O_2 inhibits nitrogenase activity, presumably by competing for reductants needed for nitrogenase activity (PARKER and SCUTT, 1960). If this hypothesis would be correct, and if the hypothesis proposed in 7.3.3 (i.e. the existence of a competiton between assimilation of ammonia and nitrogenase activity for $NADPH_2$ and /or ATP) would also be correct, then the effect of ammonium ions on nitrogenase activity should be more pronounced in the presence of excess oxygen and should be alleviated by lowering the pO_2.

To check this working hypothesis, several cultures of *A. chroococcum*, strain A40, were preincubated at pO_2 values of 0.4, 0.2 and 0.1 atm in the presence of sufficient glucose. After 6 hr of incubation, when the pO_2 values had dropped to 0.24, 0.13 and 0.08 atm, respectively, a sterile solution of ammonium acetate was supplied to a number of the cultures to obtain a concentration of 1 mM. The remaining cultures were supplied with equimolar amounts of sodium acetate. Portions of adequate amounts of acetylene were then supplied to both series of cultures and the rate of acetylene reduction of these cultures was followed by periodically analysing for ethylene produced by gas chromatography.

The results obtained in this experiment (Fig. 7.5) show, that in cultures in-

TABLE 7.1. Nitrogenase activity of living cells of *A. chroococcum*, strain A40, measured at different intervals after the addition of NH_4-acetate (5 mM) to the culture, as compared to that of cell-free extracts prepared from the same culture at the same time.

Time of incubation (hr)	Living cells			Cell-free extracts		
	Protein (1a)	Acetylene reduction (2a)	Specific activity (3)	Protein (1b)	Acetylene reduction (2b)	Specific activity (3)
0	1.67	224	134 (100)	2.12	53.6	25.3 (100)
1	1.83	21.5	11.7 (8.7)	2.35	60.5	25.7 (102)
2	2.10	12.8	6.1 (4.6)	2.19	50.1	22.8 (90)
4	2.27	10.6	4.7 (3.5)	2.07	36.9	17.9 (71)

Zero time is defined as time at which ammonium ions were added to the cultures. Values in parentheses are % of the original specific activity. All the values are averages of 3 replicates. (1a) mg per 10 ml of culture; (2a) n moles of C_2H_4 produced/10 ml/min. (3) nmoles of C_2H_4/mg protein/min; (1b) mg per ml of assay mixture; (2b) nmoles of C_2H_4 produced/ml assay mixture/min.

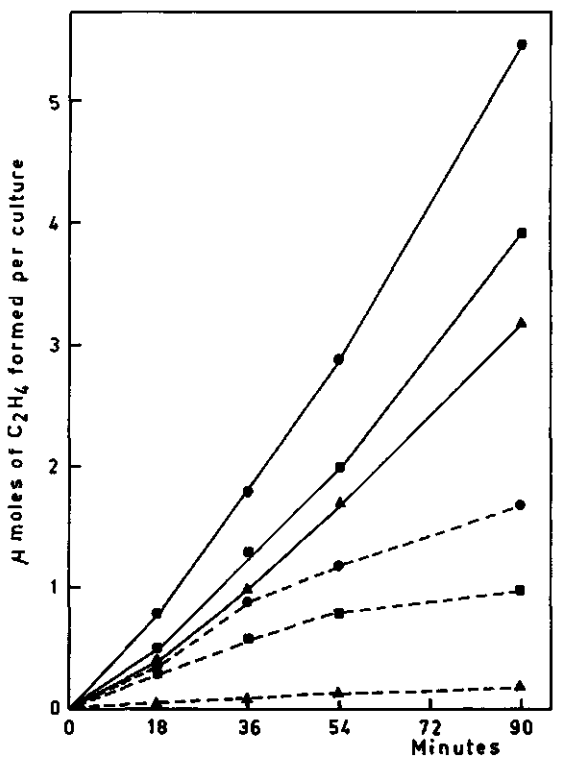


FIG. 7.5. Nitrogenase activity of *A. chroococcum*, strain A40, incubated at partial pressures of O_2 of 0.24 (▲), 0.13 (●) and 0.08 atm (■), in the absence (solid lines) or presence (broken lines) of ammonium acetate (1 mM).

cubated at a pO_2 of 0.13 atm, the effect of ammonium ions on nitrogenase activity *in vivo* was less pronounced than that at a pO_2 of 0.24 atm, apparently as a result of the higher $NADPH_2$ content of the cells incubated at the lower pO_2. However, when the pO_2 had been reduced to 0.08 atm, the adverse effect of ammonium ions on nitrogenase activity was more pronounced than that at 0.13 atm. This was presumably due to the insufficient amounts of ATP present in cells incubated at the partial pressure of 0.08 atm O_2.

7.3.5. *Effect of ammonium ions on the nitrogenase activity of* A. chroococcum *incubated in the presence of chloramphenicol*

Chloramphenicol is known to be an inhibitor of protein synthesis in procaryotic (70 S) ribosomes, by affecting chain elongation beyond the first peptide bond (HAHN, 1967). The concentration of amino acids in the pool of cells incubated in the presence of chloramphenicol is higher than that found in the pool of normal cells. However, chloramphenicol has no specific effect on the amino-acid composition of the pool (RAUNIO and ROSENQVIST, 1970).

Some authors have reported the ability of some bacteria to degrade chloramphenicol (cf. SMITH and WORREL, 1950; O'BRIEN and MORRIS, 1971). One of these bacteria is *Clostridium acetobutylicum* which reduces the aryl-nitro group of chloramphenicol via a ferredoxin-dependent enzymic reaction (O'BRIEN and MORRIS, 1971).

