THE EFFECT OF PHOSPHATE AND OXYGEN ON THE BIOCHEMISTRY AND ULTRASTRUCTURE OF *Azotobacter chroococcum*

by

PAK CHOW LEUNG

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of Master of Science in the Department of Microbiology in August, 1979.

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to my family and my friends

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Abstract

Nitrogen-fixing Azotobacter chroococcum ATCC 7493 was grown in a chemostat with vortex stirring (1750 rpm) under various oxygen and phosphorus concentrations and at different dilution rates. An in vivo Km for the oxygen-utilizing enzymes was found to be about 10% oxygen in the atmosphere, indicating the cultures would not be oxygen limited when growing in ordinary air. The respiratory index (R.I.= mmoles CO_2 produced / mg cells grown) leveled off gradually to a value of 0.05 below 15% oxygen and rose sharply above 15% oxygen in the gas phase, indicating a less efficient biomass production at higher oxygen concentrations. The amount of phosphorus required by the cells remained constant from 5 to 20% oxygen, suggesting that the higher rate of respiration was not coupled to ATP production. About 18 µg phosphorus were consumed by one mg dry weight organisms for cultures growing at oxygen concentrations in the gas phase below 5% and about 27 µg phosphorus per mg dry weight organisms for culures growing at oxygen concentrations from 5 to 20%. The growth rate of the culture was observed to play a role in the poly-β-hydroxybutyrate (PHB) accumulation in both phosphorus-limited and non-limited cultures. An accumulation of PHB up to 22% of dry weight was observed in phosphorus-limited cultures. The accumulation of PHB at the low dilution rate was about 50 fold greater than that at the high dilution rate. Electron micrographs also revealed the massive PHB accumulation at low dilution rates. Respiratory protection was shown to be present even in phosphorus-limited cultures by the higher R.I.'s under phosphorus-limited conditions. At the very low phosphorus concentration (0.07 mM) the rate of CO_2 evolution increased from 2 fold from D = 0.05 to D = 0.15 h^{-1}. At higher phosp

about 3 fold from D = 0.35 to D = 0.15 h^{-1}. At higher phosphorus concentrations the rate of CO_2 evolution remained unchanged for all dilution rates. Electron micrographs showed a larger amount of intracellular membrane vesicles at lower phosphorus concentrations. The function of the vesicles is thought to be to increase the cell surface and thus respiration. Mesosomes were thought to play a role in the increase in the amount of vesicles. Negative staining methods confirmed the membranous origin of the vesicles and revealed knob-like structures on the inside of the cell membrane.

INTRODUCTION

Introduction

The extremely high rate of respiration and the N_2-fixing ability of Azotobacter chroococcum have attracted many authors to investigate the growth and physiology of this bacterium. Dalton and Postgate (1969 a,b) were among the first to carry out studies on A. chroococcum in great detail. They found that A. chroococcum grew better at lower oxygen tensions. Carbon- and phosphate- limited cultures were more sensitive to oxygen inhibition than non-limited cultures. However, they found that carbon-limited cultures utilizing NH_4^+ seemed not to be so oxygen sensitive. Respiratory protection and conformational protection of the nitrogenase were proposed. They suggested that the carbon-limited culture did not get enough substrate to carry out respiratory protection and that the phosphate-limited culture did not have respiratory protection because the ATP/ADP ratio was affected by the lack of phosphate.

Lees and Postgate (1973) studied the behaviour of oxygen- and phosphate- limited A. chroococcum in continuous cultures. They reported that the inverse relation between biomass and dilution rate could be explained by the accumulation of poly-β-hydroxybutyrate and polysaccharide at lower dilution rates. They also confirmed the oxygen sensitivity of phosphate-limited nitrogen-fixing cultures. However, they discovered that the 'oxygen-poisoned' phosphate-limited cells were 100% viable when subsequently grown on medium containing NH_4^+. This, they thought, provided evidence for the idea that respiration protects the nitrogenase in nitrogen-fixing organisms.

Although the effect of oxygen on A. chroococcum has been extensively studied, in none of these studies were oxygen limitation and oxygen

sufficiency quantitatively defined. This inadequacy might be due to the complexity in measuring the degree of aeration in cultures (Brown, 1970). For instance, any change in the composition of the medium would change the solution rate of oxygen and so would the rate of stirring the culture. Therefore, in some cases, even though enough oxygen is supplied to the culture, the culture could still be oxygen limited if it is not stirred enough. Hine & Lees (1976) overcame this problem by growing A. chroococcum in continuous culture under vortex stirring (1750rpm). With this vortex stirring the transfer of oxygen and other gases to the cells in the culture is efficient, uniform and maximized. This is presumably due to an increase in the rate of gaseous diffusion to the cells. This gives more accurate measurements of the degree of oxygen limitation because the dissolved oxygen level measured would be very close to that of the cell surface. Using this intense rate of aeration Hine & Lees were able to determine oxygen limitation, oxygen sufficiency, and the transition between the two in terms of the respiratory index (R.I.) values (CO_2 evolved per weight of organisms produced). They showed that the amount of CO_2 evolved increased with the oxygen level and that above the R.I. value of 0.1 the respiratory protection came into function. With this stirring rate, they got a growth rate double that of Dalton & Postgate.

The aim of this investigation is to use this vortex stirring method to study the effect of phosphorus-limitation on A. chroococcum in continuous cultures under conditions of a regulated supply of oxygen by means of the vortex stirring.

HISTORICAL

Historical

Cellular nitrogen fixation is by far the most widespread process of fixing N_2 to NH_3. Molecular nitrogen is an inert gas in the atmosphere. The inertness is due to the stable triple bond in the nitrogen molecule. The outer shell of electrons of each atom in a nitrogen molecule has a neon electronic configuration, (8 electrons).

Nitrogen fixation is a part of the nitrogen cycle in nature and is quite often the limiting factor for the biological productivity in many areas, especially in agricultural land (Postgate,1978). The fixation has been known since 1888 when Hellriegel and Wilfarth proved its existence in legumes (Burns & Hardy,1975). However, active research into the process of nitrogen fixation started only about fifteen years ago when the acetylene reduction method coupled to gas chromatography was introduced to assay the nitrogenase activities (Dilworth ,1966; Koch & Evans, 1966; Hardy & Knight, 1967).

Biological nitrogen fixation is carried out exclusively by procaryotes and is found in the blue-green bacteria, the photosynthetic bacteria, the strict anaerobes and the obligate and facultative aerobes. A. chroococcum, the bacterium used throughout this investigation, is a strict aerobe, a heterotrophic nitrogen-fixer, found commonly in soil and water. It is rod shaped, about 2 x 5 µm, frequently in pairs, motile with peritrichous flagella and forms round cysts under adverse conditions (Bergey's Manual,1974).

Usually, organisms that fix N_2 are divided into two types, namely, symbiotic N_2-fixers and free-living N_2-fixers. The 'symbiotic N_2-fixers' refer to organisms that fix nitrogen only in association with their hosts,

mainly plants. Some examples of symbiotic N_2-fixers are: Rhizobium spp. with legumes, Klebsiella pneumoniae with tropical plants, Spirillum lipoferum with tropical grasses, and Frankia spp. with alder. Recently, some Rhizobium spp. have been found to fix nitrogen without the hosts (Keister,1975; Kurz & LaRue,1975; McComb et al.,1975; Pagan et al.,1975).

The study of nitrogen fixation was done on whole cells until the successful isolation of nitrogenase, the nitrogen-fixing enzyme complex, by Carnahan et al. (1960). The cell-free nitrogenase isolated was oxygen- and cold-labile. Since then nitrogenase has been isolated from about 20 microorganisms and purified from about 4 (Eady & Postgate,1974; Chatt et al.,1978). The amino-acid composition of the nitrogenases in Azotobacter, Klebsiella and Clostridium were compared (Chen et al.,1973) and that in Clostridium pasteurianum was partly sequenced (Tanaka et al., 1976). The enzyme consists of two non-heme, iron-sulfur proteins. They are called MoFe protein (also called molybdoferredoxin or protein I) and Fe protein (also called azoferredoxin or protein II) and present in the ratio of 2:1 (MoFe protein:Fe protein) in the enzyme. The overall composition of these proteins is shown in Table 1. Although the exact molecular composition is specific to each species, the basic components and most of the amino-acids constituents are very similar. Components from some organisms have been shown to cross-react to give functional enzymes. For example, Fe proteins from A. chroococcum give functional nitrogenases when mixed with the MoFe protein of Klebsiella pneumoniae (Eady & Postgate,1974). Electron micrographs show that the MoFe protein consists of four doughnut-shaped units packed together and that the Fe protein is egg-shaped (Burns et al.,

Table 1 General properties of the components of the nitrogenase (Chatt et al.,1978)

Properties	MoFe protein	Fe protein
Molecular weight	~220,000[a]	55,000-70,000[a]
Number of subunits	2x2[b]	2
Iron atoms	20-32[a]	4
Molybdenum atom	2	0
Acid labile sulfide	~20-32[a]	4

[a] exact amount depends on the source

[b] two of each of two subunit types

1974). Both MoFe and Fe proteins are required for fixing nitrogen. Neither protein can fix nitrogen on its own.

Nitrogenase can react with many substrates. These include H_3O^+, and triple-bonded substrates such as N_2, N_2O, RCN, and RCCH, where R is either a hydrogen atom or an alkyl chain. For nitrogen fixation to occur in cellular extracts an external electron donor such as sodium dithionite, or potassium borohydride, a cation such as Mg^{++} and an ATP generating system such as the creatine-phosphate/creatine phosphokinase system must be present (Bulen et al.,1965). Studies show that the complex of Mg and ATP binds to the Fe protein and the MoFe protein is reduced by the Fe protein with the involvement of Mg·ATP (Mortenson et al.,1973; Orme-Johnson et al.,1972; Shah et al., 1973; Smith et al., 1973). It was proposed that the Mo-Fe-S in the enzyme is the site for the reduction of the nitrogen molecule. The reduced Fe protein transfers its electron to the MoFe protein and then to the nitrogen molecule bound to the active site. A series of electron transfers takes place and protons are also transferred from water to the nitrogen molecule. The result is ammonia, Mg·ADP and inorganic phosphate (P_i) are produced with the nitrogenase going back to its original oxidation state. The ammonia formed is then released from the enzyme. Diazene and hydrazine are believed to be the intermediates of the reduction and are bound to the transition metal all the time at the active site. A tentative scheme of the mechanism of nitrogen fixation is shown in figure 1. There are still uncertainties in the mechanism of nitrogen fixation. The evidence for molybdenum to be the site of attachment of the nitrogen molecule is very indirect (Smith,

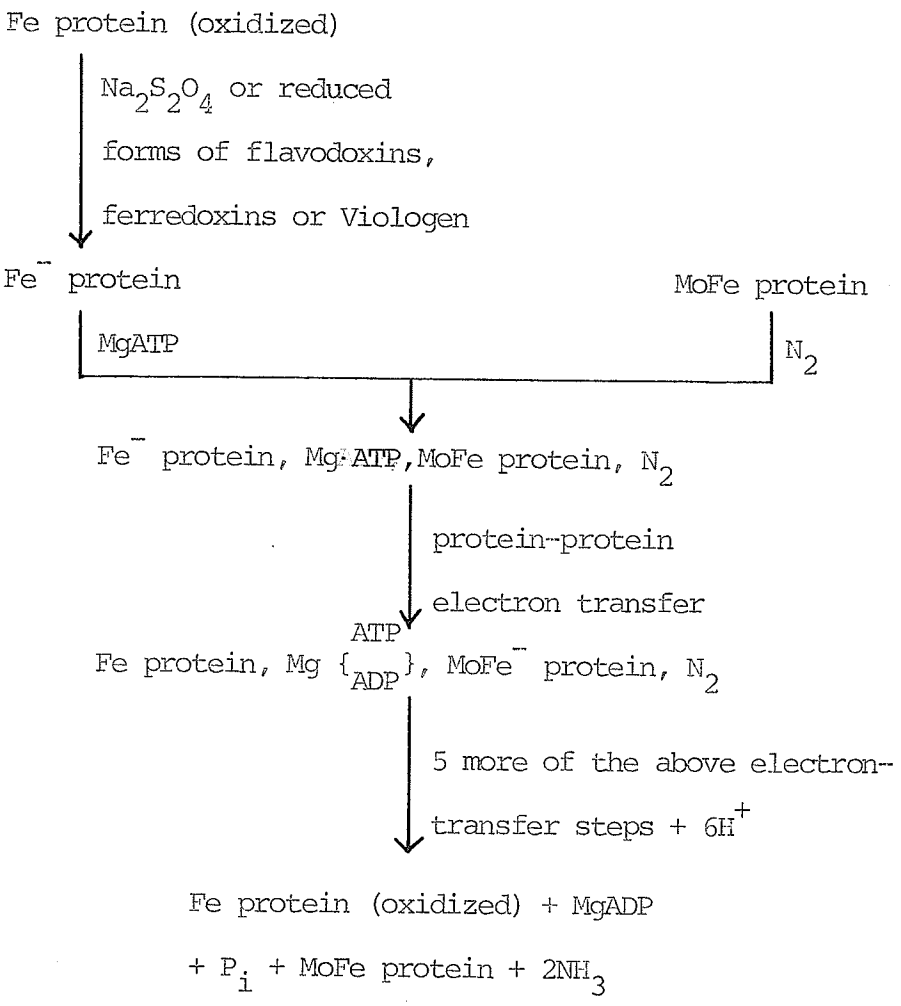


Figure 1 Simplified scheme of nitrogenase action. The scheme shows the steps believed to be involved in nitogenase action; but the sequence shown is not firmly established (Chatt et al.,1978).

1977, cited in Chatt et al.,1978). Whether the iron-sulfur clusters in the enzyme are electron donors only or also interact with the nitrogen molecule is not certain. Recently, the long hypothesized intermediate hydrazine has been isolated when the enzyme is destroyed by acid or alkali during the fixation process (Thorneley et al.,1978). However, the other hypothesized intermediate diazene has not been detected yet.

A side issue of the nitrogen fixing reaction is the production of hydrogen. Nitrogenase is a powerful reducing agent. In the presence of nitrogen, the nitrogen is reduced. However, when nitrogen is absent, H^+ in water is reduced to hydrogen. The hydrogen production is also ATP dependent and is present both in cell-free extracts (Burns & Hardy, 1975; Zumft & Mortenson,1975) and in vivo (Hamilton et al.,1964; Walker & Yates,1979). Since CO does not inhibit hydrogen production but inhibits nitrogen fixation it is believed that the catalytic site of the proton reduction is not the same as that of nitrogen reduction. Competition seems to exist between the H^+ and the N_2 for the reduction because in the presence of N_2, H_2 evolution is much reduced.

The source of electrons and ATP for the nitrogenase in Clostridium pasteurianum is the phosphoclastic breakdown of pyruvate (Evans & Phillips,1975). In strict aerobes the ATP is believed to be provided by oxidative phosphorylation (Yates & Jones, 1974). The path for the electron transfer from NADPH is not clear yet. In A. vinelandii (Benemann et al.,1971) and soybean bacteroids (Evans & Phillips,1975) the proposed sequence is: NADPH $\longrightarrow$ ferredoxin $\longrightarrow$ x $\longrightarrow$ flavodoxin $\longrightarrow$ nitrogenase. The NADPH could be generated from a number of $NADP^+$-linked dehydrogenases. The component x has not been fully identified.

Although nitrogen fixation is an exothermic reaction, a large amount of ATP is required for the process. Estimates of ATP required for nitrogen fixation *in vivo* varies from 4-5 ATP/N_2 in *A. chroococcum* (Dalton & Postgate, 1969b) to 29 ATP/N_2 in *Klebsiella pneumoniae* (Hill, 1976), while that for cell-free extract is about 15 ATP/N_2 (Silver, 1967). The exact role of ATP in nitrogenase is not known. During the fixation ATP is hydrolysed to ADP and P_i (Hardy & Knight,1966; Kennedy *et al.*, 1968). It is suggested that the binding of the ATP to the nitrogenase complex may enhance the catalytic activity of the transition metal in the complex. (Yates,1972; Zumft *et al.*,1972,1973).

Nitrogenase is extremely sensitive to oxygen, especially the Fe protein which has a half life of less than 1 minute on exposure to air (Hardy & Havelka,1975). The inactivation of the cell-free nitrogenase by oxygen is irreversible. The toxicity of oxygen has been well documented (Fridovich,1974,1975a,b,1978). The metabolism of oxygen in the cell can easily produce the highly reactive superoxide anion, O_2^-. This anion has been shown to inactivate virus, induce lipid peroxidation, damage membranes, and kill cells (Fridovich,1978). The mechanism of toxicity of the O_2^- is probably through the formation of the extremely powerful oxidant, the hydroxyl radical (OH•), when H_2O_2 is present: $H_2O_2 + O_2^- \longrightarrow OH^- + OH\bullet + O_2$. Since O_2^- and H_2O_2 are regularly produced in cells, the cells have developed enzymes to get rid of these harmful products as soon as they are formed, to prevent them from reacting with one another. The enzyme that removes O_2^- is superoxide dismutase (SOD) and the enzymes that remove H_2O_2 are catalase and peroxidase. The reactions are as follows:

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad \text{(SOD)}$$

$$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2 \qquad \text{(catalase)}$$

$$H_2O_2 + RH_2 \longrightarrow 2H_2O + R \qquad \text{(peroxidase)}$$

It is not known how nitrogenase is inactivated by oxygen. It may be due to the effect of O_2^-. However, there is much evidence that oxygen affects the physiology of the nitrogen-fixing microorganisms and that the microorganisms have developed mechanisms to protect their nitrogenases from oxygen damage. Nitrogen-fixing facultative aerobes such as Klebsiella or Bacillus and nitrogen-fixing photosynthetic procaryotes fix nitrogen only under anaerobic conditions. Some blue-green bacteria carry out nitrogen fixation in heterocysts only where the oxygen tension is low (Stewart,1978). In legumes, the plant and the bacteroids co-operate to produce leghaemoglobin to maintain a suitable oxygen-tension 10nM in the bacteroids for them to fix nitrogen. The strict aerobes face a bigger problem. They have to live in the presence of oxygen and yet they have to keep the nitrogenase in an anaerobic environment for the nitrogenase to function. The results of many investigations show that the aerobes have developed many extraordinary protective mechanisms to cope with excess oxygen supply. The following is an account of these possible protective mechanisms found in Azotobacter species.

