THE EFFECTS OF AMMONIUM IONS AND OXYGEN ON THE ULTRASTRUCTURE OF *AZOTOBACTER CHROOCOCCUM*

BY

DOROTHY E. DAWSON

A Thesis

submitted to

the Faculty of Graduate Studies and Research

UNIVERSITY OF MANITOBA

In partial fulfillment

of the requirements for the degree of

MASTER OF SCIENCE

FEBRUARY, 1977

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TO MY FAMILY

ABSTRACT

The ultrastructure of Azotobacter, as revealed by electron microscopy, has been examined in bacteria grown under differing environmental conditions. Azotobacter chroococcum (ATCC 7493), grown in an intensely agitated continuous culture ("vortex aeration", Hine and Lees, 1976), was used throughout the course of the investigation. Cells were supplied with a gas phase containing 5%, 20% or 30% oxygen and grown under nitrogen-fixing conditions or in the presence of various concentrations of NH_4^+. Each culture was allowed to stabilize for 3 days, thus achieving a steady state, before a sample was taken and prepared for electron microscopy. The bacteria were examined for general changes in their ultrastructure (in particular, the appearance of peripheral vesicles) that were correlated with changes in their culture conditions. It was noted that the concentration of oxygen as well as the concentration of NH_4^+ supplied to the culture had a marked effect on the number and distribution of such vesicles in the cell. In general, the number of these vesicles increased with the supply of both NH_4^+ and oxygen. The cytological localization of catalase was also determined. The addition of silver nitrate (10^{-4}M) to the nitrogen-fixing cultures, supplied with 5% and 20% oxygen, just prior to their preparation for electron microscopy labelled a distinct structure within the cells. A nitrogen-fixing culture grown in the presence of 20% oxygen and 2.0 ppm potassium tellurite produced cells in which the vesicles were in sharper contrast with the cytoplasm than they were in the control cultures.

ACKNOWLEDGEMENT

I would like to extend my gratitude to Dr. H. Lees for his encouragement and guidance during the course of this investigation and for his assistance during the preparation of this manuscript. I also wish to thank Mrs. Rosalie Gillespie for training me in the techniques of electron microscopy.

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LIST OF ABBREVIATIONS

AT	3-amino-1,2,4-triazole
DAB	3,3'-diaminobenzidine
Propanediol buffer	2-amino-2-methyl-1,3-propanediol
R.I.	Respiratory Index

INTRODUCTION

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During a study of Azotobacter cysts, Wyss, Neumann and Socolofsky (1961) reported the presence of "peripheral bodies" in the vegetative cells. Since these structures disappeared as encystment proceeded and reappeared upon cyst germination, these "peripheral bodies" may have served primarily to extend the cell membrane, thus increasing the surface area available to respiratory enzymes and permitting the high Q_{O_2} values characteristic of Azotobacter. All available evidence suggests that the high oxygen uptake of Azotobacter is related first to a necessarily high ATP production needed to fuel the nitrogenase activity of the cells and secondly with an oxygen-requiring "respiratory protection" mechanism that prevents oxygen access to the oxygen-sensitive nitrogenase within the cell. In these studies the cells were cultured on petri plates and were thus exposed to high (20%) oxygen throughout the experiment (Wyss et al., 1961). In 1970, Oppenheim and Marcus reported a correlation between the nitrogen source used for growth and the ultrastructure in Azotobacter vinelandii. An extensive internal membranous network was present in the cells that were grown under the nitrogen-fixing conditions whereas cells that had been provided with fixed nitrogen (eg. ammonia and amino acids) had a very much reduced membrane system confined to the cell periphery. These observations were explained in two ways: i) the presumed respiratory activity of the additional membranes may be required by the nitrogen-fixing cells to protect the nitrogenase from inactivation by oxygen ii) the extended membrane structure is necessary to contain the nitrogenase complex, since the nitrogenase generally accepted as being particulate, may be embedded in, or be a part of, the membrane.

The cells used for these experiments were grown in batch culture and were harvested in the exponential growth phase. Hill, Drozd and Postgate (1972) studied the effect of nitrogen and ammonia on A. chroococcum grown in continuous culture. They reported results comparable to those of Oppenheim and Marcus (1970) and also noted the nitrogen-fixing organisms contained 70% more phospholipid than the cells grown in the presence of NH_4^+. These results led Hill et al. (1972) to believe that the membrane content was related to the level of nitrogenase present and not to the respiratory activity. In 1973, Pate, Shah and Brill published results contrary to those reported by Oppenheim and Marcus (1970). Using batch cultures of A. vinelandii they found no difference in the extent of vesicle formation between the nitrogen-fixing cells and the cells which were provided with fixed nitrogen (NH_4Cl, NH_4acetate or $NaNO_3$). However, it was noted that cells harvested from cultures in late exponential growth contained more internal membranes than cells harvested in early exponential growth. This evidence led to the suggestion that it was unlikely that the sole function of the membranes was to protect the nitrogenase from oxygen inactivation. Pate et al. (1973) therefore proposed that the membranes were synthesized in response to decreasing dissolved oxygen tension, increasing the surface area so that enough oxygen could be taken up to allow the culture to remain in the exponential phase. Several other workers have noted the appearance of internal membrane systems and vesicle formation in Azotobacter but there has been no agreement as to what growth conditions were required for the formation of internal membranes or vesicles or what purpose such structures serve.

Recently a technique has been developed in this laboratory that

allows A. chroococcum to be grown in high cell densities without becoming oxygen-limited. This was achieved by stirring the cultures at vortex agitation rates of 1750 rpm. (Hine, 1975; Hine and Lees, 1976). This technique is being used to study the "long term effect of NH_4^+ ions on the enzyme pattern of A. chroococcum subjected to various concentrations of NH_4^+ in the medium and to various concentrations of oxygen in the gas phase" (Tsim, 1976).