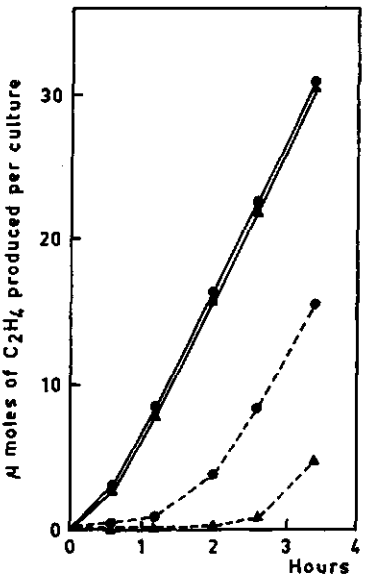
In 5.3.3 the hypothesis was proposed that chloramphenicol inhibits nitrogenase activity *in vivo* of *A. chroococcum*, presumably by competing for reduced ferredoxin. If this hypothesis would be correct, and if the hypothesis proposed in 7.3.3 concerning the effect of NH_4^+ on nitrogenase activity would also be correct, then the effect of ammonium ions on nitrogenase activity *in vivo* of *A. chroococcum* should be more pronounced when these ions are added to cells incubated in the presence of chloramphenicol.

To test this hypothesis, 14-hr old cultures of *A. chroococcum*, strain A40, were supplied with ammonium or sodium acetate (final concentration 1 mM) and their acetylene-reducing capacities were measured in the absence or presence of chloramphenicol (final concentration 20 μg of the antibiotic per ml of culture).

The results (Fig. 7.6) show that the addition of chloramphenicol to control cultures (those supplied with sodium acetate) only slightly reduced the nitrogenase activity of the microorganism. As has been discussed in 5.3.2 this weak depression of nitrogenase activity by chloramphenicol was presumably due to the relatively low concentration of the antibiotic used and to the low rate of aeration applied. However, this concentration was high enough to prolong the period of reduced nitrogenase activity caused by added ammonium ions. In the absence of chloramphenicol, nitrogenase resumed its activity about 1.5 hr after the addition of chloramphenicol, whereas in the presence of 20 μg of the antibiotic per ml of culture, this activity was restored about one hour later.

Although these results could be explained by the hypothesis proposed above, another possible explanation can not entirely be excluded. Chloramphenicol may have inhibited the *de novo* synthesis of nitrogenase in the derepressed cul-

FIG. 7.6. Nitrogenase activity of *A. chroococcum*, strain A40, incubated in the absence (●) or presence (▲) of 20 μg of chloramphenicol per ml of culture, and in the absence (solid lines) or presence (broken lines) of ammonium acetate (1 mM).



tures and thus have aggravated the repression of nitrogenase synthesis by ammonium ions.

7.4. THE EFFECT OF NITRATE ON NINHYDRIN-POSITIVE COMPOUNDS OF THE CELLS AND ON NITROGENASE ACTIVITY *IN VIVO*

It is generally believed that nitrate *per se* does not inhibit nitrogenase activity. Ammonium ions derived from the reduction of nitrate would most likely be the ultimate cause of the depression in the nitrogenase activity of nitrate-treated cultures of ntirogen-fixing microorganisms (BECKING, 1962; SORGER, 1966; THOMAS and DAVID, 1971). However, in these investigations, no efforts have been made to study the intracellular concentration of ammonium ions of nitrate-treated cells.

The results of the experiments on the effect of chloramphenicol (see 5.3) and ammonium ions (see 7.3.3) on nitrogenase activity *in vivo* of *A. chroococcum* suggest that both compounds depress nitrogenase activity by competing for reductants, reduced ferredoxin in the case of chloramphenicol, $NADPH_2$ in the case of ammonium ions. Nitrate might inhibit nitrogenase activity in a similar way: the reduction of nitrate is competing with nitrogenase activity for reductants.

To test this hypothesis, 6 flasks of 5-l capacity, each containing 900 ml of 14-hr old cultures of *A. chroococcum*, strain A40, were used. Three of these cultures received 100 ml of a sterile solution of KNO_3 giving a nitrate concentration of 4 mM. The remaining three flasks were provided with an equimolar con-

centration of KCl. At different time intervals, samples were removed and used for estimating cell yields, ninhydrin-positive compounds (n.p.c.) of the cells and acetylene-reducing capacity.

The results of two of such experiments are shown in Figures 7.7 and 7.8 and in Tables 7.2 and 7.3. It can be seen that during the first 4 hr after the addition of KNO_3, cell yields were not affected by this compound (Figures 7.7 A and 7.8 A). However, the depression of nitrogenase activity of cultures supplied with KNO_3 started immediately after the addition of KNO_3 (Figures 7.7 C and

TABLE 7.2. Cell yield, pool of ninhydrin-positive compounds (n.p.c.) and nitrogenase activity of a culture of *A. chroococcum*, strain A40, after being incubated in the absence or presence of nitrate.

Time (hr)	Cell yield[a]		N.p.c.[b]		Nitrogenase acivity[c]	
	KNO_3	KCl	KNO_3	KCl	KNO_3	KCl
0	33	33	—	—	958	1035
1	36	36	—	—	951	1247
2	38	38	—	—	763	1373
3	44	44	—	—	—	—
4	45	45	—	—	412	1876
5	56	48	156	120	198	1827
7	88	60	270	131	37	2794
9	118	71	312	181	3860	4729

[a]O.D. as % of standard, averages of 3 replicates; [b]μg per 100 ml of culture with leucine as standard, averages of duplicate values; [c]nmoles of C_2H_4 produced/10 ml of culture/30 min, averages of 3 replicates.

TABLE 7.3. Cell yield, pool of ninhydrin-positive compounds (n.p.c.), and nitrogenase activity of cultures of *A. chroococcum*, strain A40, after being incubated in the absence or presence of nitrate.

Time (hr)	Yield				N.p.c. (c)		Nitrogenase activity (d)	
	O.D. (a)		Cell protein (b)					
	KNO_3	KCl	KNO_3	KCl	KNO_3	KCl	KNO_3	KCl
0	26	26	0.3	0.3	–	–	290	290
1.5	32	32	–	–	60	40	–	–
3	–	–	–	–	–	–	120	420
4	39	39	0.9	0.9	80	50	–	–
6	–	–	–	–	–	–	12.5	730
7	84	57	1.8	1.4	163	73	–	–
9	–	–	–	–	–	–	570	700
10	133	90	2.6	1.8	273	220	–	–

(a) As % of standard, (b) mg per 10 ml of culture with bovine albumin as standard, (c) μg per 100 ml of culture with leucine as standard, (d) nmoles of C_2H_4 produced per 10 ml of culture per hour.
All of these values, except (c), are averages of 3 replicates. Values of (c) are averages of duplicates.