A. chroococcum has been shown to contain SOD and catalase and the amount of these two enzymes increases when the oxygen level supplied to the cells increases (Buchanan,1977). So these may indicate that the two enzymes are used in protecting nitrogenase from oxygen damage.

In addition to the production of SOD and catalase azotobacters

when given sufficient carbon substrate can significantly increase their respiration rate in response to the increase in oxygen supply. This augmented respiration was interpreted as respiratory protection (Dalton & Postgate,1969a,b). It was suggested that the cell increases its respiration to burn away excess oxygen so that an intracellular anaerobic condition is maintained for nitrogen fixation.

One consequence of this augmented rate of respiration is the production of excess ATP because repiration usually produces ATP. This excess ATP would upset the steady intracellular ATP:ADP ratio, which if too high would inhibit nitrogenase activities (Yates,1970). However, it was found that the respiratory chain in Azotobacter is branched and one of the branches is uncoupled with respect to ATP formation (Jones & Redfearn,1967) (figure 2). At high respiration rate electrons could be transported via the less coupled pathway, i.e., from NADH dehydrogenase to cytochrome a_2. This would enable the cell to respire at a higher rate and not to produce excess ATP. On the other hand when the oxygen level is low a pathway with high phosphorylating efficiency predominates, producing enough ATP for cellular functions and nitrogen fixation. Bergerson and Turner (1975) also reported the presence of branched electron transport system in legume bacteroid system.

Dalton & Postgate (1969a) also suggested the presence of conformational protection in Azotobacter. This means that the nitrogenase in vivo can switch from a functional and oxygen sensitive form at low oxygen tension to a non-functional, oxygen insensitive form at a sudden increase of oxygen tension and vice versa. However, much more experimental evidence is needed to confirm this idea.

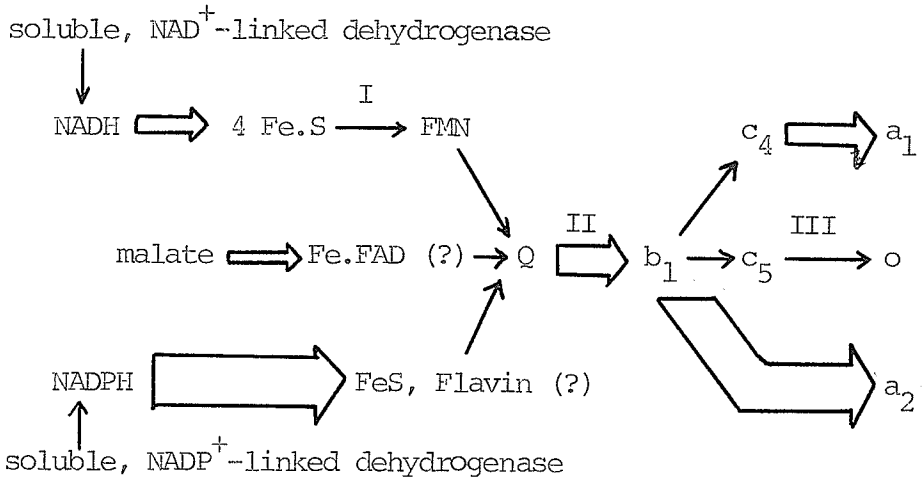


Figure 2 Cytochrome-linked electron transport pathways in A. vinelandii. I, II, III represent the three phosphorylation sites. Thickness of the arrows represents the effective electron flux through a particular pathway. (Jones & Redfearn,1967, cited in Dalton,1979)

Another protective mechanism which may be present in Azotobacter is the production of a lot of exopolysaccharide. There is evidence that the bacterial gum would slow down the rate of diffusion of oxygen (Hill et al.,1972; Hine & Lees,1976). This could protect the nitrogenase in the cell temporarily from sudden changes in oxygen tension in the environment.

The cell membrane of Azotobacter may also play a role in protecting the nitrogenase from oxygen inactivation. For instance, nitrogenase extracted from A. vinelandii is not oxygen-labile. The extract is particulate and contains membrane fragments (Bulen et al.,1964; Hardy & Knight,1966). Also, an extensive intracellular membrane network or membrane invaginations has been repeatedly reported in Azotobacter under various growth conditions. The possible function of the membrane in protecting the nitrogenase in vivo will be detailed later.

The ability of Azotobacter to accumulate large amounts of poly-β-hydroxybutyrate (PHB) is probably another protective mechanism against oxygen inactivation too. This will be discussed in more detail.

So, it seems that the Azotobacter has evolved quite a number of mechanisms, namely, SOD and catalase production, high respiratory rate, conformational change of the nitrogenase, a branched oxidative electron-transport system, exopolysaccharide production, extensive intracellular membrane network and PHB accumulation, to protect the nitrogenase from oxygen inactivation.

PHB accumulation

Many bacteria are able to accumulate PHB . Accumulation up to 70% of the cell dry weight has been reported in A. beijerinckii (Stockdale

et al.,1968). PHB was discovered by M.Lemoigne in 1927 and has a molecular weight ranging from 1,000 to 256,000 (Doudoroff,1966), depending on the growth condition the cell is in. It is a polymer of β-hydroxybutyrate and is usually accumulated into protein-coated granules (Wang & Lundgren, 1969). The compostion of a PHB granule is 98% PHB, 2% protein and trace amounts of lipid and phosphorus (Doudoroff,1966).

PHB is considered to be an intracellular reserve of carbon and/or energy that is stored up when the carbon substrate in the environment is in excess and is used when the carbon substrate in the environment is exhausted (Senior et al.,1972). Its role in endogenous metabolism has been studied extensively (Dawes & Ribbons,1964). In Azotobacter PHB is probably present in small amounts all the time (Stockdale et al.,1968). However, massive accumulation can occur when the cells get into the stationary phase of growth in the presence of excess carbon substrate (Wyss et al.,1961; Senior et al.,1972; Sadoff,1975). PHB accumulation has always been observed to precede encystment in Azotobacter. However, PHB accumulation does not necessarily result in cyst formation. In batch cultures unfavorable conditions such as mineral deficiencies would result in cyst formation in A. vinelandii (Layne & Johnson,1964) and so would the addition of n-butanol (Wyss et al.,1961). Since PHB accumulation is also observed prior to the encystment it can be said that the above mentioned unfavorable conditions may actually first effect a PHB accumulation. In continuous cultures PHB accumulation was observed in A. chroococcum at low dilution rates in phosphate-limited, N_2-limited, NH_4^+-limited and oxygen-limited cultures (Dalton & Postgate,1969b; Lees & Postgate,1973).

Senior et al. (1972) using batch and continuous cultures showed that the PHB accumulation in A. beijerinckii is due to oxygen limitation in the culture. In the presence of excess carbon substrate oxygen-limited culture accumulated PHB up to 44.6% of the dry weight while the nitrogen- and carbon- limited culture respectively accumulated less than 1.5% and 3% of the dry weight. Also, a sudden imposition of oxygen limitation on nitrogen-limited culture would result in PHB accumulation. Later experiments (Senior & Dawes, 1973) showed that when the oxygen limitation was removed, PHB accumulation stopped, even when excess glucose and N_2 were present. Furthermore, when the glucose in the medium was exhausted, the PHB was then degraded. Therefore, it was thought that the PHB serves as an 'electron-sink' for the excess reducing power built up when the cell becomes oxygen-limited and is no longer able to oxidize the NAD(P)H produced by the glucose catabolism at the high rates achieved by the nitrogen- or carbon- limited cultures.

A possible regulation scheme for PHB metabolism in A. beijerinckii is shown in figure 3. The accumulation pathway is connected to the TCA cycle. The presence of high concentration of acetyl-CoA and NAD(P)H and low concentration of free coenzyme A in oxygen-limiting condition would stimulate the synthesis of PHB and simultaneously inhibit the degradation of PHB. In high oxygen concentration, the NAD(P) increases. This stops the PHB synthesis. When the extracellular substrate is exhausted, the degradation of PHB is then stimulated, due to low level of acetyl-CoA and high level of coenzyme A.

The synthesis of PHB does not require ATP directly but requires a large amount of reducing power in the form of NAD(P)H. Thus, the PHB is actually a highly reduced intracellular carbon substrate which can

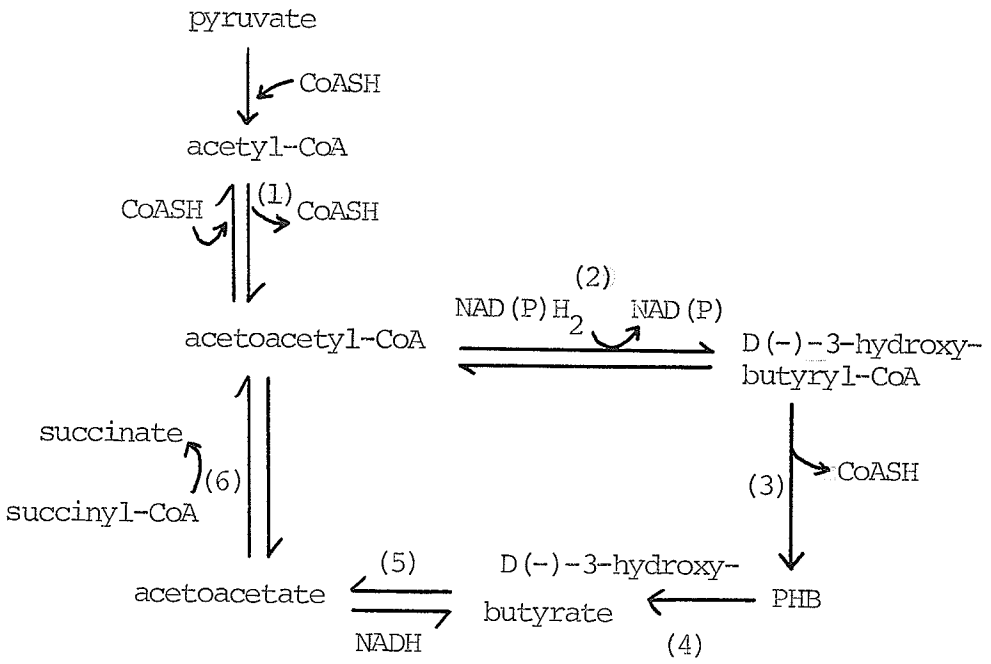


Figure 3 THe metabolism of PHB in A. beijerinckii (Dawes & Senior, 1973). In the figure: (1) β-ketothiolase, (2) acetoacetyl-CoA reductase, (3) PHB synthetase, (4) PHB depolymerase system, (5) D(-)-3-hydroxy-butyrate dehydrogenase, (6) thiophorase.

be used by the cell as carbon and/or energy in adverse conditions, to sustain the life of the microorganism as long as possible. Therefore, the storage of this compound when there is plenty of substrate would undoubtedly provide survival value to the organism. This accumulation of PHB in encystment in Azotobacter may also have the same implication, i.e., as a reserve of carbon and energy for the endergonic reactions involved in encystment and the subsequent germination of the cyst. Finally, because of the necessity of maintaining an anaerobic environment for the nitrogenase to function the accumulation of PHB may ensure a continual oxidative activity in the absence of an exogenous substrate and thereby secure respiratory protection.

The membrane vesicles

Wyss et al. (1961) first reported the presence of circular peripheral bodies along the inside of the cell membrane in A. vinelandii. These peripheral bodies disappeared at the later stage of encystment and then reappeared on cyst germination. They thought that these peripheral bodies were a network of tubules or invaginations of the cell membrane. Taking the high Q_{O_2} value of Azotobacter and the finding by Cota-Robles et al. (1958) that the respiratory enzymes of Azotobacter are associated with the cell membrane into consideration they suggested that the function of the peripheral bodies might be to offer a bigger cell surface for respiration. In the following year, electron micrographs of this extensive membrane network were published by Robrish & Marr (1962) and Pangborn et al. (1962).

Then in 1970 Oppenheim & Marcus observed that in A. vinelandii the membrane network was much more extensive in nitrogen-fixing cells than

in cells grown with fixed nitrogen sources. They thought the amount of membrane network is related to the amount of nitrogenase present in the cell. Later experiments (Oppenheim et al.,1970b) showed that membrane fragments attached to the nitrogenase extract would prevent the nitrogenase from oxygen inactivation. Thus, adding to the speculation of Wyss et al., they suggested that the membrane network in the cell actually surrounds the soluble nitrogenase and the respiratory enzymes on the membrane maintain an anaerobic condition necessary for nitrogen fixation. It was felt that this model agreed with the idea of respiratory and conformational protection proposed by Dalton & Postgate (1969a, b). The increased amount of membrane network seemed to match with the increased amount of respiration and the formation of membrane vesicles around the nitrogenase seemed to be a form of conformational protection.

However, the view that the membrane vesicles is related to respiratory activity was challenged by the findings that nitrogen-fixing cells contained 70% more phospholipid than cells grown in NH_4^+ and that the phospholipid content of the cells remained much the same at low and high oxygen concentrations (Hill,Drozd&Postgate,1972). It was then concluded that the membrane vesicles were not related to the respiratory activity but to the nitrogenase content of the cell. However, Drozd et al. (1972) later found that the previous phospholipid measurements were erroneous and that the phospholipid content remained the same for both nitrogen-fixing cells and cells grown in NH_4^+. Raczek & Burton (1979) confirmed this finding.

The more direct evidence that the nitrogenase is located on the cell membrane was provided by Stasny et al. (1973) and Raveed et al. (1973). Using ferritin-conjugated antibody Stasny et al. found that 80% of the nitrogenase was associated with the cell membrane and the

rest scattered throughout the cytoplasm. Using a similar technique Raveed et al. also found that the nitrogenase was located on the cell membrane.

The view of Hill et al. was further supported by the work of Reed et al. (1974). They isolated small membrane vesicles, from A. vinelandii, that did not contain any respiratory cytochrome but contained most of the nitrogenase of the nitrogen-fixing cell. They called these small vesicles azotophores.

However, different findings were reported by Pate et al. (1973). They found that in A. vinelandii the amount of intracellular membrane vesicles was the same when the cells were grown on N_2, NH_4^+ or NO_3^-. They also found that cells in late exponential growth contained more membrane vesicles than cells in early exponential growth. They concluded that the extensive membrane vesicles might be a means for the cell to sequester enough oxygen under oxygen-limiting condition so that the cell could remain in the exponential growth phase.

Dawson (1977) using vortex-stirring continuous cultures of A. chroococcum probably did the most detailed electron-microscopic study on the cause of the increase in the amount of membrane vesicles. She found that the membrane vesicles were present in both nitrogen-fixing cells and cells growing in NH_4^+. Above all, she found that the amount of membrane vesicles increased with the increase in the supply of oxygen or NH_4^+. Catalase was also found in the membrane vesicles.

The finding of Dawson may explain the differences in results obtained by previous authors. Since the amount of NH_4^+ present in the culture would influence the amount of vesicle formation, different authors using different concentrations of NH_4^+ to grow their cells would therefore produce different amount of membrane vesicles.

Furthermore, oxygen concentration, the primary cause in vesicle formation, played a critical role too. Different degrees of aeration in the cultures would result in different amount of membrane vesicle formation. Thus, the variation in the amount of NH_4^+ used and the degree of aeration might have caused the differences in data between various authors.

Because of the vortex stirring of the cultures (Hine & Lees,1976) Dawson's finding also gave a more accurate picture of the function of the membrane vesicles. Dawson suggested that the membrane vesicles were produced in response to a higher oxygen tension around the cells and the functions of them were to increase the amount of respiration and to get rid of the toxic products of the oxygen reduction (O_2^-, H_2O_2).

It could be a coincidence that these membrane vesicles contain nitrogenase. If so, the nitrogenase would thus also be protected from the damage by oxygen and its reactive reduction products. However, further investigation is needed to find out if the nitrogenase is actually in the membrane vesicles.

Continuous culture

The basic theory of continuous culture was proposed by Monod (1950) and independently by Novick & Szilard (1950). A continuous culture is a culture that is growing at the exponential growth phase all the time. Fresh medium is supplied to the culture at the same rate (v cm^3/h) as the culture is removed. Thus, the volume of the culture (V cm^3) is kept constant. The most commonly used continuous culture is of the flow-controlled type, often referred to as the chemostat (Herbert et al., 1956). In a chemostat with a nutrient in limiting concentration the growth rate of the microorganism in the culture is governed by the dil-

ution rate, D, of the culture, where $D = v / V$ (h^{-1}). The change in concentration of the microorganism in the culture vessel

= (growth - overflow), i.e.,

$$\frac{dx}{dt} = \mu x - Dx = x(\mu - D)$$

where x = concentration of the microorganism in the vessel at time t (mg/cm^3)

μ = specific growth rate of the microorganism at time t (h^{-1}).

At steady state:

$$\frac{dx}{dt} = 0 \text{ , and therefore } \mu = D.$$

Monod (1942,1949) observed that the specific growth rate of a microorganism is dependent on the concentration of the growth limiting nutrient in the following manner:

$$\mu = \mu_{max} \frac{s}{K_s + s}$$

where μ_{max} = the maximum value of μ (i.e. when the value of μ is not limited by s)

s = the concentration of the growth limiting substrate (mg/cm^3)

K_s = the saturation constant (equal to s at 0.5 μ_{max}).