Since differences in the biochemistry of nitrogen-metabolism had been detected under the various culture conditions (Dalton and Postgate, 1969 a&b; Drozd and Postgate, 1970 a&b; Drozd, Tubb and Postgate, 1972; Hill et al., 1972; Lees and Postgate, 1973) it was of interest to determine whether or not these differences were also reflected in the cytology of the organisms grown under vortex stirring. A systematic approach, using nitrogen-fixing conditions and multiple NH_4^+ concentrations in the medium while varying the oxygen concentration within the gas phase, had not previously been employed in determining the correlation of the ultrastructure in Azotobacter with the conditions of growth. It was in order to establish such a correlation, if any existed, that the work described in this thesis was begun.

HISTORICAL

HISTORICAL

Nitrogen, essential for the synthesis of proteins and nucleic acids, is a critical element in the metabolism of all living organisms. However molecular nitrogen (N_2), the predominant component of our atmosphere, is in a chemically inert form inaccessible to animals and uninfected plants (Lehninger, 1971).

The ability of legumes to enrich the soil has been exploited since the days of the Romans but it was not until the 19th century that scientists attempted to determine what set legumes apart from other plants. The result of this experimentation was the discovery of the biological process, nitrogen fixation, (Hellriegel and Wilfarth, 1888) which for a long time has been under increasingly intense investigation (Burns and Hardy, 1975).

The existence of nitrogen-fixing microorganisms has been established since 1892. In 1894 Winogradsky was able to isolate and characterize an anaerobic bacterium, Clostridium pasteurianum, which could use atmospheric nitrogen as its sole source of nitrogen (Dalton and Mortenson, 1972). Since this time it has become known that the ability to fix nitrogen is confined to prokaryotes (Postgate, 1974), but the property is widespread amongst these organisms since aerobes, facultative anaerobes, and anaerobes are represented (Burns and Hardy, 1975). Associations between plants and bacteria (e.g. legumes and rhizobia) and photosynthetic bacteria (cyanobacteria) are the dominant contributors to biological nitrogen fixation but nevertheless it is the studies with the free-living, non-photosynthetic organisms such as Azotobacter and Clostridium that have yielded the bulk of the information on the bio-

chemistry of nitrogen fixation (Dalton and Mortenson, 1972).

Interest in free-living, nitrogen-fixing microorganisms has been shown since the late 19th century (Mulder, 1975) but most of the understanding of the mechanism of nitrogen fixation by microbes has come in the last 16 years (Dalton and Mortenson, 1972). Before 1960, whole cells had been used to study the mechanism of nitrogen fixation and these studies had determined the need for micronutrients, especially Mo and Fe, by nitrogen-fixing organisms and had allowed the discovery and subsequent study of the inhibition of nitrogen fixation by CO, H_2, NO and N_2O. A correlation between hydrogenase and nitrogenase activity was also established (Burns and Hardy 1975). It was concluded that ammonia was probably the end product of nitrogen fixation but attempts to detect intermediates in the conversion were unsuccessful (Wilson, 1971). However, the use of whole cells severely limited the type of experiment that could be conducted. The biochemical breakthrough came in 1960 when Carnahan, Mortenson, Mower and Castle (1960) were successful in isolating cell-free preparations that consistently supported vigorous nitrogen fixation. The path was now laid for an enzymological study and accelerated progress in the understanding of the mechanism of nitrogen fixation.

The nitrogenase enzyme complex, first isolated from C. pasteurianum by Carnahan et al. (1960) has proved, fortunately, to be similar in all nitrogen-fixing organisms and to consist always of two metalloproteins: i) a tetrameric Mo-Fe protein which contains Mo, non-haem iron and acid-labile sulphur ii) a dimeric Fe protein which contains non-haem iron and labile sulphur (Dalton, 1974; Dalton and Mortenson, 1972; Eady and Postgate, 1974; Zumft and Mortenson, 1975). Although this is true

of all nitrogenase systems, the proteins from one organism are not necessarily identical with the proteins from a different organism.

The Fe protein is cold labile and shows a greater sensitivity towards oxygen than does the Mo-Fe protein (Burns and Hardy, 1975). The nitrogenase complex is responsible for catalyzing the reduction of the nitrogen atom from an oxidation number of zero (N_2) to an oxidation number of -3 (NH_3) (Zumft and Mortenson, 1975).

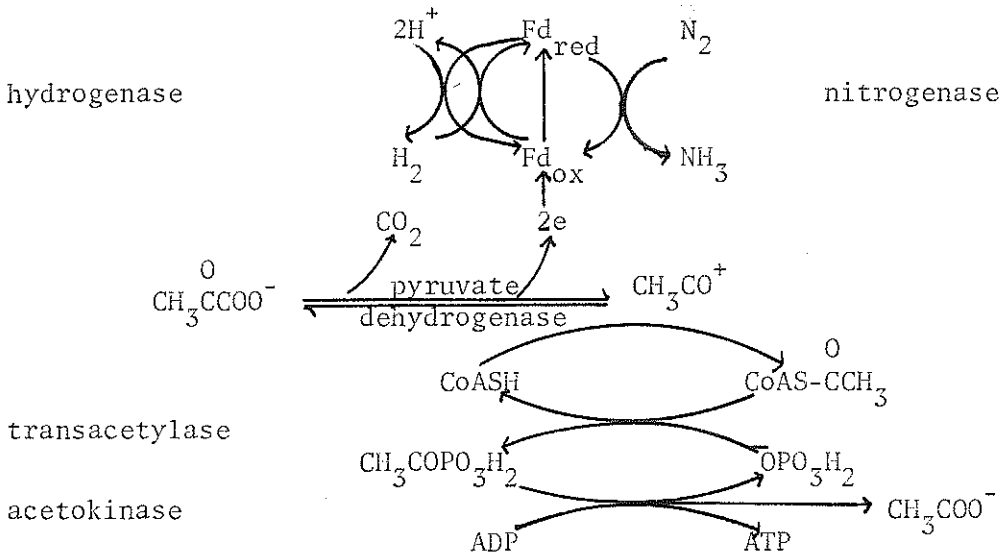
According to Kleiner and Chen (1974) the Mo-Fe protein of Azotobacter (M.W. 216,000) consists of 1 type of subunit of 56,000 daltons and contains 1.54 Mo atoms and 2.85 labile sulphur atoms per subunit. The Fe protein is comprised of two equivalent subunits of 33,000 daltons each which includes 3.45 Fe atoms and 2.85 labile sulphur atoms per subunit. It should be noted that nitrogenase components from one organism will often crossreact with components from other organisms to give functional enzymes (Eady and Postgate, 1974).