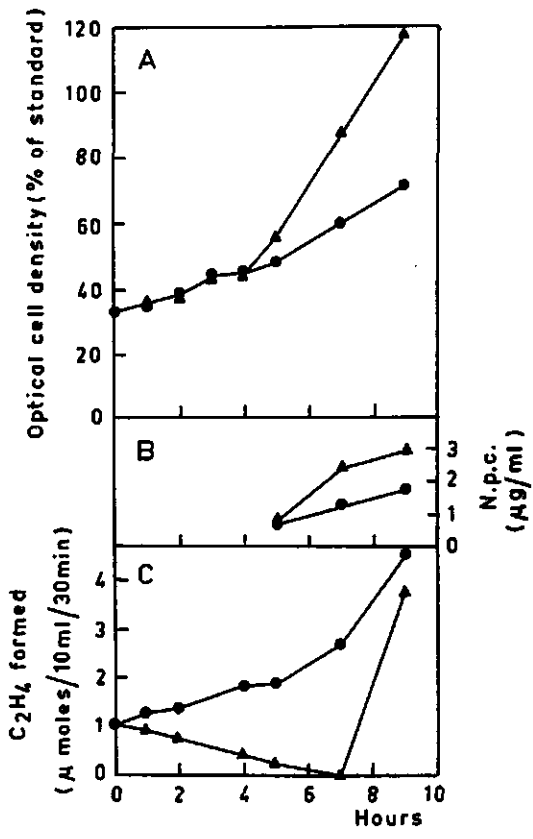


FIG. 7.7. Cell yield (A), ninhydrin-positive compounds (n.p.c.) in the pool (B), and nitrogenase activity (C) of *A. chroococcum*, strain A40, supplied with nitrate (▲), giving a final concentration of 56 µg of N per ml of culture, as compared to control cultures (●).

7.8 C) and was almost complete after approximately 7 hr when the amount of ninhydrin-positive compounds accumulated per cell was largest (Figures 7.7 B and 7.8 B). When these compounds had been used for growth, as indicated by the decrease of the rate of n.p.c. accumulation in the cell pool and by the increase in optical density of the cultures, nitrogenase activity was resumed (Figures 7.7 C and 7.8 C). These results tend to confirm the suggestion that the accumulation of certain ninhydrin-positive compounds in the cell, most possibly ammonia, was responsible for the reduced nitrogenase activity of the *Azotobacter* cells supplied with nitrate. However, the possibility that the reduction of nitrate *per se* has depressed nitrogenase activity was not entirely excluded, as it was indicated by the immediate depression of nitrogenase activity after the addition of nitrate.

7.5. DISCUSSION

Addition of ammonium ions and nitrate to growing nitrogen-fixing cultures of *A. chroococcum* caused an immediate and considerable decline of the nitrogenase

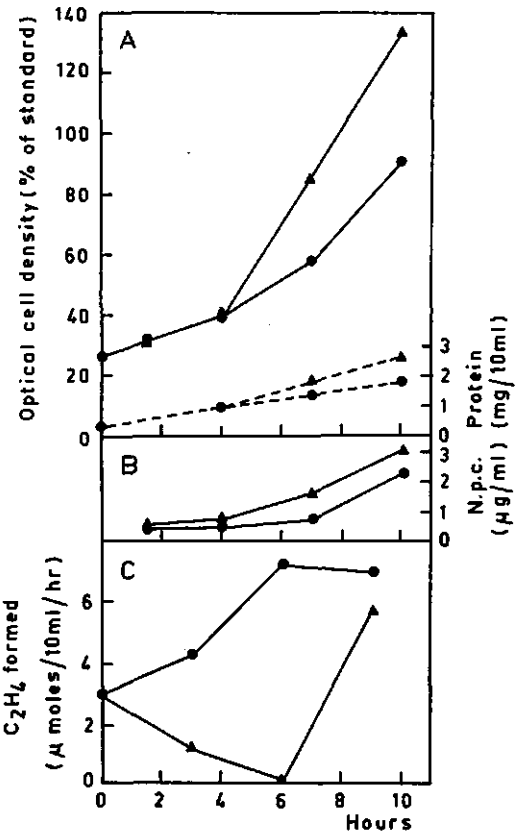


FIG. 7.8. Cell yield (A), measured as O.D. (solid lines) or as protein (broken lines), ninhydrin-positive compounds (n.p.c.) in the pool (B), and nitrogenase activity (C) of *A. chroococcum*, incubated in the absence (●) or presence (▲) of nitrate (56 μg of N per ml of culture).

activity of these cultures (Fig. 7.1). In the case of Casamino acids and amides this decline was slight (Fig. 7.2). In general it is assumed that the depression of nitrogenase activity in living cells by a supplemented nitrogenous compound depends on the readiness of conversion of this compound to ammonium ions. In the case of nitrate the possibility of a competition between the reduction of this compound and nitrogenase activity for reducing power can not be excluded. The fact that several amino acids tested separately had no effect on the nitrogenase activity of living cells (Fig. 7.1) is no evidence that these compounds do not affect nitrogenase activity, as it was shown in the present study that these amino acids were assimilated only very slowly, presumably due to a poor uptake by the cells. The slight effect of Casamino acids and amides is thought to have been due to contamination or liberation of small amounts of ammonia.

The depressing effect of small amounts of added ammonium ions on the nitrogenase activity of living *Azotobacter* cells might be due to the combination of an immediate effect, caused by either feedback inhibition or competition for reductants and/or ATP between the nitrogenase activity and assimilation of

ammonia and a slower effect caused by the repression of nitrogenase synthesis (Fig. 7.3). Experiments with cell-free extracts showed that NH_4^+ did not inhibit nitrogenase activity (Fig. 7.4).