Therefore, at steady state:

$$\mu = D = \mu_{max} \frac{s}{K_s + s}$$

or, $$s = K_s \frac{D}{\mu_{max} - D} \quad \text{.....(i)}$$

In a constant environment the rate of growth and the rate of substrate utilization are related as follows:

$$\frac{dx}{dt} = -Y \frac{ds}{dt} \text{ , and therefore}$$

$$x = Y(s_r - s) \quad \text{.....(ii)}$$

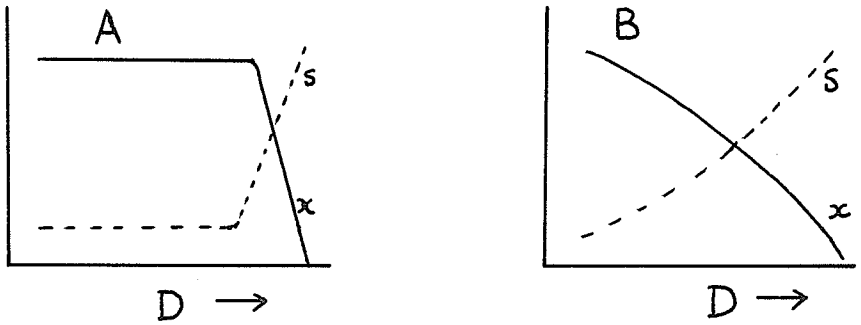
where Y = yield factor = $\frac{\text{weight of microorganism formed}}{\text{weight of substrate used}}$

s_r = concentration of the substrate in the supply.

Combining equations (i) and (ii),

$$x = Y \left(s_r - \frac{K_s D}{\mu_{max} - D} \right)$$

This is the basic growth equation of a chemostat. When D=0, the chemostat becomes a batch culture. When K_s is small compared with s_r and Y is constant, x would be quite constant with changing D, until D reaches a critical value where $D > \mu$. When this happens, the culture will be washed out (curve A). When K_s is large compared with s_r, x will then change gradually with changing D (curve B).



Variations do occur in the above basic assumptions. For instance, the yield factor, Y, usually varies somewhat with growth conditions. This is because the physiology of a microorganism may change with growth conditions such as dilution rate, oxygen availability, presence of toxic substances, pH changes, etc. In Azotobacter the accumulation of PHB at low dilution rate would tend to increase the value of Y.

Electron Microscopy

After the discovery of the particle nature of the cathode ray in 1897 by J.J. Thomson deBroglie (1924) proposed the wave properties of moving particles, implying that a beam of electron can behave as a wave and that the wavelength of the electron is inversely proportional to the momentum of the electrons. Two years later Busch (1926) discovered the focussing effect of a magnetic field on a beam of electrons and thus began the study of electron optics. A year later the first electron lens came into existence when Gabor discovered that a solenoid cased in a soft iron box had much greater electron focussing effect than just the solenoid itself. From these three discoveries the construction of the electron microscope was started. Knoll and Ruska (1932) described the first transmission electron microscope (TEM). Although these early electron microscopes were crude in many ways and resolution was below that of the optical microscope, improvement was fast and by 1934 the first picture of biological material was obtained by Ruska (1934) with his much improved electron microscope which by then contained parts that are basic to the modern electron microscope. A year later, Driest and Muller (1935),using Ruska's electron microscope, demonstrated that the electron microscope could resolve structures much better than the light microscope. In spite of all these improvements these early electron microscopes were still far from perfect, e.g. the spherical and chromatic aberration of the magnetic lenses were largely not corrected.

Since the electron microscope is operated in vacuo and the specimen is always bombarded by high velocity electrons, the validity of the image from the electron microscope was much suspected. The

first commercial electron microscope was built in 1935 with a turntable mechanism so that the specimen could be checked from time to time in an optical microscope attached to the electron microscope. Although the resolution of the electron microscope was lower than that of the light microscope, the electron microscope proved to reveal structures correctly. From then on it was not necessary to include an optical microscope in every electron microscope. Effort was then mainly aimed at obtaining better resolution with the electron microscope. In 1938 von Borrier and Ruska attained a resolution of 10 nm and in 1946 a resolution of 1 nm was achieved by Hillier. The best resolution at present obtainable with a coventional TEM is about 0.3 to 0.5 nm from suitable specimen (Barer, 1974; Horne,1976). Much effort was also put into improvement of the basic designs of the electron microscope, e.g. better vacuum pumps, more stable current through the lenses, better cooling system, better focussing of the electron beam, better electron gun and illumination system, new deflector system to get better alignment of the electron beam, better stigmatism, tilting specimen stages, etc.

Recent developments of electron microscopy include the scanning electron microscope (SEM), the scanning transmission electron microscope (STEM), the high voltage electron microscope (HVEM) and the analytical electron microscope (AEM).

The SEM produces three-dimensional images of the surface structure of microscopic objects varying widely in sizes. The depth of focus of SEM is several mm and its range of effective magnification extends from about 10x to 100,000x (Grimstone ,1977).

The STEM (Crewe et al.,1969) differs from the SEM in that the STEM produces a very small probe of electrons (0.5 nm in diameter or

smaller) and allows the electron to pass through the specimen. It has better image formation control at high resolution.

The HVEM (i.e. electron microscope with accelerating voltage greater than 200 kV) (Dupouy,1968,1973) is mostly used in metallurgical, metal-physics laboratories. HVEM up to 3 million V has been built. Because of the high penetrating power of the electron beam in the HVEM there are high hopes that thick biological sections and even living or wet specimen can be examined. It is thought this will give better three dimensional aspects of cellular organization. (Glauert & Mayo,1973)

The AEM, also referred to as electron micropscope microanalysis (EMMA), (Chandler,1973) is a modification of the conventional TEM. It makes use of the X-ray spectra produced by the components of the specimen when the specimen is irradiated by the electron beam. By analysing the X-ray spectra the chemical components in the thin section, and thus in the cell, can be located and identified.

Alongside with the development for better electron microscope interpretation and reconstruction of the image have also improved immensely. Highly sophisticated electron devices, image intensifiers, display system, video-recorder, computers, automatic mechanisms have become available as accessory items for use with the electron microscope. (Montgomery,1962; Tolles,1969; Crowther & Amos,1971; Amos,1974; Crowther & Klug,1975; Roberts & Hills,1976)

Before a specimen can be observed in a TEM the specimen has to be fixed, sectioned and stained, like those for the light microscope.

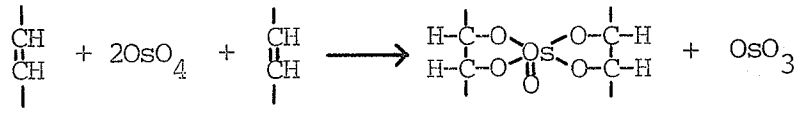
Fixation

The purposes of fixation are (1) to halt post mortem changes, and

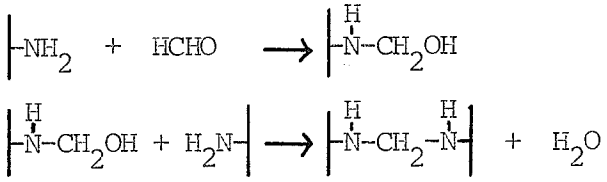
(2) to preserve the tissue in a condition which resembles the living state as closely as possible. To achieve these purposes, tissues are cut into tiny cubes of size less than 1 mm^3 to allow good penetration of the fixatives into the tissue or cells (Palade,1952). The pH of the fixation medium is usually maintained at physiological level (pH 7.3 to 7.4) (Hayat,1970). Several buffers are commonly used. Veronal acetate buffer with OsO_4 fixatives (Palade,1952), s-collidine buffer (Bennett & Luft, 1959), sodium cacodylate buffer with glutaraldehyde and OsO_4 (Sabatini, 1963), and phosphate buffers are used.

The main fixatives in electron microscopy are OsO_4, the aldehydes, and potassium permanganate.

OsO_4 mainly fixes the lipids in the cell. It is thought to cross-link double bonds in neighbouring lipid molecules as follows: (Korn,1967)



The aldehydes (formaldehyde and glutaraldehyde) mainly fix the proteins. Some enzymes remain active after the cell is fixed by aldehydes (e.g. catalase). Formaldehyde cross-links side chain amino groups in two steps:



With glutaraldehyde (a dialdehyde) cross-linkages are more complex (Richards & Knowles,1968). Glutaraldehyde was also found to be superior to other aldehydes in preserving the fine structure of the cell.

The permanganate (usually potassium permanganate) (Luft,1956)

preserves the phospholipid membrane in the cell very well. The chemistry of the fixation is still not clear.

Embedding

Because of the weak penetrating power of electrons sections must not be thicker than 0.1 μm. Also, thinner section always gives better resolution. Plastic instead of wax is used to give the fixed specimen sufficient hardness to be sectioned. The first material that came into common use was butyl methacrylate. It was soon found that by mixing butyl and methy methacrylates in different proportions different degree of hardness of the specimen could be obtained. This allowed different types of tissue to be sectioned to a thickness of 50 nm or less. However, methacrylates possess some disadvantages. They are very toxic to humans, they shrink on polymerization and sublime readily in an electron beam.

Later epoxy resins were used. Glauert et al. (1956,1958) used Araldite and Finck (1960) introduced Epon. These epoxy resins shrink little upon polymerization and are not as toxic as the methacrylates. However, they are quite viscous to work with and the penetration power into the tissues and cells is not as great as the methacrylates. In 1969 Spurr introduced a low-viscosity epoxy resin for embedding.

Polyester resins, e.g. Vestopal W, are also used for embedding medium. They are rather hard to cut but they shrink very little upon polymerization and are very stable under the electron beam. They are recommended for high resolution work (Sjöstrand,1969).

Today Araldite or Epon or various mixtures of both are routine embedding media in most laboratories.

Staining

In electron microscopy staining and fixation are almost identical. The heavier metallic fixatives, OsO_4 and permanganate, are both fixatives and stains. Examples of other stains are: uranyl acetate, lead citrate and ruthenium red. Uranyl acetate binds to both RNA and DNA (Huxley & Zubay,1961; Bernhard,1969;Monneron & Bernhard,1969). Reynold's lead citrate (Reynolds,1963) gives good contrast to cell membranes. It also stains glycogen and nucleic acids. Ruthenium red (Luft,1971a,b) is used to stain exopolysaccharide of tissue or cells.

Negative staining

When a stain does not interact chemically with the specimen but forms an electron-dense background around the specimen, the stain is called a negative stain. The stain scatters the electrons of the electron beam while the specimen does not. The resulting image appears as white area (specimen) against a dark background (stain). It is thought that the negative stains fail to react chemically with the proteins in the specimen partly because of the charge repulsion set up by the pH of the staining solution (DePamphilis,1974). The stain fills up cavities and crevices in the specimen and stays there when the rest of the stain is blotted away from the specimen. The result is that the morphological outline of the specimen is revealed when viewed under the electron microscope. The extent of detail revealed depends on the intercalation of the stain. Various contrasts can be obtained by varying the time of the staining (usually 10 seconds to 2 minutes), the pH of the staining solution (usually pH 6.8 to 7.4), and the concentration

of the stain. Phosphotungstic acid (PTA) (Brenner & Horne,1959; Horne, 1965,1975) is most widely used. However, different specimens may require different negative stains.

MATERIALS AND METHODS

Materials and Methods

The chemostat

The chemostat used was based on the design of Baker (1968) while the operation of the chemostat was essentially the same as that in Hine & Lees (1976). Basically, the chemostat consisted of an enclosed cylindrical culture vessel with an outlet on the side of the vessel to a 4-litre receiver vessel (Lees & Postgate,1973), and several ports on the top of the vessel for oxygen-electrode, pH electrode, thermometer, sampling tubing, titrant inlet and nutrient medium & gas inlet.

The temperature of the culture was continuously maintained at 30°C by an electric relay system which switched on and off an external incubation lamp as the thermometer in the culture dropped below or reached 30°C, respectively.

The medium inlet was modified to prevent backgrowth by using a capillary tubing with an internal diameter of 1 mm (Hine & Lees,1976). The rate of supply of medium was controlled by a high precision peristaltic pump (Watson-Marlow Limited, England).

The gas-flow over the culture was controlled by flowmeters (Manostat Corporation, New York). The volume of gas flowing over the culture per minute was the same as the volume of the culture. When different proportions of oxygen were required pure oxygen and nitrogen from Union Carbide Canada Limited, Toronto, were used. Gases were passed through a sterile cotton-wool filter before entering the culture vessel.

The pH of the culture was controlled by a Radiometer II titrator which added 1 M NaOH dropwise to keep the pH of the culture between

6.60 and 6.70. The pH electrode was a Radiometer combined glass-electrode.

The dissolved oxygen level in the culture was measured by a sterile Pb/Ag galvanic membrane electrode (LH Engineering Company Limited, Stoke Poges, England) connected to an oxygen meter (Western Biological, Sherborne, Dorset) coupled to a Rustrak chart recorder. The oxygen electrode was calibrated in the culture vessel above the culture. First, a steady zero reading in the O_2-meter was obtained with nitrogen only passing into the culture vessel. Then air only was passed into the culture vessel and the whole scale of the O_2-meter was used to read 20% O_2.

The culture vessel assembly, minus the pH electrode, was autoclaved at 15 psi, 120°C for 45 minutes. After autoclaving the culture vessel assembly was mounted onto the magnetic stirrer (Magnetic 9x9, Cole-Parmer Instrument Company, Chicago, Illinois) and the accessary equipment was connected. The pH electrode was standardized with commercial buffers and sterilized just before use by being immersed in 2-propanol for about 30 minutes.

Media

The B_6 medium contained (Dalton & Postgate,1969a,b) (g/l dist. H_2O): mannitol, 10; K_2HPO_4, 0.64; KH_2PO_4, 0.16; NaCl, 0.2; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; plus trace elements (mg/l): $FeSO_4.7H_2O$, 2.5; H_3BO_3, 2.9; $CoSO_4.7H_2O$, 1.2; $CuSO_4.5H_2O$, 0.1; $MnCl_2.4H_2O$, 0.09; $Na_2MoO_4.2H_2O$, 2.5; $ZnSO_4.7H_2O$, 1.2; nitrilotriacetic acid, 100. Nitrilotriacetic acid is not a source of nitrogen for A. chroococcum. It acts as a chelating agent to prevent precipitation of the mineral salts. $CaCl_2$ was autoclaved separately and added to the medium asceptically after the medium was

cooled to room temperature. When phosphorus limitation was investigated KH_2PO_4 was omitted, the amount of K_2HPO_4 was lowered accordingly and KCl was added to gave an equivalent amount of K^+ ion, e.g. in the 0.15 mM phosphorus medium the amount of K_2HPO_4 used was 0.025 g/l and the amount of KCl 0.616 g/l. B_6 agar was prepared from B_6 medium plus 2% (w/v) agar.

Organism

Azotobacter chroococcum ATCC 7493 was used throughout the course of this investigation. The stock culture was made and stored as follows: A. chroococcum was grown up in 5 ml B_6 medium for 3 days at 28°C. This 5 ml culture was spread onto B_6 agar in a Roux bottle. The Roux bottle was incubated at 28°C for about 5 days for the colonies to grow up. The colonies were then washed off the agar surface by about 30 ml of 15-20% (v/v) glycerol-dist. H_2O solution. This thick A. chroococcum suspension was then transferred to a sterile container from where it was pipetted into sterile 2 ml-plastic pro-vial (Costar Pro-Vial, Cooke Engineering Company, Alexandria, Virginia). The pro-vials were put into freezer at -48°C in an upright position and were stored there as stock culture.

To revive the stock culture the pro-vial was transferred directly from the freezer to a 40-50°C water bath and was kept there in an upright position until the content was liquefied. The content was then poured asceptically into 5 ml B_6 medium in a test-tube and was incubated at 28°C for about 3 days. This revived culture was used as inoculum after purity was checked.

Growth of culture (Hine & Lees, 1976)

About 5 ml of thick A. chroococcum suspension was used to inoculate about 200 ml sterile B_6 medium in the culture vessel. The air supply was turned on, temperature kept at 30°C and the culture was left stirring slowly. On the following day the oxygen electrode and the pH electrode were lowered into the culture. The stirring rate was turned up to maximum (1750 rpm), at which the oxygen tension in the culture rose immediately. The oxygen composition of the gas phase was then adjusted to about 5% O_2 by mixing the incoming air with nitrogen gas. The pump was turned on to give a low dilution rate of about 0.10 h^{-1}. The oxygen tension in the culture then dropped to zero in about an hour as the organism adapted to a higher respiration rate. The nitrogen gas was then reduced to give a slightly higher % of O_2 in the gas phase. The oxygen level in the culture again rose and then dropped slowly to zero again in about an hour. By repetition of this procedure, the organism could be brought to grow in a gas phase of air at vortex stirring rate (1750 rpm) with a zero dissolved O_2 tension. The dilution rate was then turned up to about 0.20 and the culture was allowed 3 days to equilibrate. After equilibration various cultural conditions were set up. In all cultures, the rest volume, i.e. the volume of the culture when stirrer was turned off, was between 240 and 250 ml. The sensitivities of the oxygen and pH electrodes were checked from time to time. The cultures were allowed two to three days for equilibration. Each experimental point represents the mean value of at least three duplicated analyses of samples taken on different days. (Each point therefore represents at least six samples.)

Sampling

A sample of culture was collected in a sterile Universal bottle (about 25 ml). Back-up pressure created by closing the outlet of the culture vessel forced the culture up the sampling tubing into the Universal bottle. Various analyses were immediately done on the sample.

Chemicals

Reagent-grade chemicals obtained from standard chemical companies were used for all media and analyses and EM-graded chemicals for electron microscopy throughout the course of this investigation.