In order that the reduction of a nitrogen molecule may be accomplished, certain precise requirements must be met. Both components of the nitrogenase complex must be present as neither of the metalloproteins has any nitrogenase activity alone (Zumft and Mortenson, 1975). ATP and Mg^{++} are necessary (Dalton, 1974) but since ADP inhibits the functioning of nitrogenase, and ADP and inorganic phosphate are the products of ATP utilization by nitrogenase, optimal nitrogenase activity is obtained only when a system generating ATP from ADP (e.g. ADP, Mg^{++}, acetylphosphate and ATP:acetate phosphotransferase) is used (Dalton and Mortenson, 1972). Reduced ferredoxin probably serves as the source of reductant <u>in vivo</u> (Yates, 1972) although sodium dithionite will serve as an artificial electron donor <u>in vitro</u> (Bulen, Burns and LeComte, 1965).

The reaction must be carried out under strictly anaerobic conditions and N_2 supplied as the substrate (Dalton and Mortenson, 1972).

No detectible intermediates are formed during the reduction (Eady and Postgate, 1974) although NH_3 is the end product (Newton, Borysko and Swerdlow, 1953; Zelitch, Rosenblum, Burris and Wilson, 1951) nor are possible intermediates (nitramide, diimide, hydrazine, hyponitrite or hydroxylamine) reduced or used as substrates in the place of N_2 (Burris, 1969). The enzyme complex will, however, reduce various triply bonded substrates such as acetylene, cyanide, isocyanide and nitrous oxide (Dalton and Mortenson, 1972; Eady and Postgate, 1974). H_2, CO and N_2O can serve as inhibitors of nitrogen fixation (Burris, 1969).

The reduction of $N_2 \longrightarrow NH_3$ is associated with a reductant-dependent ATPase activity (e. g. ATP hydrolysis requires a hydrogen-donating system) as well as an ATP-dependent hydrogenase. If a substrate (N_2) is not available, the nitrogenase will produce H_2 in the presence of ATP.



(Dalton, 1974)

In the presence of nitrogen, the nitrogenase will generate H_2 gas at a lower level since the electrons are being used to reduce N_2. CO is able to inhibit all reactions except this ATP-dependent hydrogenase function of nitrogenase (Dalton, 1974; Eady and Postgate, 1974).

Since 1927, it has been realized that growing cultures of nitrogen-fixing Azotobacter spp. displayed a marked sensitivity towards excess oxygen (Meyerhof and Burk, 1928; Postgate, 1971). Wilson (1958) reported high Q_{O_2} values of about 4000 for Azotobacteriaceae and regarded this unusually high respiration rate as a protective mechanism to prevent access of oxygen to nitrogenase. High maintenance coefficients at low oxygen tensions were also found to exist (Aiba, Nagai, Nishizawa and Onodera, 1967 a&b; Phillips and Johnson, 1961). Phillips and Johnson (1961) suggested that Azotobacter could use its respiration as an "O_2-wasting" system to maintain a low intracellular Eh.

To retain enzymatic activity, purification of the nitrogenase components from C. pasteurianum preparations must be always conducted strictly anaerobically (Bulen and LeComte, 1966; Kelly, Klucas and Burris, 1967). The in vitro reduction of nitrogen by an extract of A. vinelandii was obtained in 1964 (Bulen, Burns and LeComte, 1964). Contrary to the crude extract of Clostridium, the Azotobacter nitrogenase extract prepared from cells disrupted by a French pressure cell was stable in air and sedimented easily, suggesting it was particulate (Bulen, Burns and LeComte, 1964; Hardy and Knight, 1966). Oxygen sensitivity was not realized until the components were further purified (Bulen and LeComte, 1966; Klucas and Burris, 1967).

Using A. chroococcum grown in batch and continuous culture, Dalton and Postgate (1969a) noted that cultures supplied with a fixed source of

nitrogen showed no unusual sensitivity to oxygen whereas cultures fixing nitrogen were sensitive to oxygen, especially those which were carbon- or phosphate-limited.

These observations together with those by other workers, led Dalton and Postgate (1969a) to propose a "working hypothesis" of a respiratory and a conformational protection. These two mechanisms would then restrict oxygen access to the nitrogen-fixing site. The nitrogenase found in actively growing, nitrogen-fixing cultures of Azotobacter is in a form susceptible to oxygen damage. To protect the enzyme complex from oxygen inactivation under these conditions, the respiration rate of the cell is increased, allowing the oxygen to be scavenged from the nitrogen-fixing sites (Drozd and Postgate, 1970b). However, the enhanced respiration rate is not accompanied by an increase in the intracellular ATP concentration (Jones, Brice, Wright and Ackrell, 1973). This process which wastes substrate in order to consume potentially damaging oxygen has been termed respiratory protection.

If respiratory protection is insufficient to protect the nitrogenase the Azotobacter cells are able to shield their nitrogenase from oxygen damage by physically orientating it in such a manner that the oxygen-sensitive components are inaccessible to oxygen and are conformationally protected (Hill et al., 1972). This "conformational protection" is reversible.