To decide which part of the depression of nitrogenase activity following the addition of NH_4^+ was due to repression of nitrogenase synthesis and which part to competition for reductants and (or) ATP between nitrogenase activity and ammonia assimilation, a comparison was made between the nitrogenase activity of living azotobacters at various periods after the addition of NH_4^+, and that of cell-free extracts made of the same cells, at the same time (Table 7.1). As the specific activity of nitrogenase of cell-free preparations extracted from cells harvested one hour after the addition of ammonium ions, was slightly higher than that of cells harvested at the time of the addition of ammonium ions, it may be concluded that the repression of nitrogenase synthesis upon the addition of NH_4^+ is not immediate. However, the loss of nitrogenase activity of living cells of *Azotobacter* supplied with NH_4^+ started immediately and proceeded at a much faster rate than that of cell-free preparations of the same culture. As it was shown earlier that feedback inhibition was not involved in the depressing effect of NH_4^+ on nitrogenase activity, it can be concluded that competition between nitrogenase activity and assimilation of ammonia for reductants and (or) ATP is responsible for the immediate decline of nitrogenase activity of *Azotobacter* cells supplied with NH_4^+.

An alternative explanation has been proposed by Mr. H. Haaker, suggesting that ammonium ions might act as an uncoupler of oxidative phosphorylation in *Azotobacter* cells, thus creating shortage of energy for nitrogenase activity (personal communication). Although NH_4^+ at a concentration of about 10^{-3}M have been reported to be an effective uncoupler of non-cyclic photophosphorylation (KROGMANN, JAGENDORF and AVRON, 1959), a much higher concentration (2.5×10^{-1}M) was required to uncouple oxidative phosphorylation (GATT and RAAKER, 1959). Since the concentrations of ammonium ions used in the present investigation were much lower than those reported to be required for uncoupling oxidative phosphorylation, the suggestion of Mr. Haaker is assumed to be unattractive, but nevertheless, remains to be tested.

By considering the protein content of living cells and the specific activity of cell-free extracts at different periods after the addition of NH_4^+ (Table 7.1), it can be concluded that the total nitrogenase level of the culture remained somewhat above the initial value for at least 2 hr. Only after 4 hr a slight degradation of the nitrogenase was observed.

SUMMARY

The purpose of the present investigation was to study the effect of some chemical, physical and biological factors on growth, efficiency of nitrogen fixation and nitrogenase activity of *Azotobacter chroococcum*.

From biochemical studies with cell-free preparations of various nitrogen-fixing microorganisms reported in the literature, it can be concluded that three major requirements have to be met before N_2 fixation can proceed viz. the presence of ATP, a powerful reductant, and the appropriate enzyme system (nitrogenase). In intact (living) cells there must also be carbon skeletons for accepting the fixed nitrogen, to allow the process to continue for any length of time. Otherwise, fixed nitrogen accumulates within the cell and will impair the continuation of nitrogen fixation by repressing the synthesis of nitrogenase.

To investigate whether the degree of oxidation and the molecular size (chain length) of organic carbon sources affect the efficiency of nitrogen fixation (defined as the amount of nitrogen fixed per g of carbon compound consumed), some *Azotobacter* strains, especially those of *A. chroococcum*, were cultivated in modified Burk's liquid medium supplied with a number of hexoses, hexitols, fatty acids (as the sodium or calcium salts) and primary alcohols (chapter 3). Since the assimilability of the hexoses tested and that of the corresponding hexitols was different, the hexose-hexitol comparison could not be used for achieving the purpose of this study (Table 3.1).

Ethyl alcohol gave higher efficiency values of nitrogen fixation than acetate. Propanol and butanol were less efficient than propionate and butyrate, probably due to the inhibitory effect of these alcohols on the growth of the azotobacters at the concentrations used (Tables 3.2 and 3.3).

Increased efficiency of N_2 fixation occurred with increased length of the carbon chain of fatty acids up till four carbon atoms (Table 3.5). The efficiency of the nitrogen fixation of *Azotobacter* cultures supplied with butyrate was found to be considerably higher than that of cultures with glucose. This higher efficiency might be due to the greater reducing capacity of the former compound and/or the production of more ATP per weight unit of butyrate. Since azotobacters have very high Q_{O_2}-values, it is more likely that these organisms suffer from insufficient reducing capacity and carbon skeletons than from insufficient energy for growth. Therefore, the greater reducing capacity per weight unit of butyrate was obviously the cause of the higher efficiency of N_2 fixation with this compound as compared to that with glucose.

The results of the experiments reported in chapter 4 show that increased O_2 supply of *A. chroococcum* up to a certain level favoured the nitrogenase activity of the cells (Figures 4.1 B and 4.2). This was presumably due to the improved supply of ATP and $NADPH_2$ resulting from an increased catabolism of carbon compounds including respiration.

Upon further increase of the O_2 supply of the cells, nitrogenase activity decreased, presumably as a result of competition between respiration and nitrogenase activity for reductants. If azotobacters were exposed to excess oxygen for a short time, and then were returned to the optimum pO_2, the cells immediately resumed their optimum nitrogenase activity (YATES, 1970). If this exposure to excess oxygen was continued for a prolonged period, return to the optimum pO_2 gave only a poor recovery of the nitrogenase activity (Fig. 4.3). This low recovery was presumably due to the inactivation of the oxygen-sensitive component of the nitrogenase.

The optimum level of O_2 supply for nitrogenase activity was dependent on the cell density of the *Azotobacter* cultures. Those with high cell densities fixed N_2 (and grew) at an O_2 supply which inhibited N_2 fixation (and growth) of cultures with low cell densities, apparently due to a more efficient removal of oxygen (achieved by cell respiration) in the former culture.

In agreement with results of earlier investigations reported in the literature (cf. DALTON and POSTGATE, 1969), in the present study it was found that lowering the pO_2 increased the cell yield of *A. chroococcum* grown with N_2 as the source of nitrogen. Such an effect was not observed when urea had been supplied as the nitrogen source (Table 4.1). These results suggest that the decreased cell yield of *A. chroococcum*, grown at high pO_2 with N_2 as the nitrogen source was due to the increased utilization of carbon compounds for respiratory protection of nitrogenase.