Culture purity check

Culture was checked weekly to ensure that only A. chroococcum was present. A sample from the culture was directly streaked on commercial trypticase-soy agar, B_6 agar, B_6 agar with mannitol replaced by rhamnose or starch. The last three tests were specific for A. chroococcum (Bergey's Manual,1974). A. chroococcum exhibits pleomorphism and this was allowed for in the purity check. Identification tests were carried out on different colony types. A sample of the culture was also observed under a phase-contrast microscope to help in checking the purity of the culture.

Biomass measurement

A sample taken directly from the culture was centrifuged immediately at 0 - 5°C at 12,000xg for 5 minutes, washed once in cold dist. H_2O

and centrifuged again as before. The resulting cell pellet was washed with dist. H_2O into a pre-dried and pre-weighed aluminium foil weighing pan and left overnight in an oven at 98°C. On the following day the dried aluminium weighing pan was weighed after it was cooled in a desiccator under vacuum. Enough sample (10 to 50 ml) was always taken to give a good dry-weight reading.

CO_2 measurement

CO_2 evolution from the culture was measured in a percolator with glass beads as described by Lees & Postgate (1973). Briefly, gas leaving the culture forced the 0.1 M $Ba(OH)_2$ to percolate with the gas. The percolator was pre-equilibrated with the 0.1 M $Ba(OH)_2$ and phenolphthalein indicator and the exact concentration of the $Ba(OH)_2$ was measured in terms of the amount of 0.10 M HCl used to neutralize 5.0 ml of the $Ba(OH)_2$. After percolating for about an hour or more, depending on the amount of CO_2 evolved, the concentration of the $Ba(OH)_2$ was again determined by titrating with 0.10 M HCl . The amount of CO_2 that had reacted with the $Ba(OH)_2$ was calculated from the difference between the amount of 0.10 M HCl used for the two titrations.

Respiratory Index (R.I.)

$$R.I. = \frac{\text{mmoles } CO_2 \text{ evolved / h / 1 culture}}{\text{mg dry wt organisms / h / 1 culture}} \quad , \text{ (Hine \& Lees,1976).}$$

R.I. is a dimensionless number and is a quantitative measure of the degree of respiration of the culture.

Total nitrogen

The nitrogen content of the cells was determined by microKjeldhal method (Steyermark,1951) followed by steam-distillation of the ammonia (Markham,1942) into 1% boric acid containing methyl red and cresol green indicators plus methylene blue. These indicators changed from red to green with an easily detected mid-point (brown) at pH 4.7. The boric acid was back-titrated by 1/140 M HCl.

Cell suspension containing about 500 µg N was centrifuged down and washed with dist. H_2O. The washed cell pellet were transferred to a Kjeldhal digestion flask. Conc. H_2SO_4 1 ml and a tiny pinch of catalyst mixture (SeO_2 : $CuSO_4$: Na_2SO_4 (1:1:1, v:v:v)) were added to the cells. Digestion was carried out until about 10 minutes after the digest turned clear. When the digest was cooled, it was diluted to 10 ml with dist. H_2O. An aliquot (2 ml) of this diluted digest was steam distilled with 2 ml of 10 M NaOH. The ammonia distilled over was collected by 5 ml of 1% boric acid containing indicator. The amount of ammonia, and thus the amount of N, was determined by back-titrating the boric acid with 1/140 M HCl. A control with dist. H_2O only and a standard of known nitrogen content (bovine serum albumin) were also treated similarly to test the accuracy of the method.

Polysaccharide measurement

Freshly prepared anthrone reagent (5 ml of 0.2 g anthrone and 5 ml ethanol in 100 ml of 75% v/v H_2SO_4) was added to 0.2 ml suspension of cells, washed in dist. H_2O, at $0^{\circ}C$ with shaking. The tube was covered, heated in a boiling water bath for 10 minutes and then cooled rapidly at $0^{\circ}C$. The absorbance at 620 nm was read. Standards of glucose and a

water blank were treated similarly.

PHB measurement

PHB was assayed on a glass fibre disc as described by Ward & Dawes (1973). New glass fibre discs (2.4 cm GF/A, Whatman) were cleaned in hot, concentrated H_2SO_4, ethanol and ether and dried. Washed cell suspension (0.2 ml, equivalent to 1 mg dry wt or less) was applied to each disc and dried at 80 - 110°C. The dried discs were then treated with 0.2 ml NaOCl for 1 h and dried again. Hot chloroform (50 - 60°C) was applied for several times onto the discs which were then incubated at 37°C in NaOCl solution for 1 h. The discs were then washed twice with each of the following: dist. H_2O, ethanol, acetone and diethyl ether. When dried, each disc was heated with 5 ml concentrated H_2SO_4 in a test-tube in a boiling water bath for 10 minutes. The tubes were then cooled under the tap and the contents were mixed thoroughly but gently. Absorbance at 235 nm was read. A blank with concentrated H_2SO_4 only and standards of β-hydroxybutyrate in 5 ml concentrated H_2SO_4 were heated similarly without the NaOCl and chloroform treatment.

Phosphorus measurement

The method used was that of Chen et al. (1956) described by Cooper (1977). The supernatant of the culture, 1 ml, diluted if necessary, was mixed thoroughly with 4 ml of reagent which was freshly prepared and contained: 1 vol. 3 M H_2SO_4, 2 vol. dist. H_2O, 1 vol. 2.5% w/v ammonium molybdate solution and 1 vol. 10% w/v ascorbic acid solution. The tubes were covered with Parafilm, incubated at 37°C for 2 h, and

cooled to room temperature. Absorbance at 820 nm was read. Standards using K_2HPO_4 and a blank with dist. H_2O were treated similarly.

The amount of phosphorus consumed by the cells was determined from the difference between the phosphorus content of the supply medium and that of the supernatant.

Electron microscopy

The procedures for the electron microscopy were essentially those in Dawson's M.Sc. Thesis (1977).

Fixation

Cells directly from continuous culture were washed once in 0.1 M sodium cacodylate buffer pH 7.3 and were then resuspended for 4 h at 4°C in the fixation mixture containing: 0.15 ml acrolein, 0.60 ml 25% glutaraldehyde, 1.75 ml dist. H_2O and 2.50 ml 0.2 M sodium cacodylate pH 7.4. The cells were then washed four times in 1 h with 0.1 M cacodylate buffer pH 7.3 and then embedded in 2.5% w/v warm liquid noble agar. The agar block was cut into cubes of size less than 1 mm^3. The cubes were left in 1% OsO_4 in 0.1 M cacodylate buffer for 2 h at 4°C and then washed 4 times with dist. H_2O for 1 h before being left overnight at 4 °C in 0.5% w/v uranyl acetate solution.

Dehydration

The agar cubes left in 0.5% uranyl acetate solution were washed in dist. H_2O for 10 minutes and were then dehydrated in an increasing percentage of ethanol: 15 minutes in each of the following concentrations of ethanol : 30%, 50%, 70%, 90%, and then two 30 minutes in absolute

ethanol.

Embedding

After dehydration the agar cubes were soaked in 1:1 (v:v) Spurr-absolute ethanol mixture. Spurr refers to the low-viscosity epoxy resin embedding medium introduced by Spurr (1969). It contains 5 g vinyl cyclohexene dioxide, 3 g diglycidyl ether of propylene glycol, 13 g nonenyl succinic anhydride and 0.2 g dimethylaminoethanol (DMAE). To prepare Spurr the DMAE was added only after the first three ingredients were mixed thoroughly. After soaking for half an hour, fresh Spurr of volume equal to that of the mixture was added and mixed. Then after 1 h the Spurr-alcohol solution was decanted and fresh pure Spurr was added. The Spurr was changed after 1 h. Then the agar cubes in the pure Spurr were left overnight at room temperature on a slow rotor for complete penetration of Spurr into the cells in the agar cubes.

Polymerization

One agar cube was added to each plastic capsule filled to about 4/5 full with Spurr. When the agar cube had sunk to the bottom of the capsule it was adjusted to the centre of the bottom with a fine glass needle. The capsules were then left at room temperature for a few hours and were then put into an oven at 60^{o}C under vacuum for at least 12 hours. The capsules were then left at room temperature for 24 hours before sectioning.

Sectioning

The hardened epoxy resin block was removed from the capsule and the tip, where the agar cube was lodged, was trimmed so that only a small face of the agar cube was sectioned. The face was trimmed with a new and clean razor blade to a trapezoid shape with the longer of the parallel edges at the bottom. A chosen freshly cut glass knife was used for sectioning. Sections were floated on water in a boat made from vinyl plastic tape attached to the glass knife. Only silvery or gray sections were used. They were further spread on the water surface by xylene vapour and were then picked up on a 300 mesh copper grid previously washed in acetone and dist. H_2O. The microtome used was Reichert, Austria, OM U2 Ultramicrotome.

Post-staining

A grid with sections was placed with sections facing down on a drop of lead citrate stain (Reynolds,1963) on wax for exactly 1 minute. The grid was then immediately washed with dist. H_2O and then blotted dry on a filter paper.

Electron microscope examination

Stained sections were examined and photographed either in AEI model EM 6B or AEI model EM 801 electron microscope, with an accelerating voltage of 60 kV. The pictures were taken on Kodak EM film.

Negative staining

One drop of cellular preparation was applied onto a carbon-coated 160 mesh copper grid. Enough time (10 seconds to 1 minute, depending on the thickness of the preparation) was allowed for cells or cellular material to settle onto the grid. The grid was then blotted dry and 1 drop of 1% w/v phosphotungstic acid (PTA) in water , pH 7.0 (pH adjusted by 1 M KOH), was applied onto the grid for about 10 seconds. After it had been blotted dry the grid was observed under the electron microscope. The best results were obtained by trying different percentages of PTA (1 to 2 %) at different pH values. Controls with buffer instead of cellular preparation were treated in the same way.

(a) Membrane preparation for negative staining: A. chroococcum from continuous culture was treated with lysozyme-EDTA according to the method of Kaback (1971). An appropriate amount of cells was taken from the culture and was washed twice with 10 mM Tris.HCl, pH 8.0 at 0 ^{o}C. The cells were then resuspended in 30 mM Tris .HCl, pH 8.0, containing 20% sucrose and swirled. Sodium EDTA, pH 7.0 and lysozyme were added to final concentrations of 10 mM and 0.5 mg/ml, respectively. After 30 minutes at room temperature the suspension was centrifuged at 16,000xg until the supernatant was clear. The cells were then resuspended in the smallest possible amount of 0.1 M K_2HPO_4 buffer pH 6.6, containing 20% sucrose and were used for negative staining. Since the cell walls were digested by lysozyme the cells burst under the vacuum in the electron microscope, exposing the inside of the membrane.

(b) Broken cells preparation for negative staining : A. chroococcum from continuous culture was washed and resuspended in 50 mM potassium

phosphate buffer pH 7.0. The suspension was then passed through a French Press Cell (American Instrument Co., Maryland) at a pressure of 17,000 psi in the Cell. The resulting cellular preparation was used for negative staining.

Localization of catalase

Cellular catalase was localized by precisely the method used by Dawson (1977). The results, figure 15C, precisely confirm what she found.

RESULTS

Results

A. chroococcum was grown in continuous culture in a chemostat at 30°C (Hine,1975). The pH of the culture was controlled by a Radiometer II titrator which added 1 M NaOH dropwise to keep the pH between 6.60 and 6.70. The culture was always stirred at a vortex stirring rate, i.e. the maximum stirring rate of the magnetic stirrer,1750 rpm. This vortex stirring rate maintained a maximum and uniform rate of transfer of oxygen to the cells of the culture. The dissolved oxygen level in the culture was most of the time nominally zero (below 1 μM O_2) or nearly so except at very low phosphorus concentrations, in which case the oxygen level rose somewhat to about 3 μM.

The variation of biomass with oxygen concentration in the gas phase above the culture, as shown in figure 4, is a biphasic curve with a faster rate of increase of biomass from zero to two percent oxygen and a slower rate of increase of biomass from two to twenty percent oxygen. This seems to indicate that the efficiency at which oxygen was used to produce cellular material became less as the oxygen in the gas phase increased and reached an apparent maximum at about 20% oxygen , i.e. the oxygen concentration of ordinary air.

Figure 5 shows the amount of carbon dioxide evolved under different oxygen concentrations in the gas phase above the culture at a dilution rate of 0.20 h^{-1}. The amount of carbon dioxide evolved increased with the oxygen concentration. This suggests, as it were, a greater usage of substrate when the oxygen is increased (c.f. figure 4).

Figure 6 shows the mmole CO_2 evolved per mg of cell growth. This is termed as Respiratory Index (R.I.) (Hine & Lees,1976). The R.I. rose

Figure 4 Variation of biomass of nitrogen-fixing A. chroococcum grown in continuous culture under different percentages of oxygen in the gas phase. D = 0.20 h^{-1}

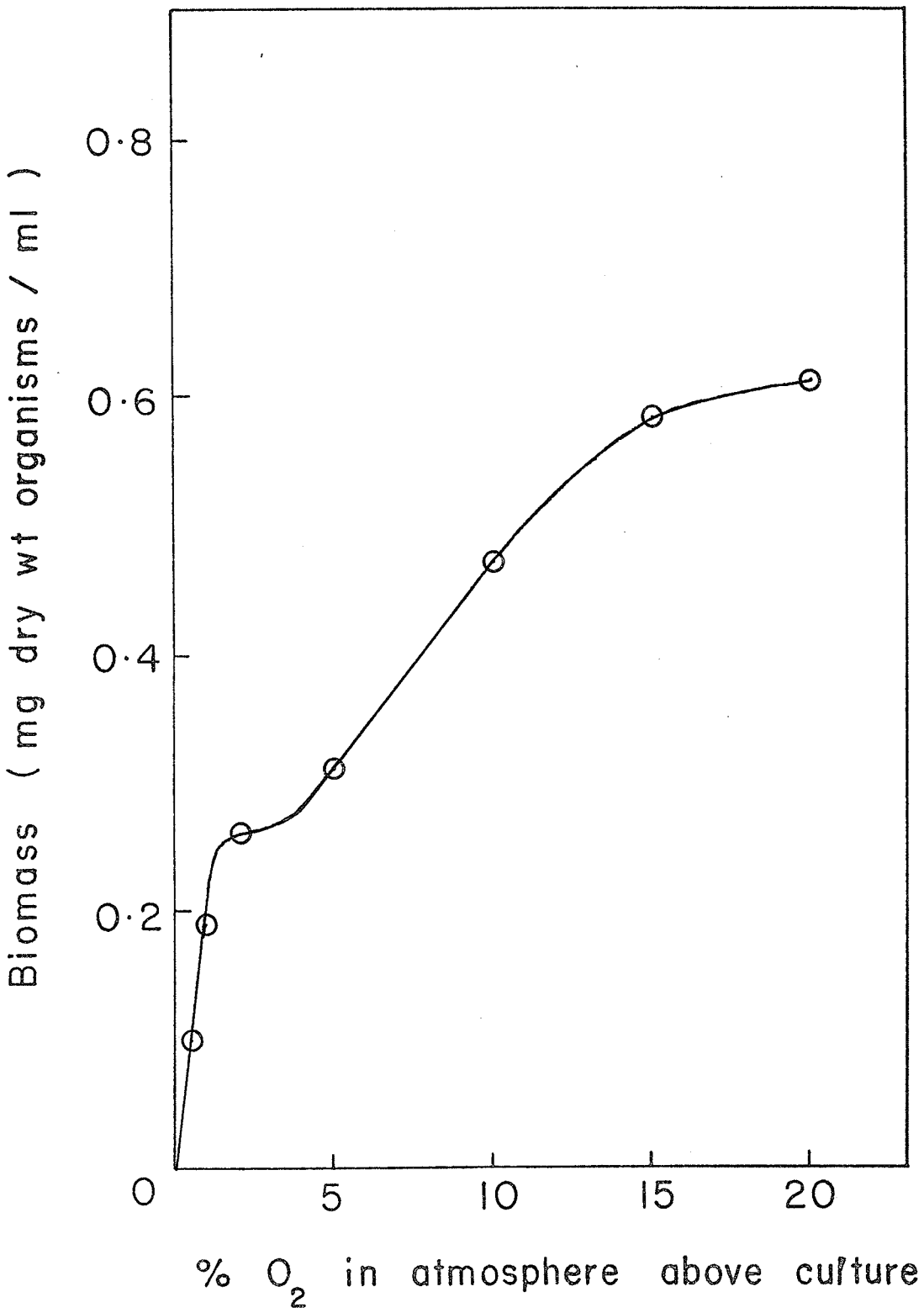

Biomass (mg dry wt organisms / ml)
0·8
0·6
0·4
0·2
0
5
10
15
20
% O_2 in atmosphere above culture

Figure 4

Figure 5 Carbon dioxide evolved by nitrogen-fixing A. chroococcum grown in continuous culture under different percentages of oxygen in the gas phase. D = 0.20 h^{-1}