The terms "switched-off" and "switched-on" refer to the ability of the cell to inactivate its nitrogenase rapidly and reversibly. When a nitrogen-fixing culture is vigorously shaken in air, the acetylene-reducing activity of the nitrogenase is lost or "switched-off" because under these circumstances respiratory protection is no longer sufficient

to shield the nitrogenase from oxygen damage. If the culture is now gently shaken in air respiratory protection can take over and the acetylene-reducing activity of the nirogenase is restored or "switched-on" (Drozd and Postgate, 1970).

Respiratory protection is usually able to permit Azotobacter to grow satisfactorily at any point within a fairly wide range of oxygen supply rates as these nitrogen-fixing organisms are able to respire away the excess oxygen through high respiratory activity using increased amounts of the carbon substrate. A high aeration rate of a high pO_2 will cause nitrogen fixation to become very inefficient in terms of the carbon substrate consumed per unit N_2 fixed. A breakdown of respiratory protection will occur if the oxygen supply is too great, the supply of organic carbon is too little or the culture is subjected to phosphate limitation (why phosphate limited cells behave in this way is not clear). The second system, conformational protection, takes over when respiratory protection can no longer protect the nitrogenase from oxygen inactivation. In this state the nitrogenase is temporarily inactive and inaccessible to oxygen (Dalton and Postgate, 1969a).

In 1970, Oppenheim and Marcus reported that an extensive internal membranous network was present in A. vinelandii when the cells were fixing nitrogen; the network was greatly reduced and appeared primarily at the cell periphery when the cells were grown in the presence of a fixed nitrogen source such as ammonium or nitrate ions. If the nitrogenase system was extracted from such nitrogen-fixing cells by osmotic lysis, it behaved similarly to the nitrogenase of C. pasteurianum and showed oxygen lability in contrast to the extract obtained by the French pressure cell which was particulate and oxygen stable (Oppenheim,

Fisher, Wilson and Marcus, 1970).

These observations seemed to suggest that nitrogenase could be structurally modified to protect the nitrogenase from oxygen inactivation (Dalton and Postgate, 1969a). It was therefore proposed that the internal membranes surround the soluble nitrogen-fixing enzyme system and that the respiratory enzymes contained in the membranes maintain the conditions conducive to nitrogen fixation. The membrane system could be analogous to other structures such as heterocysts and root nodules which allow an aerobic organism to carry out an anaerobic process. If this were so then conformational protection might be no more than the operation of an internal respiratory protection mechanism closely associated with the nitrogenase molecules themselves.

It was felt that this model complemented the earlier proposal of respiratory and conformational protection by Dalton and Postgate (1969 a&b). Indeed, the idea was incorporated by Postgate and his colleagues (Hill et al., 1972). They saw the internal membranes as being the site of conformational protection, viewing the amount of membrane present as being related to the nitrogenase content and not to respiratory activity. The particulate (encapsulated) form of the nitrogenase might then correspond to the "switched-off" state while the soluble, oxygen sensitive form would correspond to the "switched-on" state suggested by Drozd and Postgate (1970b).

Further studies by Postgate and his colleagues have provided more evidence to support their hypothesis. It is generally recognized that most aerobic nitrogen-fixing bacteria and cyanobacteria that have been studied fix nitrogen optimally at sub-atmospheric pO_2 values (Postgate, 1971). If the rate of nitrogen-fixation by an aerobic culture grown in

air is assessed against a range of pO_2 values by the acetylene test, a bell-shaped curve results (Biggins and Postgate, 1969; Drozd and Postgate, 1970a; Stewart, 1969).

Drozd and Postgate (1970b), using A. chroococcum, demonstrated that the shape of the curve varied when a culture adapted to a low pO_2 and a culture adapted to a high pO_2 were subjected to various pO_2 values. The higher the Q_{O_2} of the culture, the greater the oxygen concentration the population could withstand before inhibition of acetylene reduction appeared.

Lees and Postgate (1973) demonstrated that phosphate-limited, nitrogen-fixing cultures of A. chroococcum exposed to oxygen stress for several hours would not grow on nitrogen free medium but remained perfectly viable if tested on a medium containing NH_4^+; this suggested that the primary damage caused by oxygen stress was confined to the nitrogenase systems of the cells. Earlier, Dalton and Postgate (1969a) indicated oxygen stress had a lethal effect on such cultures but had not included NH_4^+ in the medium used for the viability tests. These findings suggest the nitrogenase, or a component closely associated with it, was affected by the oxygen stress, providing evidence for respiratory protection as advanced by Postgate and his co-workers (Hill et al., 1972).

However, Kurz, LaRue and Chatson (1975) have put forth the idea that the "switch-off, switch-on" phenomenon described by Dalton and Postgate (1969 a&b) may not be an oxygen protection mechanism but may be due to the sensitivity of the nitrogenase to changes in the environment since an addition of $5x10^{-2}$M ammonium acetate or potassium acetate will initially give comparable drops in nitrogenase activity.

Unlike the results of Oppenheim and Marcus (1970), those of Pate et

al. (1973) showed no difference in the quantity of intracellular membrane present and suggested the magnitude of the network may reflect the availability of oxygen. Under limited oxygen conditions more membrane material could be synthesized, increasing the surface area available to take up oxygen and to allow the culture to remain in the exponential part of the growth curve.

Reed, Toia and Raveed (1974) reported the purification of nitrogenase containing "azotophore" membranes distinct from the cytochrome containing respiratory membranes. These "azotophore" membranes may well be the same as, or similar to, the membranes seen by Oppenheim and Marcus (1970). This indicated that the azotophores may exist as small vesicular membranes that are independent of the respiratory membranes. Perhaps the azotophore membranes provide protection against oxygen inactivation of the nitrogenase. Reed et al. (1974) have defined azotophores as being small nitrogenase containing membranes that have been isolated from A. vinelandii.

During a study of Azotobacter cysts, Wyss et al. (1961) reported a network of invaginations in the cell membrane. These structures disappeared as encystment proceeded but reappeared upon cyst germination. The authors suggested that perhaps the invagination (peripheral bodies) extended the cell membrane and supplemented the respiratory enzymes thus giving rise to the high Q_{O_2} values that characterize Azotobacter. However this suggestion, although logical and reasonable, has so far received little firm biochemical support.