Although it is generally believed that nitrogen fixation in *Azotobacter* cultures is confined to proliferating cells, there are some indications reported in the literature concerning the ability of non-growing azotobacters to fix N_2 (see 5.1). These reports, however, are not supported by critical quantitative studies on cell growth.

In chapter 5, various methods have been described to stop the growth of azotobacters. These methods include: application of chloramphenicol, depriving the bacteria of K^+ or Ca^{2+}, and incubating the cells under air containing 10% C_2H_2.

Although the elimination of cell growth by the addition of chloramphenicol was found to be accompanied with the suppression of N_2 fixation (Table 5.3), the latter process was mostly less severely depressed than cell proliferation (Tables 5.1 and 5.2).

To explain the reduced N_2 fixation by non-growing chloramphenicol-treated azotobacters, it should be considered that although the synthesis of nitrogenase was prevented, the activity of the enzyme in the non-growing cells could have been retained for a prolonged period. This would lead to the accumulation of soluble nitrogenous compounds including NH_4^+, which are interfering with the nitrogenase activity of living cells (7.3.1).

Although the addition of chloramphenicol to cell-free extracts did not inhibit the activity of nitrogenase (Fig. 5.4), a ready decline of this activity was observed when the antibiotic was added to living cells. This was the case both under

air (where N_2 fixation was possible; Table 5.4) and under air containing 10% C_2H_2 (where N_2 fixation was prevented; Figs. 5.1 and 5.2). These results suggest that the depressing effect of chloramphenicol on the N_2 fixation by living, non-growing azotobacters was due to a competition between the antibiotic and nitrogenase for reductants. The presence of accumulated NH_4^+ may aggravate this effect (7.3.1).

Depriving *Azotobacter* cultures of potassium ions, reduces and finally stops the growth of the cells by preventing protein synthesis. Although nitrogen fixation follows this tendency, similar to that of chloramphenicol-treated cells it is less severely affected by K deficiency than growth (Table 5.5). This indicates that the effect of K deficiency on nitrogenase activity is indirect viz. by effecting the accumulation of soluble nitrogenous compounds, including ammonia, which depresses nitrogenase activity (see 7.3.1). Decreased metabolic activity of the cells, resulting in shortage of reductants and energy, may also be involved in the reduced nitrogenase activity of K-deficient cells.

In contrast to *Azotobacter* cells deprived of K^+, which continue to fix small amounts of N_2, even when cell growth is almost completely arrested, Ca-deficient cells under similar conditions cease to fix N_2 (Table 5.5). The cause of the suppression of N_2 fixation in Ca-deficient cells in unknown, but is most probably due to the decreased metabolic activity of such cells.

That non-growing cells of azotobacters are able to preserve a high nitrogenase activity for a prolonged period was clearly shown by incubating cultures of nitrogen-fixing cells under air containing 10% acetylene. Under such conditions, owing to the high affinity of nitrogenase for C_2H_2, the fixation of N_2 is completely suppressed, resulting in the existence of non-growing cells which do not fix N_2 and as a consequence do not accumulate ammonia in their pool. This enables the nitrogenase to function for a prolonged period (Fig. 5.5).

In the present study, it was shown for the first time that living cells of azotobacters are able to produce H_2 when being incubated under air containing 10% acetylene. Although cell-free extracts of azotobacters are able to evolve H_2 when incubated under argon with C_2H_2 or N_2, H_2 evolution has never been observed to take place in living cells incubated under air (Tables 5.6 and 5.7). These differences presumably depend on the fact that part of the reductants and the ATP which in growing N_2-fixing cultures are used for the assimilation of fixed nitrogen (ammonia) are available for H_2 production when the cells are incubated under air containing 10% acetylene.

Azotobacter cells are known to excrete small amounts of nitrogenous compounds into the medium (cf. RUBENCHIK, 1960). In an experiment with 5 different strains of *Azotobacter*, 7–13% of the total nitrogen fixed was excreted by the cells (Table 6.1). Chapter 6 describes two efforts to remove the excreted nitrogenous compounds and to study the effect of this removal on the nitrogenase activity of cultures of *A. chroococcum*. These efforts include: (1) harvesting, washing, and resuspending growing cells in a fresh medium containing the same amount of sugar as present in the discarded supernatant, and (2) growing

A. chroococcum in the presence of a *Rhodotorula* sp.

The first method gave a slight, but consistent increase in nitrogenase activity (Fig. 6.1). *Azotobacter* cells grown with yeast cells achieved a higher efficiency of nitrogen fixation than pure cultures only when subjected to excess oxygen (Table 6.2). This beneficial effect was apparently due to the consumption by the yeast cells of excess oxygen, thus creating better conditions for nitrogen fixation, rather than to the removal of excreted ammonia as was suggested when starting these experiments. Similar findings have been reported by KALININSKAYA (1967) in an association of a nitrogen-fixing *Mycobacterium* sp with a yeast.

The effect of combined nitrogen on the synthesis and the functioning of nitrogenase in *A. chroococcum* is described in chapter 7. Addition of various forms of combined nitrogen to growing N_2-fixing cultures showed (Figs. 7.1 and 7.2) that (a) ammonium ions and nitrate caused a considerable decline of the nitrogenase activity, (b) Casamino acids and amides slightly reduced this activity, and (c) amino acids had no effect on the nitrogenase activity presumably due to the poor uptake of these compounds by the cells. In general it is assumed that the reduction of the nitrogenase activity of living *Azotobacter* cells by a supplemented nitrogenous compound depends on the readiness of conversion of this compound to ammonium ions. In the case of nitrate the possibility of a competition between the reduction of this compound and the nitrogenase activity for reductants can not be excluded (Figs. 7.7 and 7.8).

The decline in nitrogenase activity of *Azotobacter* cells upon the addition of NH_4^+ was assumed to be due to the combined effect of (1) competition between nitrogenase activity and ammonia assimilation for reductants and/or ATP, and (2) repression of nitrogenase synthesis (Fig. 7.3). If the amount of NH_4^+ added to the culture was large enough, eventually degradation of the enzyme also took place (Table 7.1). From the absence of an effect upon the addition of NH_4^+ to cell-free extracts (Fig. 7.4) it was concluded that feedback inhibition did not participate in the decline of nitrogenase activity *in vivo* caused by the addition of NH_4^+ to living cells.