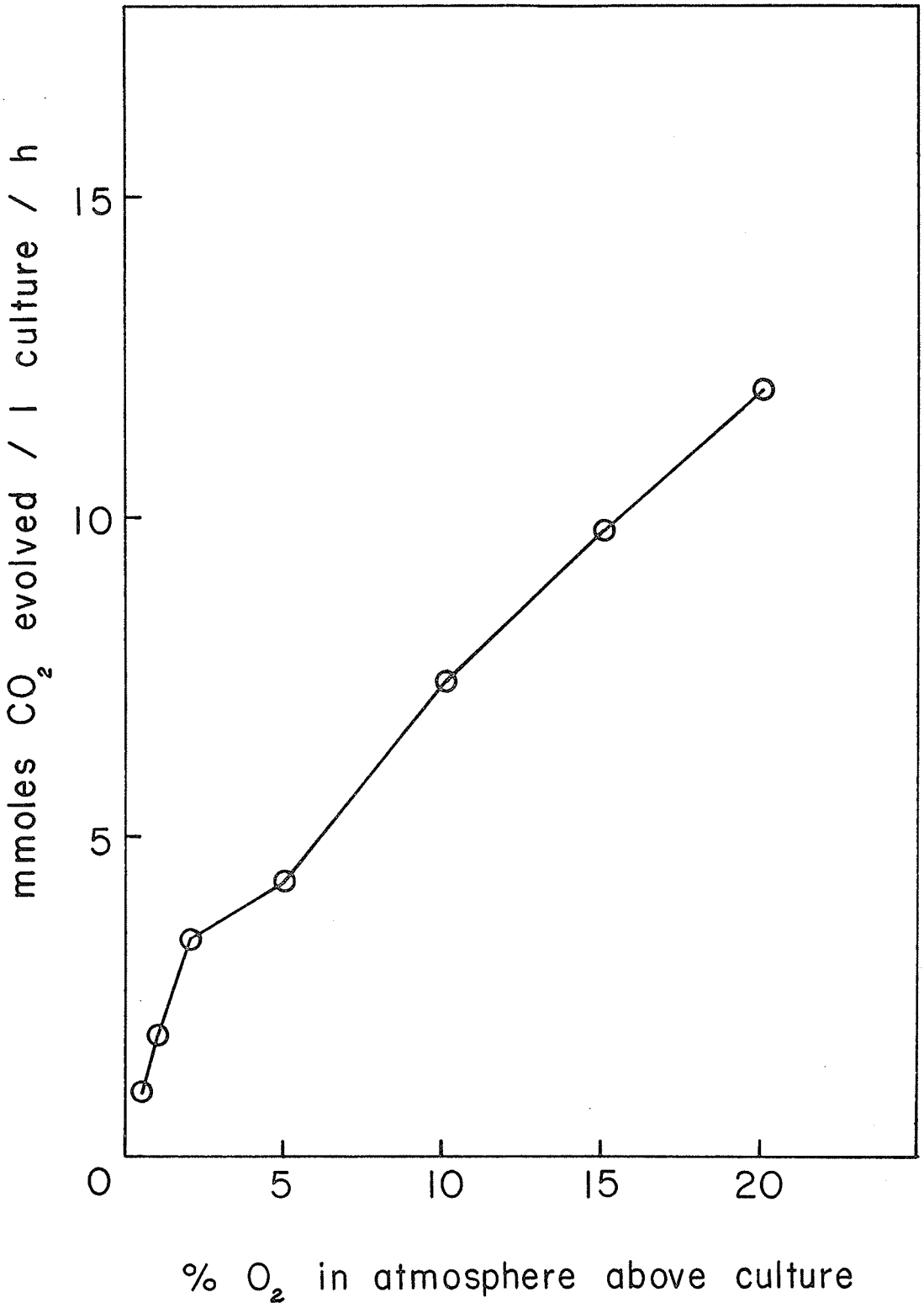

mmoles CO_2 evolved / l culture / h
0
5
10
15
0
5
10
15
20
% O_2 in atmosphere above culture

Figure 5

Figure 6 Variation of Respiratory Index of nitrogen-fixing A. chroococcum grown in continuous culture under different percentages of oxygen in the gas phase. $D = 0.20\ h^{-1}$

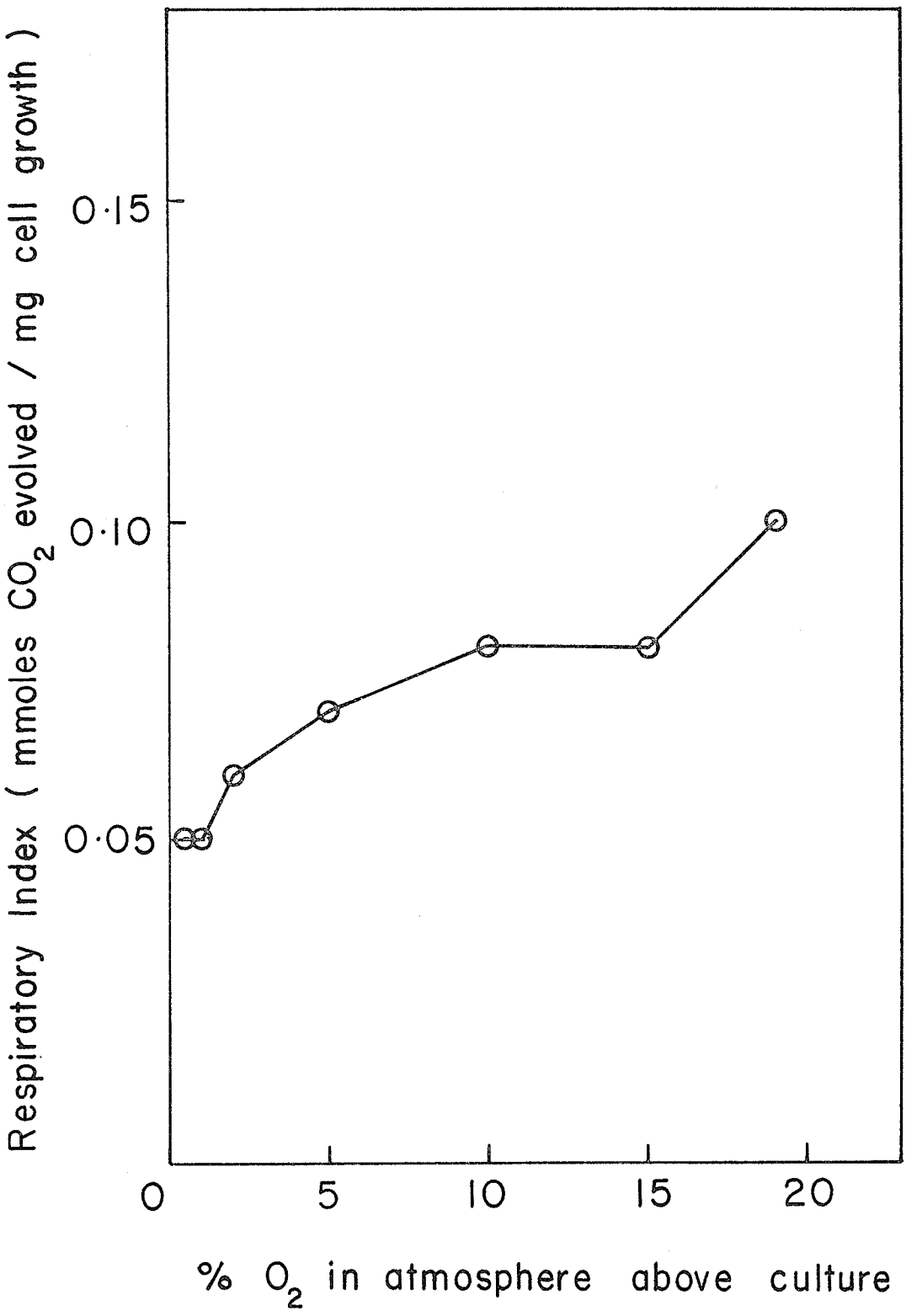

Respiratory Index (mmoles CO_2 evolved / mg cell growth)
0·15
0·10
0·05
0
5
10
15
20
% O_2 in atmosphere above culture

Figure 6

as the oxygen concentration increased as would be expected from figures 4 and 5 . This rise was consistent with the R.I. plot of Hine & Lees. Above 15% of oxygen in the gas phase the culture began producing more CO_2 per unit weight of cells. This augmented rate of respiration which represented a protection of the cells against excess oxygen was termed 'respiratory protection' by Dalton & Postgate (1969). It was suggested that the cell tried to maintain a low oxygen concentration inside it by respiring the excess oxygen away as carbon dioxide. The flattening of the R.I. plot at oxygen concentrations lower than 15% seems to indicate that most of the oxygen was utilized by the cells in biomass production.

Figure 7 is a Lineweaver-Burk plot of oxygen concentration and the oxygen-utilizing enzyme of A. chroococcum in vivo from 5% to 20% oxygen in the gas phase. Although the oxygen-utilizing enzyme or enzymes are not specified, the plot does show that the cells utilizing oxygen show Lineweaver-Burk kinetics with respect to oxygen. Furthermore, the Km of 10% oxygen is a very reasonable one for cells living in natural water or near the surface of the soil. The biomass was the product of oxygen utilization since in figure 4 it was shown that biomass production was related to the oxygen concentration in the gas phase. The graph also indicates that when the cells are growing in air the cells should not be oxygen limited.

Figure 8 shows the amount of phosphorus consumed per mg dry wt organisms when A. chroococcum was grown under various percentages of oxygen in the gas phase. There were two levels of phosphorus consumption. More phosphorus was required when the cells were grown at or above 5% oxygen than at or below 2% oxygen. This greater uptake

Figure 7 Lineweaver-Burk plot of biomass and percentages of oxygen in the gas phase. $D = 0.20\ h^{-1}$

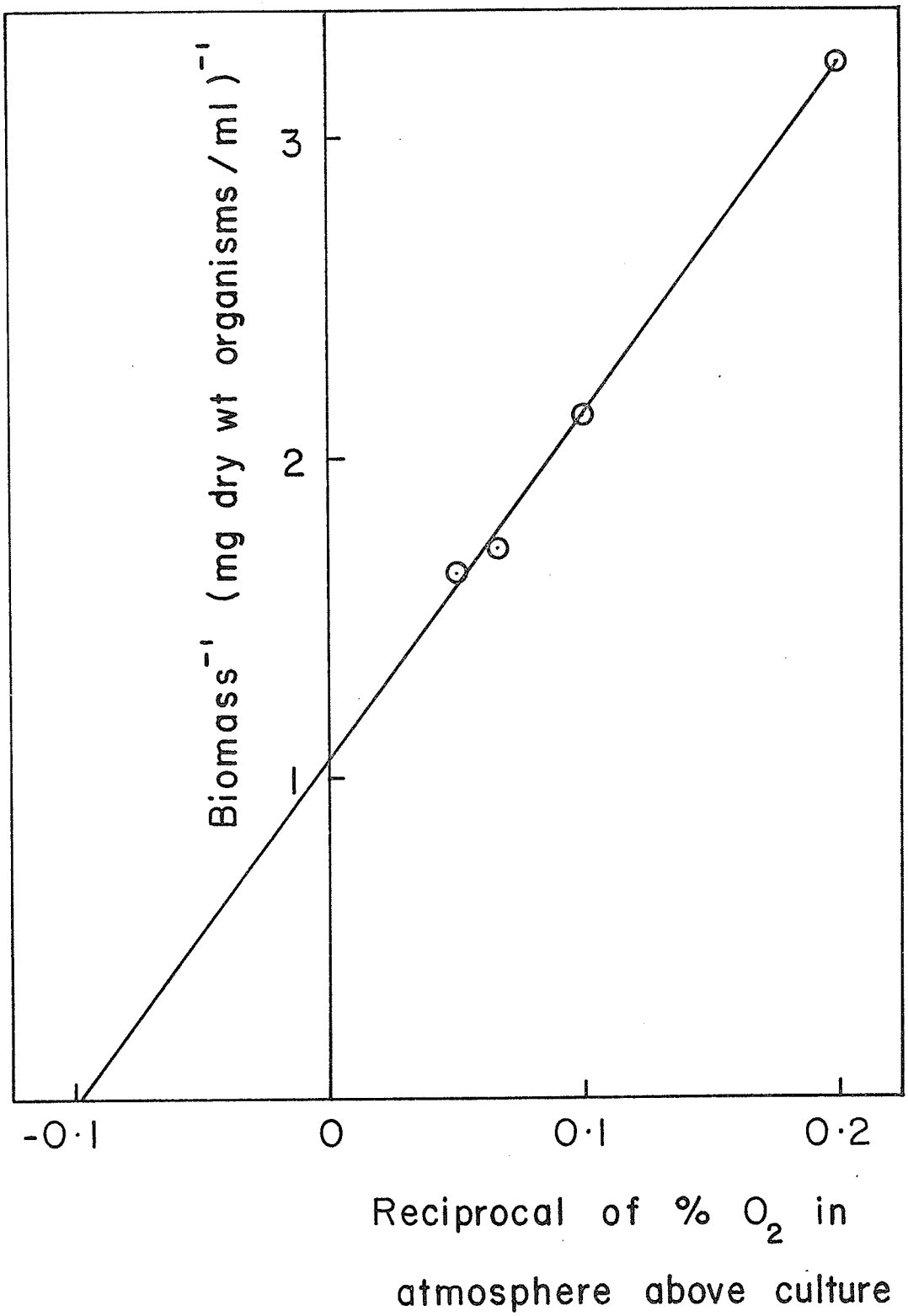

Biomass^{-1} (mg dry wt organisms / ml)$^{-1}$
3
2
1
-0·1
0
0·1
0·2
Reciprocal of % O_2 in
atmosphere above culture

Figure 7

Figure 8 Amount of phosphorus consumed by nitrogen-fixing A. chroococcum grown in continuous culture under different percentages of oxygen in the gas phase. $D = 0.20\ h^{-1}$

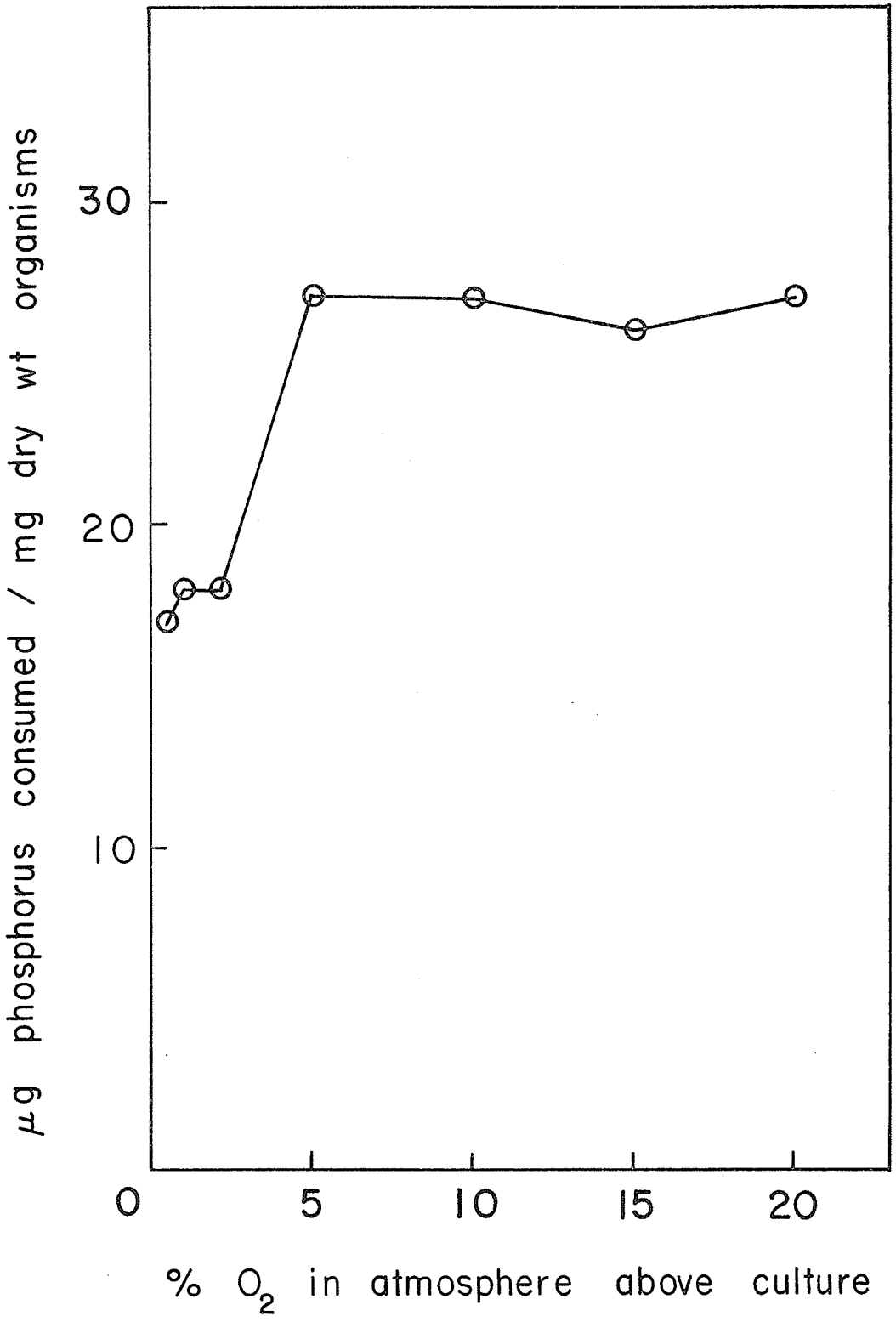
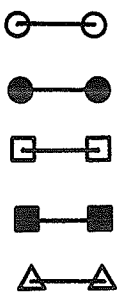

μg phosphorus consumed / mg dry wt organisms
30
20
10
0
5
10
15
20
% O_2 in atmosphere above culture

Figure 8

of phosphorus seems to indicate a change in cellular phosphorus metabolism which might have been the increase in ATP or RNA production. It is interesting to note that although there was an increase in the rate of respiration (figure 6), there was not a corresponding increase in phosphorus uptake. Since respiration in the usual sense is equivalent to ATP production from ADP and P_i, this uniform uptake of phosphorus would indicate uniform production of ATP in the cell. This observation tends to support the idea that in A. chroococcum the increased rate of respiration at high oxygen tensions is not coupled to ATP production (Nagai & Aiba,1972). Also, the fact that the curve (figure 8) does not drop to zero µg phosphorus consumption below 5% O_2 but levels off at about 18 µg phosphorus consumed/mg dry wt indicates that a minimal amount of phosphorus was required for cell maintenance. The minimal amount in this case was about 18 µg phosphorus per mg dry wt organisms. The higher level of phosphorus consumption was about 27 µg per mg dry wt organisms which was about 3% of the dry weight of the cell and was the same as the phosphorus composition of Escherichia coli (Luria,1960).