Some aspects of continuous culture

The development of microbial populations in batch cultures is self-

limiting either through the depletion of the available nutrients or through the accumulation of toxic metabolic products. Four distinct stages are recognized under such growth conditions; the lag phase, exponential, stationary, and death phases (Stanier, Doudoroff and Adelberg, 1970).

All continuous cultures begin as freshly inoculated batch cultures (Stanier et al., 1970; Tempest, 1970) and once the growth medium has been inoculated the lag phase persists until the cells can resume division and proceed into the exponential phase. One can now start the influx of fresh medium and by controlling the influx rate maintain the cell population in a steady-state condition of exponential growth. This method avoids the stationary phase and eliminates the death phase inherent in batch cultures and, more importantly, provides a continuous supply of cells that is of constant overall composition and also in a predetermined physiological state suitable for experimental use (Stanier et al., 1970).

The steady-state of the system depends upon the fact that one substrate alone in the supply medium is made to be the "growth-limiting factor" and it is therefore important when dealing with aerobic organisms to make sure that the culture is adequately aerated so there is no unsuspected oxygen limitation (Herbert, Elsworth and Telling, 1956).

Once the cells have entered the exponential growth phase, dilution of the culture vessel can begin. The rate of dilution can be expressed as w/v; where "w" = influx rate in ml/hr and "v" = volume of the culture in ml; this dilution rate is commonly written as D and has the dimensions of t^{-1} (usually hr^{-1}). The size of the population within the culture vessel is subjected to two opposing factors once dilution has begun; i) a constant increase in the population due to growth ii) a constant

decrease in the population caused by the outflow of culture from the culture vessel since the culture volume is kept constant (Stanier et al., 1970).

The rate of these two opposing factors can be described mathematically.

$xt = \mu x$ growth equation

$\frac{dx}{dt} = \mu x$ instantaneous growth rate of the population

$\frac{dx}{dt} = -Dx$ rate of loss of cells by washing out

therefore

$\frac{dx}{dt} = \mu x - Dx = (\mu - D)x$ the net rate of change of the population size within the culture vessel

where

x = the concentration of organisms (mgm/ml)

μ = the instantaneous growth rate constant (hr^{-1})

D = the dilution rate

When increasing the dilution rate, one must bear in mind the growth-rate constant, μ, which can not exceed a certain maximal value, μ_{max}. The actual growth rate is given as

$$\mu = \mu_{max} \frac{S}{K_s + S}$$ Herbert et al., 1956; Tempest, 1970)

where

μ_{max} = the maximum value of μ (e.g. when S is no longer growth limiting)

S = the concentration of the growth-limiting substrate

K_s = the saturation constant (equal to S at $0.5\mu_{max}$), exactly analagous to K_m in the Michaelis - Menten enzyme equation, that is characteristic of a particular substrate and the organism under consideration.

If D, the dilution rate, exceeds μ_{max} the cells will not be able to maintain themselves within the culture vessel and wash out will result (dx/dt will be negative). By restricting D to a submaximal value of μ, dx/dt will be positive and the population size will increase (Stanier et al., 1970). This increase in population will cease when some component of the inflowing medium becomes limiting. A decline in the growth rate will be noted until the growth rate equals the dilution rate. At this point a steady state will have been reached as the bacterial growth is self-regulating, keeping the size of the population within the culture vessel constant.

$$\frac{dx}{dt} = 0$$ (Herbert et al., 1956; Tempest, 1970)

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

A constant dilution rate determines the rate of influx of fresh medium into the culture vessel and thus the steady-state concentration of the limiting nutrient which is inversely related to the population within the culture vessel.

If we call the steady state value of the growth limiting nutrient "$\bar{s}$", then a rearrangement of the last equation shows

$$\bar{s} = K_s \frac{(D)}{\mu_{max} - D}$$ (Herbert et al., 1956; Tempest, 1970)

Over any finite period of time during the exponential growth phase

$$Y = \frac{\text{the weight of organisms produced}}{\text{the weight of substrate consumed}}$$

where

Y = the yield factor

If the relation between the rate of growth and of substrate comsumption is constant (Monod, 1942) it can be expressed as

$$\frac{dx}{dt} = -Y \frac{ds}{dt}$$ (Herbert et al., 1956; Tempest, 1970)

Therefore, for any given dilution rate, the concentration of organisms, $\bar{x}$, is given by

$$\bar{x} = Y(S_r - \bar{s}) = Y\left[S_r - K_s \frac{(D)}{\mu_{max} - D}\right]$$ (Herbert et al., 1956; Tempest, 1970)