To decide which part of the depression by NH_4^+ was due to 'competition' and which part to repression of the nitrogenase synthesis, a comparison was made between the nitrogenase activity of living cells, measured at various periods after the addition of NH_4^+, and that of cell-free extracts made from the same cells, at the same time (Table 7.1). As the loss of the nitrogenase activity in living cells started immediately and proceeded at a much faster rate than that of cell-free preparations of the same culture, it is concluded that competition for reductants and/or ATP between nitrogenase activity and NH_4^+ assimilation was responsible for the immediate decline of nitrogenase activity of *Azotobacter* cells supplied with NH_4^+. The repression of nitrogenase synthesis upon the addition of NH_4^+ was not immediate and proceeded at a relatively slow rate.

Although the immediate decline of the nitrogenase activity in living cells supplied with NH_4^+ might be due to uncoupling of the oxidative phosphorylation, the concentrations of NH_4^+ used in this investigation were much lower than the values reported in the literature to be responsible for that effect.

SAMENVATTING

Het doel van het hier beschreven onderzoek was het nagaan van de invloed van een aantal chemische, fysische en biologische factoren op de groei, de efficiency van de stikstofbinding en de nitrogenase-activiteit van *Azotobacter chroococcum*.

Uit biochemisch onderzoek met celvrije preparaten van verschillende stikstofbindende microorganismen dat in de literatuur is beschreven, kan worden geconcludeerd dat bij de N_2-binding aan drie hoofdeisen moet worden voldaan, nl. de aanwezigheid van ATP, van een sterk reducerend systeem, en van het bij de stikstofbinding betrokken enzymsysteem (nitrogenase). In levende cellen moeten bovendien koolstofverbindingen aanwezig zijn om de gebonden stikstof op te nemen zodat het stikstofbindingsproces voor onbeperkte tijd kan worden voortgezet. Anders zal de gebonden stikstof in de cel ophopen en verdere stikstofbinding onmogelijk maken door repressie van de synthese van nitrogenase.

Om na te gaan of de efficiency van de stikstofbinding (d.i. de hoeveelheid N_2 gebonden per g verbruikte koolstofverbinding) bepaald wordt door de mate van oxydatie en de moleculaire ketenlengte van de organisch koolstofbron, werden enkele *Azotobacter*-stammen, in het bijzonder die van *A. chroococcum*, gekweekt in Burk's vloeibaar medium, voorzien van een aantal hexosen, hexitolen, vetzuren (als natrium of calciumzouten) en primaire alcoholen (hoofdstuk 3). Aangezien de assimileerbaarheid van de geteste hexosen en de daarmee overeenkomende hexitolen verschillend was, konden deze verbindingen niet voor het genoemde doel worden gebruikt (Tabel 3.1).

Ethanol gaf hogere waarden voor de hoeveelheid gebonden stikstof dan acetaat. Propanol en butanol gaven veel lagere waarden dan propionaat en butyraat, vermoedelijk omdat deze alcoholen in de gebruikte concentraties schadelijk waren voor azotobacters (Tabellen 3.2 en 3.3).

Bij vetzuren nam de efficiency van de N_2-binding door *Azotobacter*-culturen toe met stijgende ketenlengte van het molekuul, tot en met een lengte van vier koolstofatomen (Tabel 3.5). Butyraat gaf aanzienlijk hogere waarden dan glucose. Dit zou kunnen worden toegeschreven aan het grotere reductievermogen van de eerstegenoemde verbinding en(of) de productie van meer ATP per gewichtseenheid butyraat. Aangezien azotobacters zeer hoge Q_{O_2}-waarden bezitten, is het meer waarschijnlijk dat deze organismen een tekort hebben aan reducerend vermogen en aan koolstofverbindingen voor celsynthese dan aan onvoldoende ATP. De grotere efficiency van de stikstofbinding bij boterzuur was waarschijnlijk een gevolg van het grotere reducerende vermogen van deze verbinding, in vergelijking met dat van glucose.

De resultaten van de proeven vermeld in hoofdstuk 4 laten zien dat verhoging van de O_2-voorziening tot een bepaald niveau de nitrogenase-activiteit van de cellen bevorderde (Figuren 4.1 B en 4.2). Dit was waarschijnlijk een ge-

volg van de verbeterde voorziening met ATP en $NADPH_2$ afkomstig van een versterkte afbraak van C-verbindingen die ook in de verhoogde ademhalingsintensiteit tot uiting kwam.

Bij een verdere verhoging van de O_2-voorziening van de cellen, verminderde de nitrogenase-activiteit van de bacteriën, waarschijnlijk als gevolg van een competitie tussen ademhaling en nitrogenase-activiteit voor reducerende verbindingen. Indien azotobacters voor een korte tijd aan overmaat zuurstof werden blootgesteld, en daarna weer werden geplaatst bij een optimale pO_2, dan kregen de cellen hun optimale nitrogenase-activiteit onmiddellijk terug (YATES, 1970). Werd de incubatie bij overmaat zuurstof langer voortgezet dan vond herstel van deze activiteit slechts in geringe mate plaats (Fig. 4.3). Dit was waarschijnlijk een gevolg van inactivering van de zuurstofgevoelige component van de nitrogenase.

De optimale O_2-voorziening voor de nitrogenase-activiteit hing af van de celdichtheid van de *Azotobacter*-cultures. Bacteriesuspensies met grote celdichtheid bonden N_2 (en groeiden) bij een zuurstofvoorziening die de stikstofbinding (en de groei) van cultures met een geringe dichtheid remden, blijkbaar als gevolg van het elimineren van de te hoge O_2-spanning door de eerstgenoemde cultures.