Figure 9 shows the variation of biomass at different dilution rates when A. chroococcum was grown in air at various phosphorus concentrations in the supply medium. The criterion used to determine whether the culture was phosphorus limited was that the biomass increased proportionally to the amount of phosphorus supplied at a given dilution rate. It should be pointed out that the irregularity in biomass at $D = 0.15\ h^{-1}$ with changing phosphorus concentration (figure 9) was probably due to the growing influence of 'batch-culture' type of growth characteristic of such normally fast growing organisms at such a low dilution rate (Hine &

Figure 9 Variation of biomass of nitrogen-fixing A. chroococcum growing in air in continuous culture at different dilution rates with different phosphorus concentrations in the medium supplied to the culture.



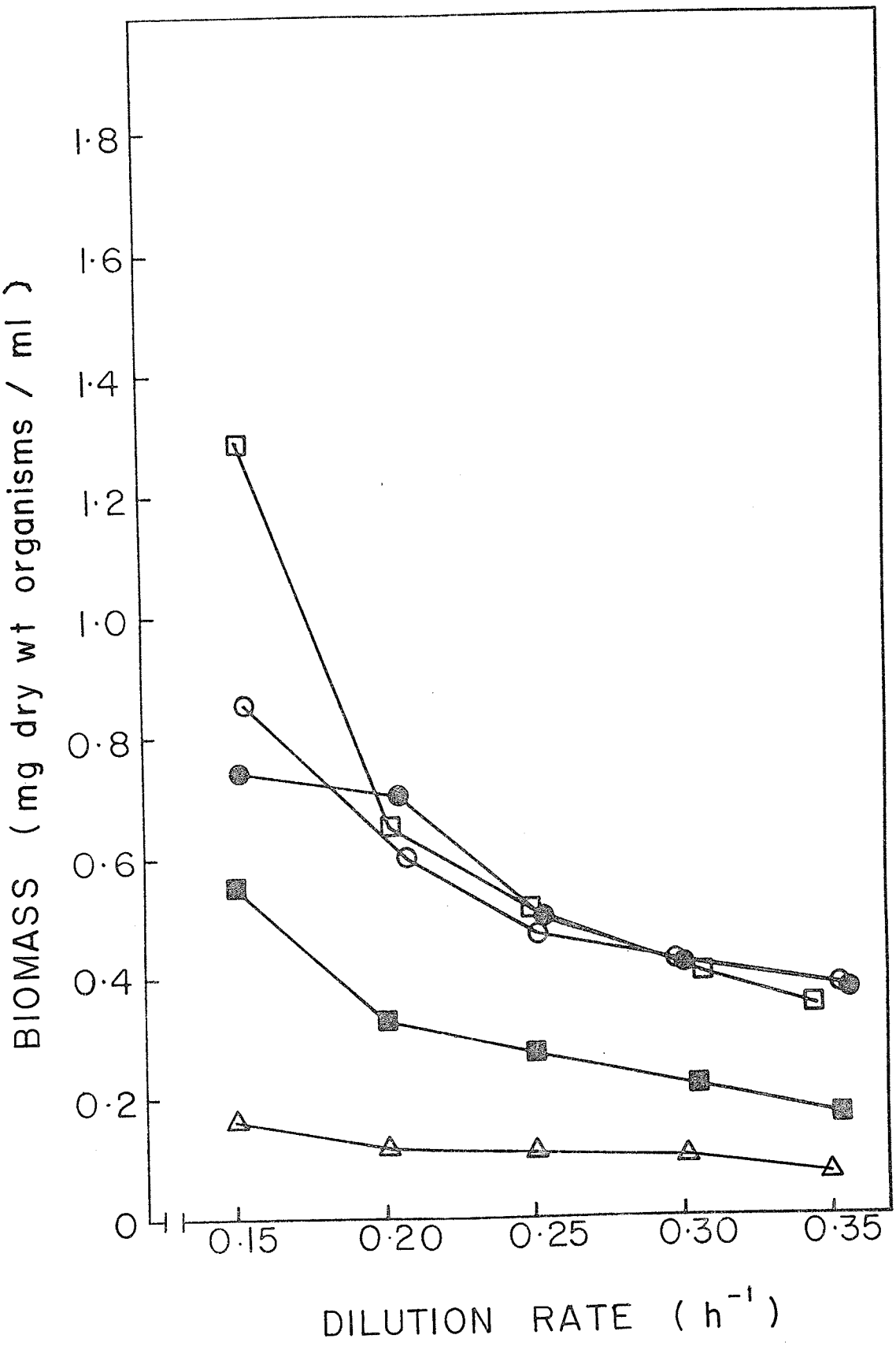

BIOMASS (mg dry wt organisms / ml)
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0·15
0·20
0·25
0·30
0·35
DILUTION RATE (h⁻¹)

Figure 9

Lees,1976). As a result, the growth at D = 0.15 h^{-1} was not taken into consideration in the assessment of phosphorus limitation. Figure 9, therefore, shows at every dilution rate above D = 0.15 h^{-1} the cultures could proportionally increase their biomass when they were grown at 0.07 and 0.15 mM phosphorus while there was no further increase in the biomass from 0.29 to 4.84 mM phosphorus. Accordingly, the cultures grown at 0.07 and 0.15 mM phosphorus were considered phosphorus limited while those at 0.29, 0.58 and 4.84 mM phosphorus were considered not phosphorus limited.

Figure 9 also shows that for every phosphorus concentration the biomass of the culture increased inversely with dilution rate. Under non-phosphorus limiting conditions Lees & Postgate (1973) found the increase in biomass could be accounted for by the accumulation of polysaccharide and PHB in the cell. However, under phosphorus limiting condition the increase in biomass was found mainly due to the accumulation of PHB (Table 2). The PHB accumulation varied from 0.4% of the dry weight at D = 0.35 to 22% at D = 0.15. The polysaccharide content of the cell remained more or less constant for most dilution rates. The total nitrogen of the cell increased with the dilution rates. This agrees with Lees & Postgate (1973) who found an increase in proteins under the same conditions.

Electron micrographs of the accumulation of PHB by A. chroococcum

Table 2 Composition of A. chroococcum growing in air in continuous culture in 0.15 mM phosphorus in the supply medium at various dilution rates.

D (h^{-1})	Total nitrogen	Polysaccharide	PHB
	(µg / mg dry wt organisms)		
0.15	71	19	222
0.20	96	37	98
0.25	96	31	27
0.30	117	42	6
0.35	108	41	4

at low dilution rates under phosphorus limiting condition are shown in figure 10. The large, white, shadowy, polyhedral bodies inside the cell at dilution rates 0.20 and 0.15 h^{-1} are granules that are apparently composed of PHB. (Tchan et al.,1962; Wyss et al.,1961; Lin et al.,1978; Lundgren et al.,1964; Ellar & Lundgren,1968) The PHB measurement in Table 2 plus the fact that PHB, being a polyester, is not very soluble in water also strongly suggest that the granules are PHB granules. These granules were also seen as refractile spherical aggregates inside the cell under phase contrast microscope.

The micrograph at D=0.15 h^{-1} shows, beside massive PHB accumulation, signs of an azotobacter cell going into a resting stage. The cell was more spherical. The nuclear material was not conspicuous. The membrane vesicles on the periphery of the cell were decreasing in number. (Wyss et al.,1961; Sadoff,1975) However, the facts that the culture was not washed out and extensive respiration was present (figure 12,R.I.>0.1) showed that the cells in the culture were growing, i.e., they were not resting cells or cysts. At higher dilution rates (D = 0.30 or 0.35 h^{-1}) when the cells were growing faster there was no PHB granules, indicating the cells were not accumulating PHB to any great extent at all (figures 10 D, E; Table 2).

The same trend of PHB accumulation in A. chroococcum was also observed under electron microscope at other phosphorus concentrations.

Figure 11 shows the mmoles of CO_2 evolved by A. chroococcum grown in air at various dilution rates and in various phosphorus concentrations in the supply medium. The amount of CO_2 evolved was more or less the same for all dilution rates under each phosphorus concentration

Figure 10 Electron micrographs of nitrogen-fixing A. chroococcum grown in air in continuous culture at various dilution rates and in 0.15 mM phosphorus in the supply medium. Bars ≡ 1 µm ; w = cell wall; m = cell membrane; c = cytoplasm; n = nuclear material; s = mesosome; v = vesicle; PHB = poly-β-hydroxybutyrate granule; u = unidentified component.

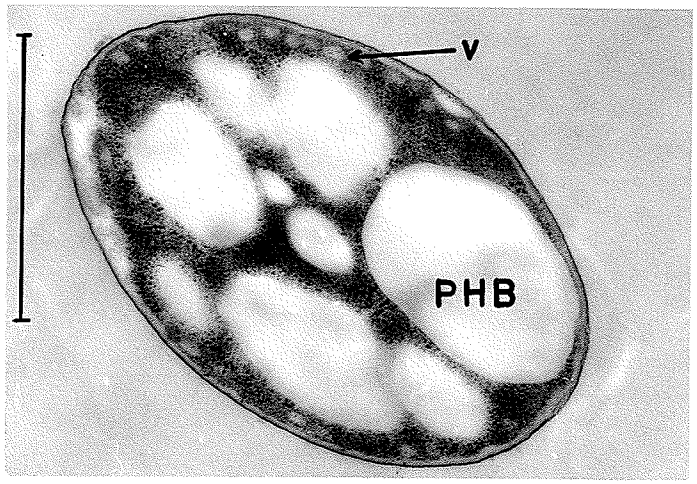
A : D = 0.15 h^{-1}

B : D = 0.20 h^{-1}

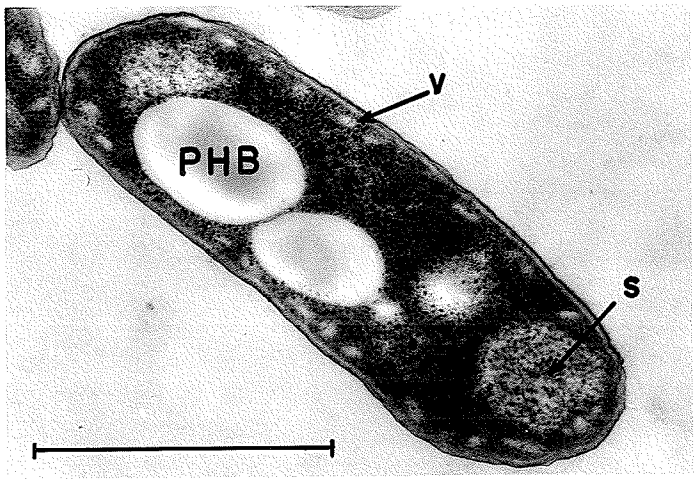
C : D = 0.25 h^{-1}

D : D = 0.30 h^{-1}

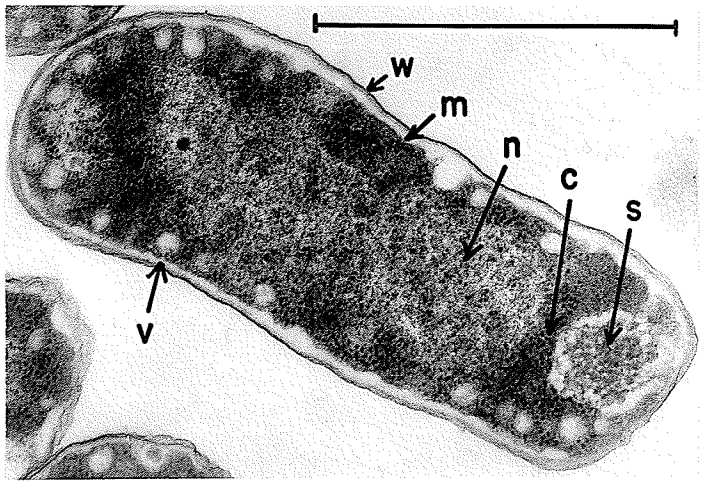
E : D = 0.35 h^{-1}



A



B



C

Figure 10

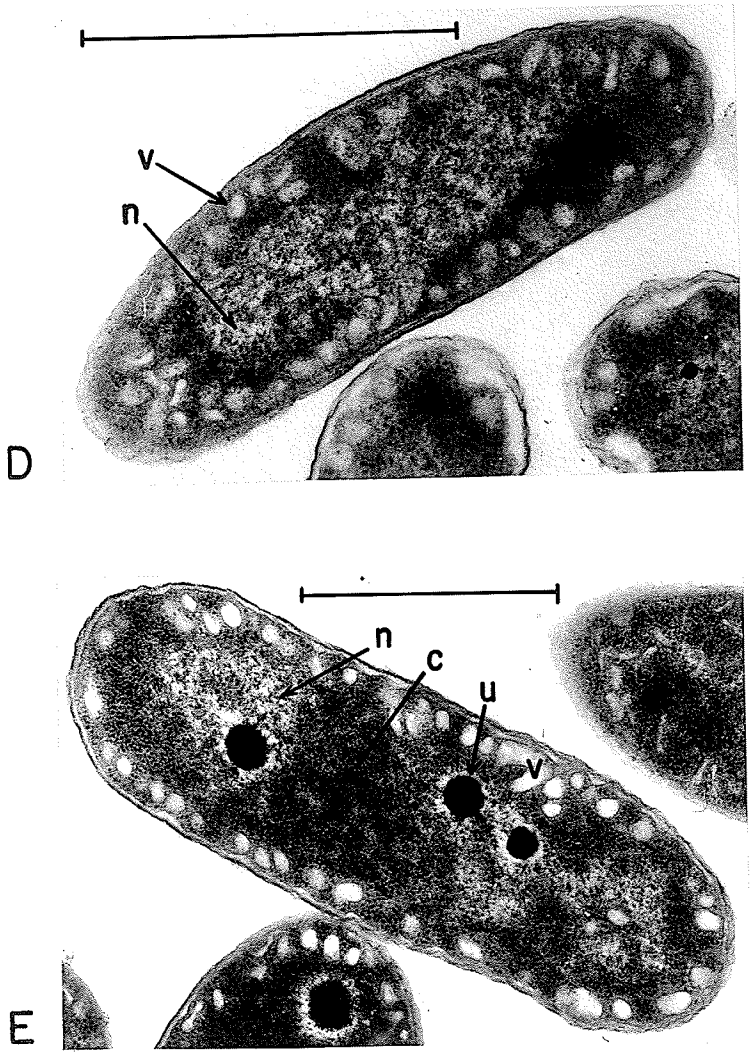
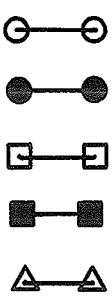


Figure 10

Figure 11 Amount of carbon dioxide evolved by nitrogen-fixing A. chroococcum grown in air in continuous culture at different dilution rates with different phosphorus concentrations in the medium supplied to the culture.



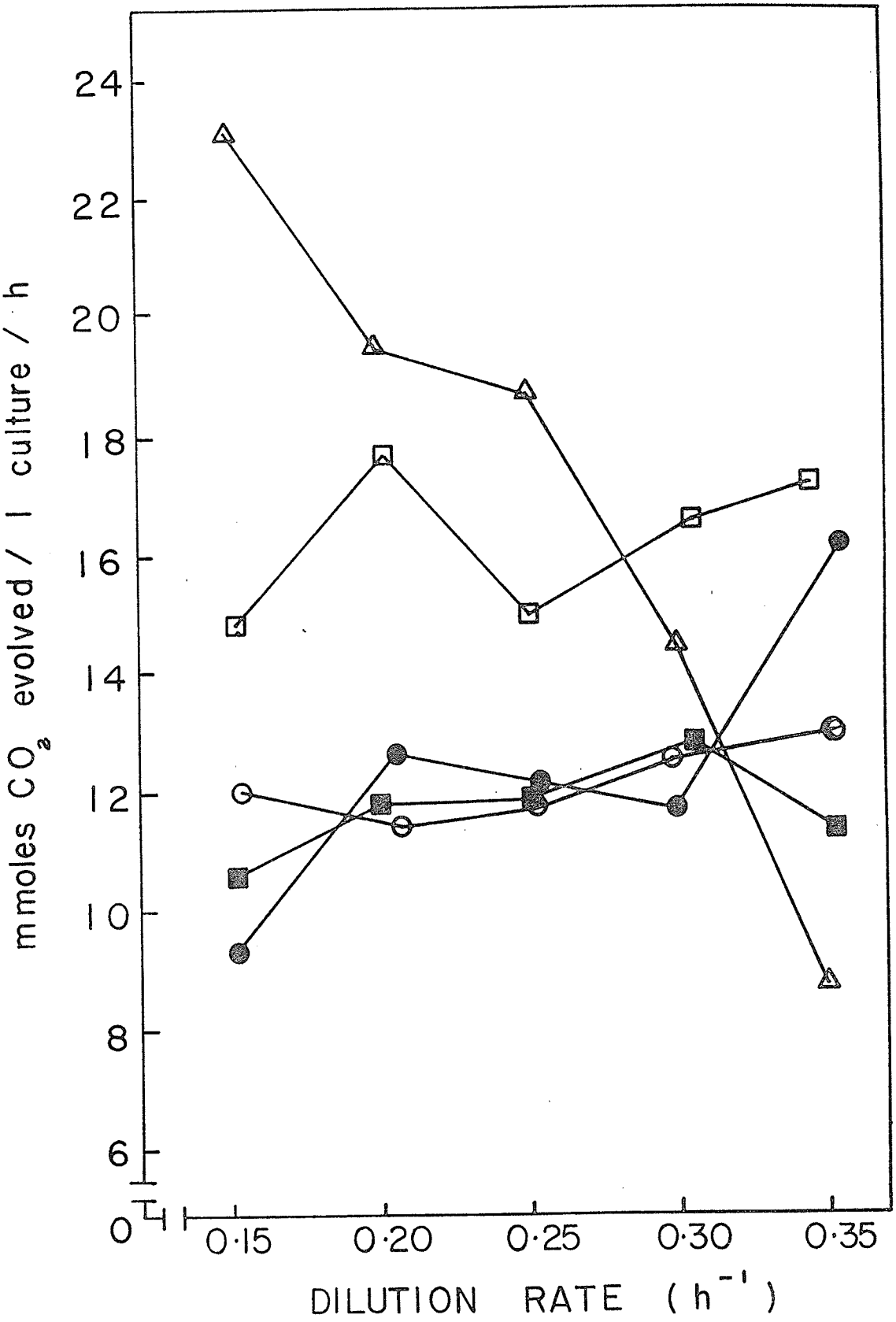
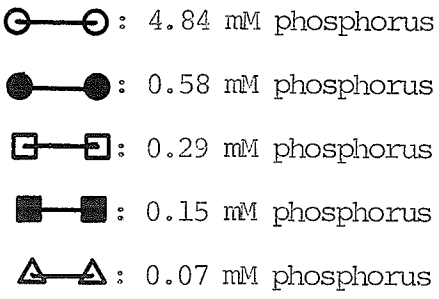

24
22
20
18
16
14
12
10
8
6
0
mmoles CO_2 evolved / l culture / h
0·15
0·20
0·25
0·30
0·35
DILUTION RATE (h^{-1})

Figure 11

except at 0.07 mM phosphorus. At this very low phosphorus concentration the rate of CO_2 evolution increased about 3 fold from D = 0.35 h^{-1} to D = 0.15 h^{-1} .