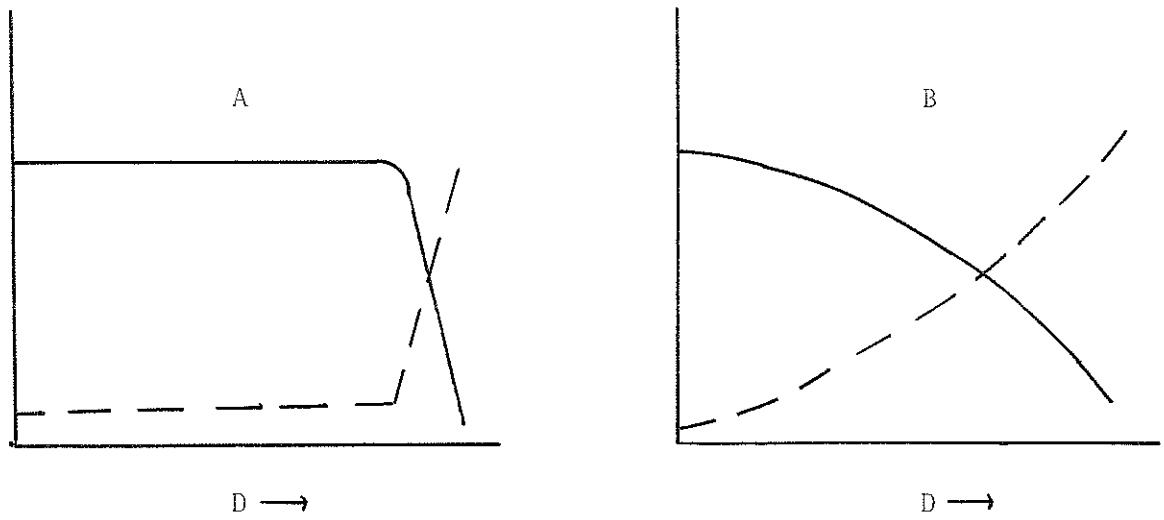
where

S_r = the concentration of the substrate supplied to the culture from a reservior

$\bar{s}$ = the concentration of the substrate within the culture

Since μ_{max}, K_s and Y may all be assumed to be constant over a fairly wide range of dilution rates, the main effect of varying the dilution rate is to alter $\bar{s}$, the growth-limiting substrate concentration within the culture. Since $\bar{s}$ is directly proportional to μ, the specific growth rate of the organisms will be affected by D (Tempest, 1970).

These equations indicate when K_s is small relative to S_r, varying the dilution rate will yield curves of type A. When K_s is large relative to S_r, a curve represented by type B will result.



(Herbert et al., 1956; Tempest, 1970)

The above derivations are illustrative, not rigid, and contain several unexpressed assumptions especially at low and high dilution rates.

The mean generation time, G, under steady-state conditions in a chemostat can be derived from the dilution rate (Stanier et al., 1970)

$$\mu = \frac{w}{v}$$

$$G = \frac{1}{k} = \frac{1}{0.69\mu} = \frac{V}{0.69w}$$

where

$0.69 = \ln 2$

$\frac{w}{v}$ = the flow rate

μ = the instantaneous growth rate

$\frac{1}{k}$ = the mean doubling time

Therefore, every time a volume equal to 0.69V flows through the culture vessel when the culture is in a steady state the population within the vessel doubled.

Electron microscopy

In 1876 Ernest Abbe showed that the maximum resolution in light microscopy had been achieved because no matter how perfect the physical optics may be the ultimate resolution depends, in practice, on the wavelength of the light used. In theory this resolution can be enormously increased if electrons, which may be considered to have a wavelength inversely related to their momentum, are used to replace photons as a structural probe. However, it was not this realization that was responsible for the development of the electron microscope, it was the indirect result of an attempt to understand the cathode ray tube in respect to geometric optics (Mulvey, 1967).

The construction of the first commercially built electron microscope, the Metropolitan Vickers E.M. 1, began in 1935. Because this machine was equipped with a turntable mechanism that allowed viewing of the specimen both by an electron beam and by a light beam (to reassure the operator he was not being misled by the instrument) the resolving power did not surpass that of the optical microscope. However, this instrument served a useful purpose since it was no longer necessary to include an optical microscope in subsequent electron microscopes. Therefore work was now able to proceed unhindered on the development of a production electron microscope that was capable of achieving high resolution. Such a microscope was built in 1938, based on the design of von Borries and Ruska (Mulvey, 1967).

To ensure the effient use of the electron microscope, it was necessary to improve the earlier histological techniques. Attempts at ultrathin sectioning were unsuccessful until 1948 when Pease and Baker used the plastic "Parlodion" in combination with a carnauba-paraffin

wax mixture which was sufficiently hard to yield usable sections. Subsequently Newman et al. (1949) introduced a butylmethylacrylate embedding technique which became the standard method since the embedded specimen cut well and the polymer impregnated the tissues easily and reliably. Then the introduction of glass knives gave sections of much higher quality than steel knives (Lotta and Hartmann, 1950) and microtomy was further aided when the sections were floated away from the knife edge on a fluid surface (Gettner and Hillick, 1950).

Although osmium tetroxide fixatives were used initially, reasonably consistent results were not obtained until Palade in 1952 introduced a slightly alkaline buffered osmium tetroxide solution. Since then it has been noted that certain aldehydes coupled with osmium tetroxide as a secondary fixative serve as excellent cytological fixatives (Pease, 1964). The now classical technique uses osmic acid, versene buffer and uranyl acetate (Kellenberger, Ryter and Sechaud, 1958) as stain and fixative; this technique is the basis for most electron micrographs produced today.

The localization of catalase

While it seems inconceivable that oxygen, which is necessary to support aerobic growth, could regularly generate a substance (O_2^-, the superoxide anion) so toxic that all aerobic organisms have a defence mechanism against it, such is nevertheless the case (Fridovich, 1975). All aerobic organisms possess the enzymes superoxide dismutase, peroxidase and catalase to protect themselves from the toxic intermediates involved in the reduction of oxygen to water.

The superoxide dismutases, which are oxidoreductases, catalyze

the dismutation of O_2^- to H_2O_2.

$$2\ O_2^- + 2H^+ = H_2O_2 + O_2$$

This activity appears to be present in all respiring cells (Fridovich, 1975).

The haemoproteins, catalase and peroxidase, are responsible for the removal of H_2O_2 by reducing it to 2 H_2O.

Peroxidases (donor: hydrogen peroxide-oxidoreductase E.C. 1.11.1.7) catalyze the reduction of H_2O_2 using a reductant other than H_2O_2.

$$H_2O_2 + H_2R \longrightarrow 2\ H_2O + R$$ (Essner, 1974; Fridovich, 1975)

Catalases (hydrogen peroxide:hydrogen peroxide-oxidoreductase E.C. 1.11.1.6) are able to reduce H_2O_2 to H_2O using H_2O_2 as the reductant.