In overeenstemming met literatuurgegevens (DALTON en POSTGATE, 1969) werd gevonden dat verlaging van de pO_2 de celopbrengst van *A. chroococcum* verhoogde indien de bacterie met N_2 als stikstofbron groeide, maar niet met ureum (Tabel 4.1). Deze resultaten wijzen erop dat de verlaagde celopbrengst bij hoge pO_2 en met N_2 als stikstofbron een gevolg was van een verhoogd gebruik van koolstofverbindingen om via een verhoogde ademhaling de nitrogenase te beschermen tegen overmaat zuurstof ('respiratory protection').

Hoewel algemeen wordt aangenomen dat stikstofbinding door azotobacters beperkt is tot groeiende cellen, zijn er enkele aanwijzingen in de literatuur betreffende het vermogen van niet-groeiende bacteriën om N_2 te binden (zie 5.1). Kwantitatieve gegevens over celgroei ontbreken echter in deze mededelingen.

In hoofdstuk 5 zijn verschillende methoden beschreven om de groei van azotobacters te stoppen, nl. toevoeging van chlooramfenicol, weglaten van K^+ of Ca^{2+}, en incuberen van de cellen onder lucht met 10 % C_2H_2.

Hoewel het onderdrukken van de celgroei door de toevoeging van chlooramfenicol vergezeld ging van de onderdrukking van de N_2-binding (Tabel 5.3), werd laatstgenoemd proces meestal minder sterk beïnvloed dan de celgroei (Tabellen 5.1 en 5.2).

Om de verlaagde N_2-binding door niet groeiende met chlooramfenicol behandelde azotobacters te verklaren, moet rekening worden gehouden met de mogelijkheid dat, hoewel de synthese van nitrogenase was onderdrukt, de activiteit van het enzym in de niet-groeiende cellen bleef bestaan. Maar dit zou leiden tot de ophoping van oplosbare stikstofhoudende verbindingen, waaronder NH_4^+, dat de nitrogenase-werking in levende cellen ongunstig beïnvloedt (7.3.1).

Hoewel de toevoeging van chlooramfenicol aan celvrije extracten de activiteit van de nitrogenase niet remde (Fig. 5.4), werd een snelle vermindering van deze activiteit verkregen indien het antibioticum aan levende cellen werd gegeven. Dit was het geval zowel onder lucht (waar N_2-binding mogelijk was; Tabel 5.4) als onder lucht met 10% C_2H_2 (waar de N_2-binding was onderdrukt; Figuren 5.1 en 5.2). Deze resultaten doen vermoeden dat de snelle vermindering van de N_2-binding in levende, niet groeiende azotobacters door chlooramfenicol een gevolg was van een competitie van het antibioticum met nitrogenase voor reducerende verbindingen. De aanwezigheid van opgehoopte NH_4^+ kan dit effect versterken (7.3.1).

Onthouden van kalium-ionen aan *Azotobacter*-cultures, vermindert de groei en doet deze uiteindelijk stoppen als gevolg van het onderdrukken van de eiwitsynthese. De stikstofbinding vertoont ook een dergelijke tendens, maar is, evenals bij toediening van chlooramfenicol, minder gevoeling dan de groei (Tabel 5.5). Dit wijst erop dat de invloed van K-gebrek op de nitrogenase-activiteit indirect is; door de ophoping van oplosbare N-verbindingen, waaronder ammoniak, wordt de nitrogenase-werking onderdrukt (7.3.1). Verminderde metabolische activiteit van de cellen die leidt tot een tekort aan reducerend vermogen en energie, is vermoedelijk eveneens betrokken bij de verminderde nitrogenase-activiteit van bacteriën met K-gebrek.

In tegenstelling tot azotobacters met K-gebrek die doorgaan met het binden van kleine hoeveelheden N_2, zelfs indien de celgroei vrijwel volledig is opgehouden, stoppen cellen met Ca-gebrek onder dergelijke omstandigheden met het binden van stikstof (Tabel 5.5). De oorzaak van de onderdrukking van de N_2-binding in cellen met Ca-gebrek is onbekend, maar hangt waarschijnlijk samen met de verminderde metabolische activiteit van zulke cellen.

Dat niet-groeiende *Azotobacter*-cellen in staat zijn hun nitrogenase-activiteit gedurende geruime tijd op peil te houden, bleek bij het incuberen van N_2-binbindende cultures onder lucht met 10% acetyleen. Door de hoge affiniteit van nitrogenase voor C_2H_2 wordt de binding van N_2 onder zulke omstandigheden volledig onderdrukt. Hierdoor ontstaan niet-groeiende cellen die geen N_2 binden en als gevolg hiervan geen ammoniak in hun cellen ophopen. Dit stelt het nitrogenase-systeem in staat gedurende geruime tijd te blijven functioneren (Fig. 5.5).

In dit onderzoek werd voor het eerst aangetoond dat levende azotobacters in staat zijn H_2 te produceren indien ze worden geïncubeerd onder lucht die 10% acetyleen bevat. Hoewel celvrije extracten van azotobacters in staat zijn H_2 te produceren indien ze onder argon worden geïncubeerd, al of niet bij aanwezigheid van C_2H_2 of N_2, is H_2-productie door levende cellen onder lucht nooit waargenomen (Tabellen 5.6 en 5.7). Deze verschillen berusten waarschijnlijk op het feit dat een deel van het reducerende systeem en van de ATP die in groeiende, N_2-bindende, cultures worden gebruikt voor de assimilatie van de gebonden stikstof (ammoniak) beschikbaar zijn voor de H_2-productie indien de cellen onder lucht met 10% acetyleen worden gebracht.

Het is bekend dat azotobactes kleine hoeveelheden stikstofhoudende verbindingen in het groeimedium uitscheiden (zie RUBENCHIK, 1960). In een proef met 5 verschillende *Azotobacter*-stammen werd 7–13% van de totale hoeveelheid gebonden stikstof door de cellen uitgescheiden (Tabel 6.1). In hoofdstuk 6 zijn twee proeven beschreven om de uitgescheiden stikstof te verwijderen en om het effect van deze verwijdering op de nitrogenase-activiteit van cultures van *A. chroococcum* na te gaan. Deze proeven omvatten: (1) oogsten, wassen en opnieuw suspenderen van groeiende cellen in een vers medium dat evenveel suiker bevatte als aanwezig in het verwijderde supernatant, en (2) het laten groeien van *A. chroococcum* in aanwezigheid van een *Rhodotorula* sp.