Figure 12 shows the Respiratory Index of A. chroococcum grown in air at various dilution rates and various phosphorus concentrations in the supply medium. The R.I. increased inversely with the phosphorus concentrations, indicating that the cells respired more at lower or even phosphorus-limiting conditions. At each phosphorus concentration, except at 0.07 mM, the R.I. remained quite constant. The drop in the R.I. at D=0.15 h^{-1} for both 0.15 mM and 0.29 mM phosphorus concentrations was probably due to the increase in biomass as a result of massive PHB accumulation (figure 9). At 0.07 mM phosphorus concentration the R.I. dropped with increasing dilution rates although the R.I.'s were much higher than those of other phosphorus concentrations. Since the biomass at 0.07 mM phosphorus were quite constant (figure 9) the decrease in the R.I.'s was mainly due to the decrease in CO_2 production of the culture (figure 11).

Figure 12 Respiratory Index of nitrogen-fixing A. chroococcum grown in air in continuous culture at different dilution rates with different phosphorus concentrations in the medium supplied to the culture.



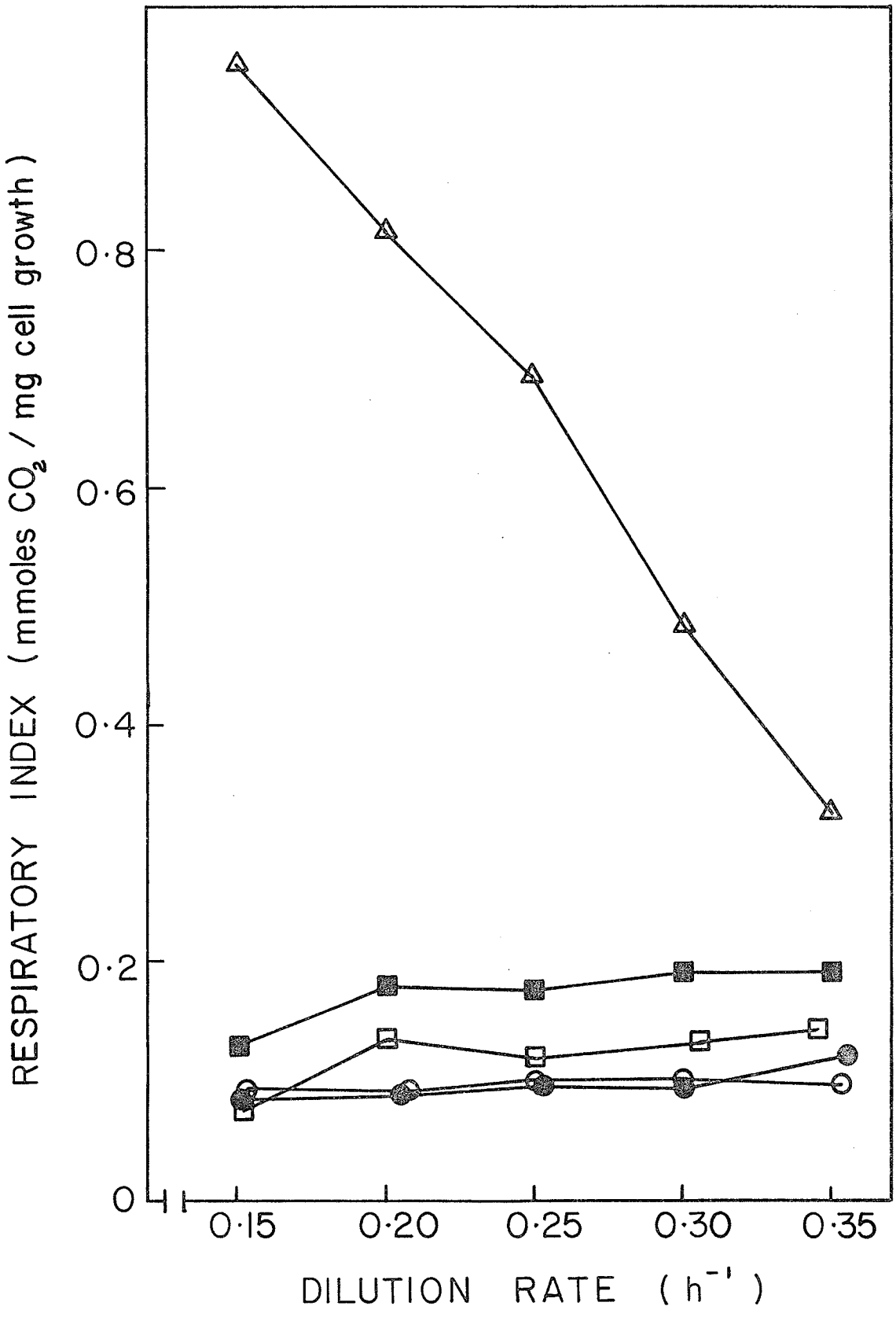

RESPIRATORY INDEX (mmoles CO_2 / mg cell growth)
0·8
0·6
0·4
0·2
0
0·15
0·20
0·25
0·30
0·35
DILUTION RATE (h^{-1})

Figure 12

Figure 13 shows the intracellular morphological change in A. chroococcum when it was grown in air under different phosphorus concentrations at D = 0.30 h^{-1}. The outstanding change was in the amount of vesicles on the periphery of the cells. There were many more vesicles in the cells grown near phosphorus limiting conditions (D, E, F,) than in cells grown under non-phosphorus limiting condition (A, B, C). At very low phosphorus condition (0.07 mM) these vesicles in the cell became small tubules (F).

Although the cells were grown under phosphorus-limiting condition, the nitrogen and polysaccharide of these cells were the same as those of cells grown in non-phosphorus limiting condition (Table 3), indicating the former cells were just as 'healthy' as the latter cells.

This increase in the number of vesicles in A. chroococcum cell was also observed in other growth condition (Dawson, 1977). Thus, cells grown in the presence of a high percentage of O_2 in the gas phase (figure 14B) or in the presence of high concentration of NH_4^+ in the medium (figure 14C) or in phosphorus-limiting condition (figure 14D) had same morphological change. They produced many more vesicles than cells grown in normal air (figure 14A).

The vesicles are shown in figure 15A to be invaginations of the plasma membrane of the cell. Figure 15B seems to show the presence of knob-like particles on the inside of the membrane and figure 15C shows the presence of catalase in the vesicles.

Figure 13 Electron micrographs of nitrogen-fixing A. chroococcum grown in air in continuous culture under various phosphorus concentrations in the supply medium. D=0.30 h^{-1} Bars ≡ 1μm. w = cell wall; m = cell membrane; c = cytoplasm; n = nuclear material; v = vesicle.

A : 4.84 mM phosphorus

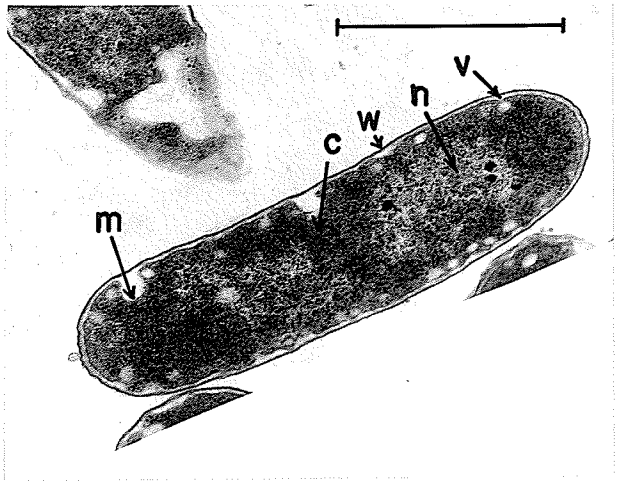
B : 1.16 mM phosphorus

C : 0.58 mM phosphorus

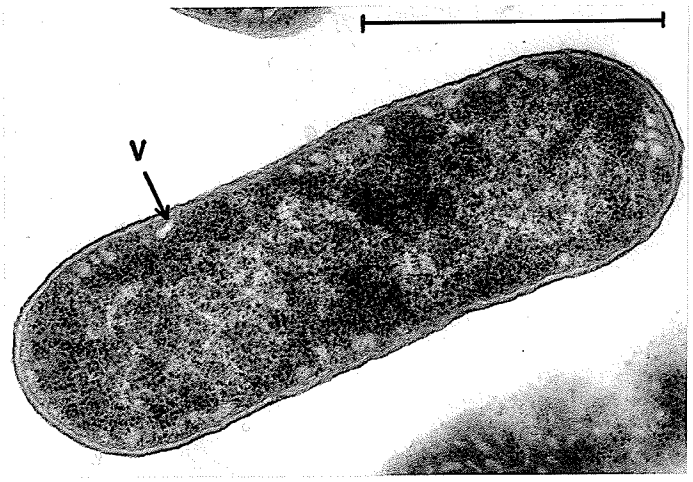
D : 0.29 mM phosphorus

E : 0.15 mM phosphorus

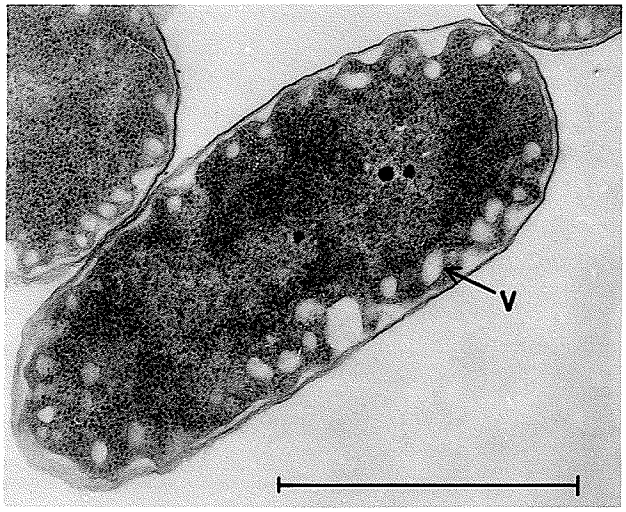
F : 0.07 mM phosphorus



A



B



C

Figure 13

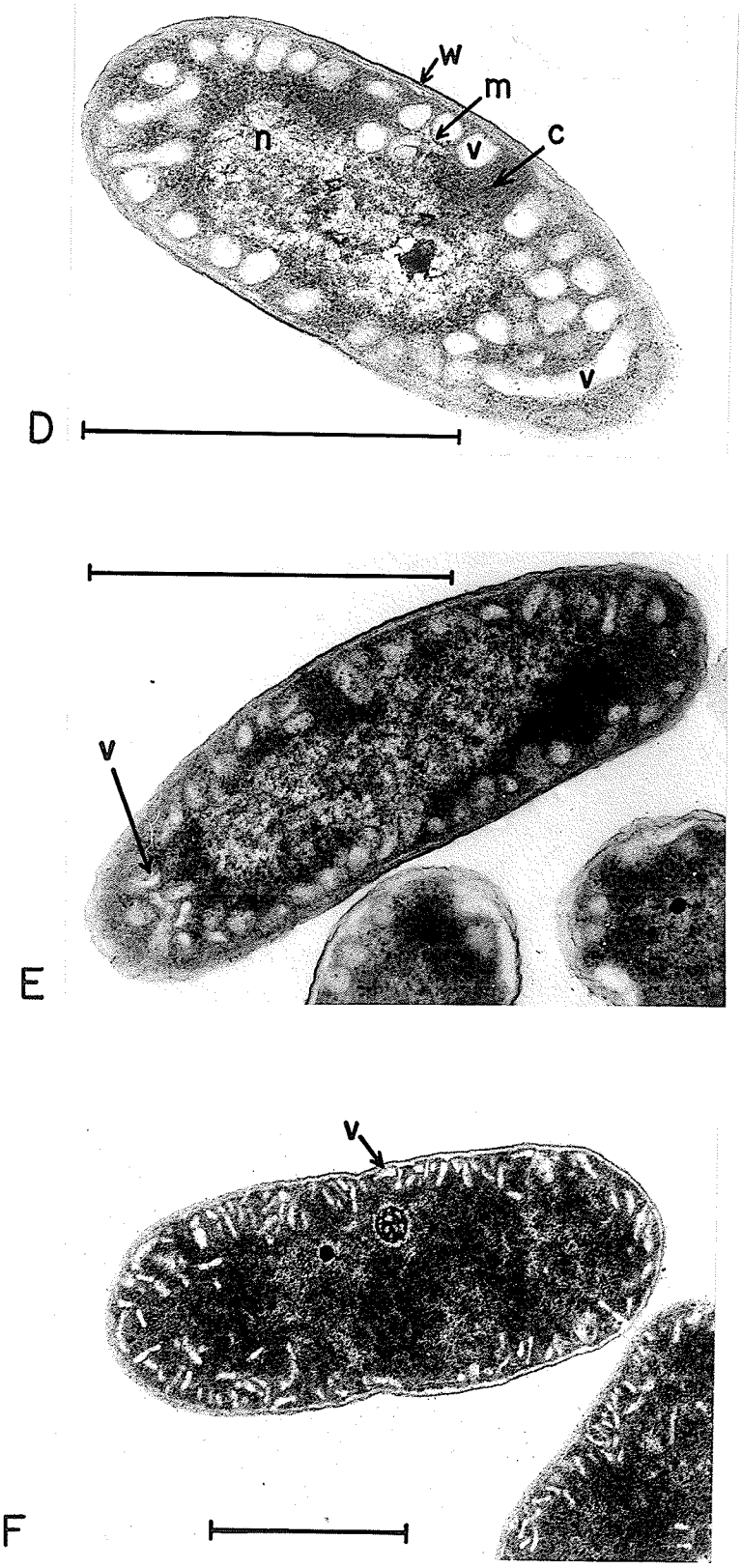


Figure 13

Table 3 Nitrogen and polysaccharide of A. chroococcum growing in continuous culture at dilution rate 0.30 h^{-1} and at various phosphorus concentrations in the supply medium.

Phosphorus concentration (mM)	total nitrogen (µg / mg dry wt organisms)	polysaccharide
4.84	118	43
0.58	123	43
0.29	103	33
0.15	117	42
0.07	116	35

Figure 14 Comparison of the amount of vesicles in A. chroococcum grown under various conditions in continuous culture.

v = vesicle; Bars ≡ 1 µm .

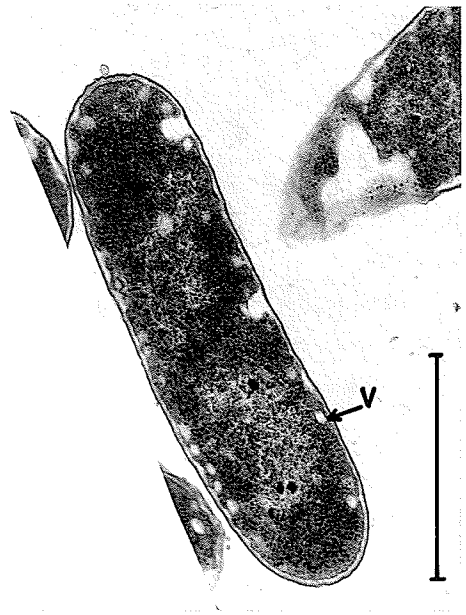
Micrographs B and C were prepared by Dawson (1977).