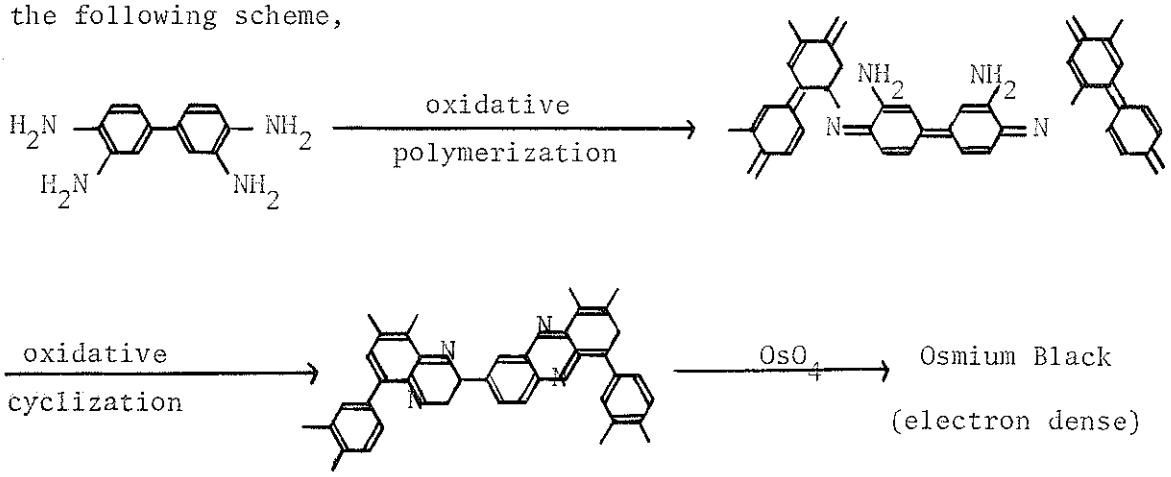
$$H_2O_2 + H_2O_2 \longrightarrow 2\ H_2O + O_2$$ (Essner, 1974; Fridovich, 1975)

In 1966 Graham and Karnovsky used 3,3'-diaminobenzidine (DAB) to show where the peroxidase activity was located in the tissue as viewed through the electron microscope. Before this time, benzidine (first employed by Fischel in 1910) had been used extensively to show the presence of haemoproteins by light microscopy. Mitsui, in 1960, had also used benzidine to localize the peroxidase activity at the ultrastructural level. However, benzidine proved to be an unsuitable substrate in electron cytochemistry since the deposits obtained after incubation in the benzidine medium were generally of irregular size and shape, and had a relatively low electron density (Goldfischer, 1969; Graham and Karnovsky, 1966a; Mitsui, 1960).

Oxidized DAB, easily seen in light and electron microscopy, has now been used extensively to localize haemoproteins in animal cells and

tissues (Essner, 1974), plant cells (Frederick and Newcomb, 1969) and in yeast (Van Dijken, Vienhuis, Vermeulen and Harder, 1975; Williams and Stewart, 1976).

The high resolution possible in the ultrastructural localization of haemoproteins is due to the unique properties of DAB. Graham and Karnovsky, (1966a) had noted the high electron density of the deposits and suggested that there was some reduction of OsO_4 by the reaction product (whose chemical structure was unknown). In 1968, Seligman, Karnovsky, Wasserkrug and Hanker suggested the opacity could be explained by the following scheme,



(Seligman et al., 1968)

in which the oxidized DAB was polymerized to form a noncoalescing, osmiophilic and amorphous macromolecule of high electron density. This product is not solublized during dehydration or by embedding agents, and exhibits minimal diffusion under the usual conditions of incubation (Essner, 1974).

The DAB medium which Graham and Karnovsky (1966a) developed was suited to localization of small amounts of peroxidase in cells. Novikoff and Goldfischer (1969) modified the original medium so that the presence of catalase could be detected. Optimal staining of catalase

required an alkaline pH, an elevated concentration of DAB and a increased incubation temperature ($37^{o}C$). Vigil (1969) also found high levels of H_2O_2 (0.06%) may sometimes be necessary to detect catalase. The alkaline pH is effective in dissociating the catalase into peroxidase subunits which are able to oxidize the DAB at neutral pH (Essner, 1974).

To confirm that the staining is due to catalase activity, a control with 3-amino,1,2,4-triazole (AT) has been used (Fahimi, 1968; Hirai, 1968; Novikoff and Goldfischer, 1969). AT, an effective inhibitor of catalase (Heim et al., 1956) binds irreversibly to the carboxyl groups in the active centre of the enzyme through an amide linkage. KCN is also used as a control since it inhibits staining due to both peroxidase and catalase (Novikoff and Goldfischer, 1969).

DAB is straw-coloured when freshly prepared but on standing at room temperature becomes increasingly darker due to autooxidation enhanced by formation of an intermediate and a partially oxidized end product, DAB oxide, which has an affinity for the active site of various haemoproteins (Hirai, 1968 & 1971). If incubation in the DAB medium extends for periods longer than the usual 60 minutes it is important that the medium be changed hourly, since little autooxidation occurs in medium exposed to air for less than 1 hour, this will prevent accumulation of the DAB oxide.

MATERIALS AND METHODS

MATERIALS AND METHODS

Organism

Azotobacter chroococcum (ATCC 7493) was used throughout the course of this study.

Growth and maintenance of the cultures

A modified nitrogen-free Burk's medium ("mannitol B_6") described by Dalton and Postgate (1966 a&b) was used as the medium for the continuous culture of the organism.

The continuous culture apparatus used was based on the design of Baker (1968) and subsequently modified as described by Hine (1975) and Tsim (1976). The nitrogen-fixing cultures were maintained at 27°C and a suitable gas mixture was supplied at a rate of 150 ml/min.