De eerste methode gaf een geringe, maar duidelijk waarneembare, verhoging van de nitrogenase-activiteit (Fig. 6.1). Azotobacters die in aanwezigheid van gistcellen groeiden, bereikten een hogere efficiency van stikstofbinding dan reincultures alleen bij overmaat zuurstof (Tabel 6.2). Deze gunstige werking was blijkbaar een gevolg van het verbruik door de gistcellen van de overmaat O_2, dus van het scheppen van betere omstandigheden voor de N_2-binding, en niet van het verwijderen van uitgescheiden NH_4^+ zoals bij het begin van het onderzoek werd verwacht. Soortgelijke resultaten zijn vermeld door KALININSKAYA (1967) voor een associatie van een stikstofbindende *Mycobacterium* sp. en een gist.

De invloed van gebonden stikstof op de synthese en het functioneren van de nitrogenase bij *A. chroococcum* is beschreven in hoofdstuk 7. Toevoeging van verschillende stikstofverbindingen aan groeiende N_2-bindende cultures leerde (Figuren 7.1 en 7.2) dat (a) ammonium-ionen en nitraten een aanzienlijke daling van de nitrogenase-activiteit te weeg brachten, (b) Casaminozuren en amiden deze activiteit in geringe mate deden verlagen, terwijl (c) aminozuren geen effect hadden op de nitrogenase-activiteit, waarschijnlijk als gevolg van de slechte opneming van deze verbindingen door de cellen. In het algemeen wordt aangenomen dat de verlaging van de nitrogenase-activiteit van levende *Azotobacter*-cellen door een toegevoegde stikstofverbinding berust op de snelheid van omzetting van deze verbinding in ammonium-ionen. Bij toevoeging van nitraat moet de mogelijkheid van competitie tussen de reductie van deze verbinding en de nitrogenase-activiteit, voor reducerende verbindingen niet worden uitgesloten (Figuren 7.7 en 7.8).

Aangenomen werd dat de daling van de nitrogenase-activiteit van *Azotobacter*-cellen na toediening van NH_4^+ een gevolg was van de gecombineerde werking van (1) competitie tussen nitrogenase-activiteit en ammonia-assimilatie voor reducerende verbindingen en (of) ATP, en (2) repressie van de synthese van nitrogenase (Fig. 7.3). Indien de hoeveelheid toegevoegde NH_4^+ groot genoeg was, vond waarschijnlijk ook afbraak van het enzym plaats (Tabel 7.1). Uit het feit dat toevoeging van NH_4^+ aan celvrije extracten geen invloed op de nitrogenase-activiteit uitoefende (Fig. 7.4), werd geconcludeerd dat eindproductremming niet betrokken was bij de daling van de nitrogenase-activiteit *in vivo* na toediening van NH_4^+.

Om na te gaan welk gedeelte van de verlaging van de nitrogenase-activiteit een gevolg was van 'competitie' en welk gedeelte van repressie van de nitrogenase-synthese, werd een vergelijking gemaakt tussen de nitrogenase-activiteit van levende cellen, gemeten op verschillende tijdstippen na de toevoeging van NH_4^+, en die van celvrije extracten, gemaakt op dezelfde tijdstippen van dezelfde cellen (Tabel 7.1). Aangezien de daling van de nitrogenase-activiteit in levende cellen onmiddellijk begon en met veel grotere snelheid voortschreed dan die van celvrije preparaten van dezelfde cultures, werd geconcludeerd dat competitie voor reducerende verbindingen en/of ATP tussen nitrogenase-activiteit en NH_4^+-assimilatie verantwoordelijk was voor de onmiddellijke verlaging van de nitrogenase-activiteit van *Azotobacter* cellen waaraan NH_4^+ was toegediend. De repressie van de nitrogenase-synthese na toevoeging van NH_4^+ begon niet onmiddellijk en schreed met betrekkelijk geringe snelheid voort.

Hoewel de verlaging van de nitrogenase-activiteit, die onmiddellijk na de toevoeging van NH_4^+ in levende cellen optreedt, een gevolg zou kunnen zijn van ontkoppeling van de oxydatieve fosforylering, zijn de concentraties van NH_4^+ die in dit onderzoek gebruikt werden, veel lager dan de waarden die volgens literatuurgegevens voor de genoemde ontkoppeling nodig zijn.

ACKNOWLEDGEMENTS

The investigations have been carried out at the Laboratory of Microbiology of the Agricultural University, Wageningen, The Netherlands.

I would like to express my sincere gratitute to my promotor Prof. Dr. Ir. E. G. Mulder for his invaluable guidance, help and encouragement throughout this investigation and his criticism, suggestions and help during the preparation of the manuscript.

I am also much indebted to Miss Dr. J. Ruinen for kindly providing two of the *Azotobacter* strains used in this study and for encouragement in general, to Mr. J. Antheunisse for providing the other *Azotobacter* strains used and for his help in solving various technical problems, to Dr. A. D. L. Akkermans for his valuable suggestions and advice in the gas-chromatographical analysis, to Dr. T. A. Lie and Drs. E. H. R. van den Berg for their interests and valuable suggestions, and to Dr. W. J. Middelhoven who kindly read the manuscript.

I would like to express my thanks to Mrs. C. Möller, Miss C. M. van der Putten and Mrs. J. I. Philippi for their kind help in typing and correcting the manuscript. Thanks are also due to Messrs. J. C. van Velzen for drawing the figures, A. Wessels for developing the photographs, M. van der Kolk and C. F. Schouten for their help in sterilizing the media and utensils.

My sincere gratitude is due to all my colleagues in the Laboratory of Microbiology at Wageningen for their help, encouragement and kindness during may stay with them.

I thankfully acknowledge the scholarships received from the Ministry of Education and Science (through the mediation of Prof. Dr. M. H. van Raalte), NUFFIC, and Netherlands Organization for the Advancement of Pure Research (Z.W.O.), which made this study possible.

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