A : cells grown in air, fixing nitrogen

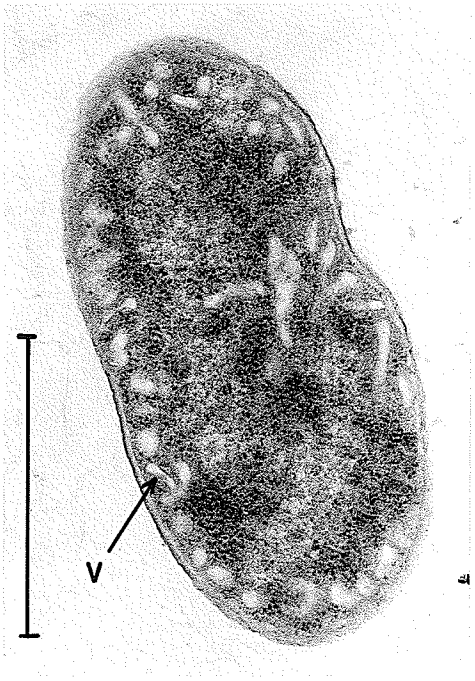
B : cells grown in 30% oxygen, fixing nitrogen

C : cells grown in air with 20 mM NH_4^+ in the supply medium

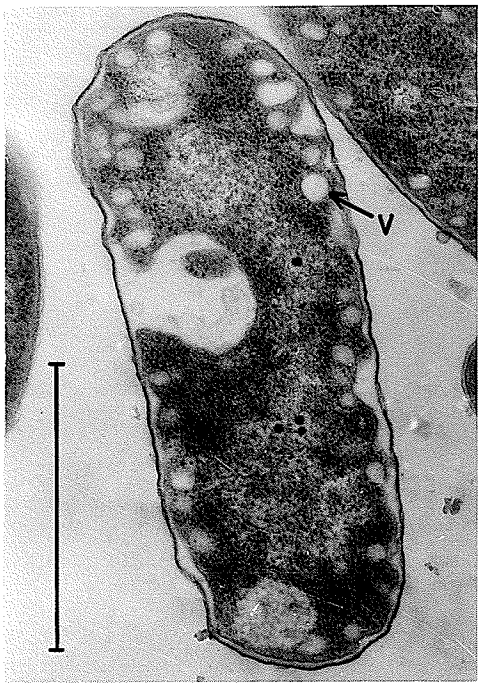
D : cells grown in air with 0.29 mM phosphorus in the supply medium, fixing nitrogen.



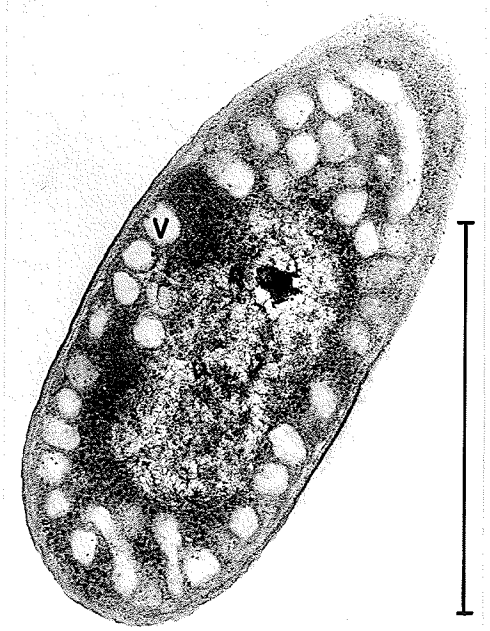
A



B



C



D

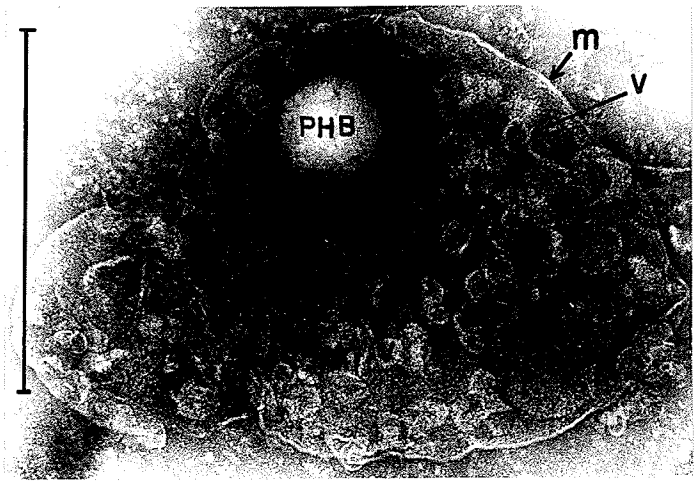
Figure 14

Figure 15 Electron micrographs. Bars ≡ 1 µm

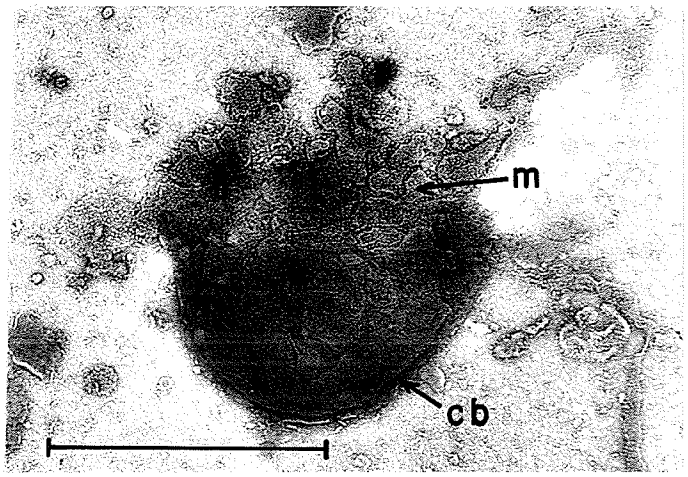
A : Negative staining of membrane fragment of A. chroococcum. PHB = poly-β-hydroxybutyrate granule; v = membrane vesicle; m = cell membrane.

B : Negative staining of broken cells of A. chroococcum m = folded plasma membrane with knob-like particles; cb = fragmented cell body.

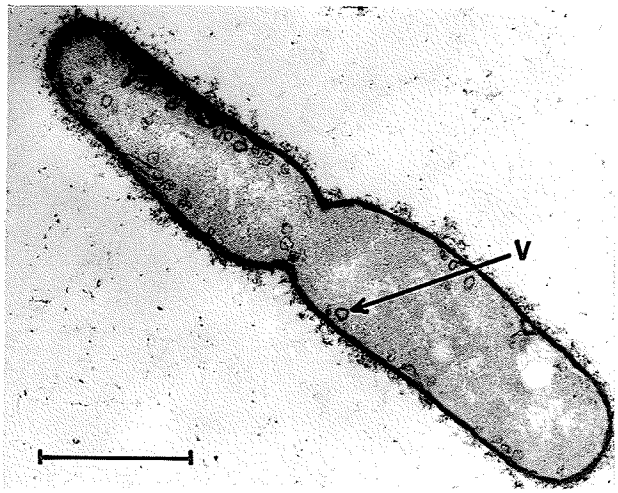
C : Localization of catalases by means of DAB staining method. Section of dividing A. chroococcum. v = DAB stained vesicle showing the presence of catalase. (DAB = 3,3'-diaminobenzidine)



A



B



C

Figure 15

DISCUSSION

Discussion

PHB accumulation

Senior et al. (1972) observed that A. beijerinckii in batch culture started to accumulate massive amount of PHB at about the end of the exponential phase of growth. At the same time the oxygen tension in the culture was observed to drop to zero. Further studies in continuous cultures showed that carbon- and N_2- limited cultures accumulated PHB less than 3% and 1.5% of dry weight, respectively, at every dilution rate, while oxygen-limited cultures accumulated PHB from 19.6% to 44.6% of dry weight from dilution rates 0.225 to 0.053 h^{-1}. These results showed that oxygen limitation is undoubtedly the crucial factor in the PHB accumulation. It was suggested that PHB acts as an electron sink for the excess reducing power produced at low oxygen concentration in the presence of excess carbon and energy substrate. The removal of the reducing power keeps the TCA cycle functioning as the reducing power, mostly as $NADH_2$, is inhibitory to the TCA cycle (Jackson & Dawes,1976).

The criterion used by Senior et al. (1972) for oxygen limitation was zero detectable oxygen tension (d.t.o.) in their cultures. This does not seem to be a good criterion because a zero d.t.o. does not necessarily indicate that the culture is oxygen-limited. A zero d.t.o. only indicates that the cells in the culture are using up oxygen just as fast as the oxygen can dissolve into the culture. One of the extra-ordinary characteristics of Azotobacter cells is their high respiration rate which is believed to be a protective mechanism for maintaining an anaerobic environment for the nitrogenase to function properly. Thus,

whether the cells are oxygen sufficient or oxygen deficient, with respect to energy production, they will respire away as much oxygen as possible. All the cultures, except those at 0.07 mM phosphorus , in this investigation have zero d.t.o. The fact that they were not oxygen-limited in air is supported by the following findings. (i) Once over 20% oxygen the growth of the cultures did not improve. In figure 4 the difference in biomass between 1% and 20% oxygen was about 0.5 mg dry weight organisms while in figure 3 of Hine & Lees (1976) the difference between 20% and 40% oxygen was about 0.25 mg dry weight organisms. (ii) The R.I. increased much faster above 20% oxygen (figure 7 of Hine & Lees,1976) than below 20% oxygen (figure 6, this thesis). Therefore, excess oxygen started to be present in the culture grown at 20% oxygen in the gas phase above the culture. (iii) The Lineweaver-Burk plot for oxygen and the oxygen utilizing enzymes in vivo gave a Km of about 10% oxygen in the gas phase above the culture (figure7). Thus, there was enough oxygen when the cultures were growing in air.

It is noticeable that in all the studies of Azotobacter in continuous cultures very massive PHB accumulation always occurred at very low dilution rates (Senior et al.,1972; Dalton & Postgate,1969b; Lees & Postgate,1973). Figure 9 shows the biomass of A. chroococcum was highest at D = 0.15 h^{-1}. Therefore, it appears that the growth rate, which corresponds to the dilution rate of the culture, may also play an important role in the PHB accumulation. Azotobacter species can form dormant and resistant cysts at the end of the exponential growth phase (Socolofsky & Wyss,1961,1962). Just before the encystment process the cells pass into a slow growing stage, become round and accumulate

large amount of PHB (Wyss et al.,1961; Stevenson & Socolofsky,1966). In A. chroococcum culture the accumulation of massive amounts of PHB by the cells at the low dilution rate ($D = 0.15\ h^{-1}$) matched quite well with the events before the onset of encystment. It appears that this slowing down of the growth rate, induced by the low dilution rate, shifted the cellular metabolism into the accumulation of large amounts of carbon reserve in the form of PHB as if the cells were preparing for encystment, although cysts were never formed in any of the cultures. The effect of dilution rate upon PHB accumulation is especially clear in phosphorus-limited nitrogen-fixing cultures (Table 2). The accumulation of PHB at the low dilution rate was about 50 fold greater than that at the high dilution rate. These phosphorus-limited nitrogen-fixing cells were not oxygen limited as shown by the high respiration rate in figure 12. Therefore, beside the effect of oxygen limitation, dilution rate probably also plays a role in the massive accumulation of PHB. Also, the two factors could work together to intensify the PHB accumulation so that an accumulation of up to 50% of dry weight is possible(Senior et al.,1972;Lees & Postgate,1973).

Phosphorus-limited cultures

The nitrogen-fixing phosphorus-limited A. chroococcum cultures reported by Dalton & Postgate (1969a,b) were oxygen sensitive in the sense that the growth of the culture stopped in overaerated culture and the 'oxygen-poisoned' cells were not viable on nitrogen-free medium. Lees & Postgate (1973) again observed that when oxygen supplied to the culture increased from 20% to 40%, the nitrogen-fixing phosphorus-limited A. chroococcum, unlike that in normal B_6 medium, could not increase its respiration rate to maintain a low oxygen tension in the culture and

the cells stopped growing. However, it was discovered that the oxygen-poisoned cells in the phosphorus-limited culture were completely viable on NH_4^+-supplemented medium. So, it seems that a deficient supply of phosphorus results in the breakdown of the respiratory protection mechanism so that the cells can not cope with the sudden increase of oxygen tension. The excess oxygen inactivates the nitrogenase system and the cells are no longer able to fix nitrogen.

The results in this investigation show that the reported lack of respiratory response in phosphorus-limited nitrogen-fixing culture is not applicable to cells growing in air. In figure 12, the R.I. increased as the amount of phosphorus supplied to the culture decreased and the most phosphorus-limited culture (0.07 mM) has the highest R.I., showing that the phosphorus-limited cells can increase their respiration rate. This rise in the R.I. was probably due mainly to the decrease in biomass, which resulted in an increase of oxygen load to each cell. An additional cause could be a higher oxygen solution rate at lower biomass as shown by the rise in CO_2 evolution in phosphorus-limited conditions (0.07&0.28 mM phosphorus, figure 11) (for biomass $\underline{v}$ O_2 solution rate, see Hine & Lees, 1976).

Thus, nitrogen-fixing phosphorus-limited cells do have protective respiratory response although this respiratory response is broken down by a sudden huge increase of oxygen as reported by Lees & Postgate. This finding agrees well with the natural habitat of A. chroococcum. In soil, phosphorus is rarely a limiting nutrient. Therefore, the non-phosphorus-limited nitrogen-fixing A. chroococcum can cope with the oxygen levels in the soil, which is almost always less than 20%. The results in figure 8 also support the above reasoning. Figure 8 shows that extra phosphorus is not required for increased respiratory activities between 5% and 20% oxygen (figure 6). This would indicate that the

excess respiration in A. chroococcum is not coupled to ATP production and thus as long as the cells are getting enough phosphorus for the normal activities of the cells the excess repiration is not disturbed. This also indicates that limiting the supply of phosphorus limits the production of biomass only, not the respiratory ability.

The membrane vesicles

The intracellular peripheral membrane vesicles in A. chroococcum have been studied extensively by Dawson (1977) with vortex stirred continuous cultures. The appearance of these vesicles was found to be related directly to the amount of respiration, which in turn is directly related to the oxygen tension in the culture. Under nitrogen-fixing conditions an increase in oxygen tension increased the R.I. (Hine & Lees,1976) and the amount of vesicles (figure 14B). In NH_4^+-grown cells the disappearance of the nitrogen-fixation process lessened the cellular consumption of oxygen for ATP production. This unused amount of oxygen in the culture mimicked a rise in oxygen tension in the gas supply. The cells responded by increasing the rate of respiration and the amount of membrane vesicles (figure 14C).

The same increases in the respiration rate (figure 12) and the amount of membrane vesicles (figure 14D) were also observed in nitrogen-fixing phosphorus-limited cultures. This lends further support to Dawson's suggestion. As the phosphorus decreased, the R.I. increased and the amount of vesicles increased. At the very low phosphorus concentration (0.07 mM) the vesicles became tubular, indicating a further increase in the surface of respiration. Thus, it appears that oxygen is the only

factor that affects the formation of the membrane vesicles and the primary function of the vesicles is to increase the surface of respiration, regardless of the source of nitrogen. The relation of the membrane vesicle with oxygen utilization is further shown by the presence of catalase in the vesicles (figure 15C).

Whether these membrane vesicles provide any protection to the nitrogenase is not certain, mainly because the nitrogenase, although found to be located on the cell membrane (Stasny et al.,1973; Raveed et al.,1973), has not yet been shown to be present in the vesicles. However, it appears that the inside of each vesicle is anaerobic and could therefore be a logical location of the nitrogenase. Furthermore, the role of the cell membrane in protecting the nitrogenase from oxygen inactivation can be appreciated by the fact that in a membrane associated with cell-free nitrogenase extract the nitrogenase is not oxygen labile (Bulen et al.,1964; Hardy & Knight,1966).

The membranous origin of these vesicles is shown by the negatively stained cell membrane of A. chroococcum (figure 15A). The vesicles arose from invaginations of the cell membrane. The production of these membrane vesicles does not seem to require extra phospholipid production (Drozd et al.,1972; Reczek,1977). This is probably due to the mesosome in the cell. Mesosome is a system of cell membrane tubules present in many bacteria. It may perhaps serve as a membrane reserve in A. chroococcum. The electron micrographs in figure 10 also seem to show the absence of mesosomes in cells with a large number of membrane vesicles.

Also, more membrane vesicles are seen at higher dilution rates

than at lower dilution rates (figure 10). This is somewhat contradictory to the results of Pate et al., (1973) who observed that more membrane vesicles were present in cells growing in the late exponential phase than in cells in the early exponential phase. In continuous culture the growth at low dilution rates may be considered as approaching late exponential phase because the cells remain much longer in the culture. Pate et al. suggested that the cells at late exponential growth produced more membrane so as to get more oxygen to maintain themselves in the exponential growth phase. However, they did not further justify their suggestion.

Since these membrane vesicles are related to respiration, the possibility of their relating to ATP production was investigated under the electron microscope. Knob-like structures were found around the interior of the membrane invaginations by negative staining technique (figure 15B). Whether these knob-like structures are related to the knob-like ATPase particles on the cristae of the mitochondrion of eukaryote was not determined.

Therefore, phosphorus-limited nitrogen-fixing A. chroococcum has respiratory protection. This highly aerobic respiration rate can be looked on as a means of generating an anaerobic environment. The requirement of an anaerobic environment for many cellular functions is well illustrated by the nitrogen-fixing process. Inside nitrogen-fixing root nodules the oxygen tension is 10 nM. In nodules this very low oxygen tension is achieved by high respiration and is indicated by the absorption spectrum of leghaemoglobin. In A. chroococcum a high respiration rate may well achieve the same low oxygen tension

but there is no method known of measuring the oxygen tension within a single Azotobacter cell, yet the low oxygen tension in nitrogen-fixing nodules makes such a low oxygen tension a reasonable possibility. This low internal oxygen tension would require an excess of carbon substrate (e.g. mannitol) and a reasonably low oxygen supply to the cell. Higher oxygen tensions would result in a less efficient growth yield, a state of affairs indicated by a higher R.I. as has been shown in this thesis.

The use of a vortex stirring rate which keeps the dissolved oxygen in solution at very low concentrations in the chemostat in our laboratory has enabled us to take a more direct look into the effect of oxygen on the nitrogen-fixing process in Azotobacter.

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