A sample of inoculum (approximately 50 ml) was aseptically added to a sterilized continuous culture vessel containing between 150-200 ml of medium and maintained at 30°C. The culture was gently stirred in a gas phase of air overnight to allow the organism to grow. The following morning the addition of fresh medium was begun at a very low dilution rate, the stirring rate was brought to vortex (1750 rpm) and sufficient nitrogen added to the gas phase to maintain a minimal dissolved oxygen tension of zero (0-3 μM) within the culture. The 200 ml culture was then left to adjust to the increased agitation rate and to grow. The amount of nitrogen added to the gas phase was decreased at intervals so that the dissolved oxygen tension within the culture would rise to approximately 10 μM oxygen. The culture was then allowed to return the dissolved oxygen

concentration to a nominal zero after which the procedure was repeated. Eventually the culture was able to maintain itself at full vortex stirring rate with a dissolved oxygen tension of zero even though the gas phase was air or 30% oxygen. The dilution rate was then gradually increased to the desired value. All cultures were allowed to stabilize for 3 days before they were sampled. Cultures grown in the presence of $(NH_4)_2SO_4$ were kindly supplied by A. Tsim, grown according to the method given in his MSc. thesis (1976).

Measurement of the dissolved oxygen concentration in the culture

A steam sterilizable galvanic oxygen electrode attached to an oxygen meter (both obtained from the L. H. Engineering Co. Ltd., Bells Hill, Stoke Poges, Bucks, England) was used to measure the dissolved oxygen concentration within the culture. The meter was connected to a Rustrak recorder (Rustrak Instrument Div., Gulton Industries Inc., Manchester, N. H., U.S.A.) which recorded the concentration of oxygen on chart paper set to move at 1 inch per hour. Calibration of the oxygen electrode was either done within the specially designed vessel used to house the electrode during sterilization and storage or within the culture vessel itself using the method described by Tsim (1976). The electrode was tested periodically for its sensitivity by aseptically raising it above the culture and noting if the needle responded correctly to the concentration of oxygen in the gas phase. If the sensitivity of the electrode had been lost it was replaced (aseptically) with a fresh electrode.

Sampling procedure

If a small sample was required, it was removed directly from the culture and used immediately. If larger volumes were necessary, the cells were collected overnight in a receiver set in ice and were used the following morning.

Chemicals

Unless otherwise indicated, reagent-grade chemicals obtained from standard chemical companies were used in all media and reagent-grade or EM-grade chemicals were used in all electron microscopy.

Carbon dioxide measurement

Determination of the carbon dioxide output was measured in the manner described by Lees and Postgate (1973).

Electron microscopy

Preparation of cells

If only a small volume of cells was required for fixation, a sample of the culture was taken directly from the culture vessel. However, when a large volume was necessary the cells were collected overnight in a receiver set in ice.

Fixation procedure

Hess Method (Hess, W.M. 1966)

a) The cells were collected by centrifugation and suspended in the

the fixation mixture which consisted of the following:

0.2M cacodylate buffer pH 7.4	5.0 ml.
distilled water	3.5 ml.
25% glutaraldehyde	1.2 ml.
acrolein	0.3 ml.

The sample was then allowed to fix for 4 hours in the cold.

b) Wash twice, 15 mins. each in 0.1M cacodylate buffer pH 7.4.

c) The suspension was centrifuged at 1,000xg. for 5 mins. and the pellet was mixed with warm agar. The agar was then spread onto a glass slide and allowed to harden. This suspension was cut into tiny cubes.

d) Wash twice, 15 mins. each in 0.1M cacodylate buffer pH 7.4.

e) Fix for 4 hours in 1% OsO_4 in 0.1M cacodylate buffer pH 7.4 in the cold.

f) Wash 4 times, 15 mins. each in 0.1M cacodylate buffer pH 7.4.

g) Place in 0.5% aqueous uranyl acetate overnight.

h) Wash once in distilled water for 5 mins.

Dehydration

The specimens were dehydrated by allowing them to stand in 50%, 70% and 90% ethanol solutions for 10 mins. each and in 2 changes of absolute ethanol for 30 mins. each.

Embedding

ERL-4206 (Vinyl Cyclohexene Dioxide) - based Epoxy Resin (Spurr, 1969)

ERL-4206 (Vinyl cyclohexene dioxide)	10 gms.
D.E.R. 736 (Diglycidyl ether of propylene glycol)	6 gms.

NSA (Nonenyl succinic anhydride)	26 gms.
S-1 (Dimethylaminoethanol of DMAE)	0.4 gms.

The first three compounds were added in order and mixed thoroughly. The solution was allowed to stand until any air bubbles had surfaced (15-20 mins.) and then the DMAE was added. This mixture will be referred to as "Spurr".

a) The specimen was placed in a small volume of absolute ethanol to which an equal volume of "Spurr" was added. This was mixed thoroughly and allowed to stand 30 mins.

b) Double the volume with fresh "Spurr"
Mix thoroughly and allow to stand 30 mins.

c) Decant and add a fresh volume of "Spurr"
Allow to stand 30 mins. Repeat at least once more.

d) Decant and add a fresh volume of "Spurr"
Allow to stand 2 hours.

e) Decant and add a fresh volume of "Spurr"
Allow to stand overnight.

Polymerization procedure

a) The capsules were filled 3/4's full with "Spurr" and an agar cube was added to each.

b) The filled capsules were allowed to stand for 12 hours.

c) The samples were allowed to polymerize in a vacuum oven at 60^{o}C for 12 hours.

After the capsules had cooled, silver-gray sections were cut on a Reichert OM U2 ultramicrotome using a glass knife and were mounted on 300 mesh copper grids.

Post-staining

The stain used was lead citrate prepared according to Reynolds (Reynolds, 1963).

Lead citrate	1.33 gms.
Sodium citrate	1.76 gms.
Distilled water	30.0 ml.

This mixture was shaken vigorously for 1 min. and intermittently for another 30 mins. 8.0 mls. of 1N NaOH was added to dissolve the precipitate. Distilled water was added to bring the volume to 50.0 ml.

The sections were post-stained in lead citrate under a nitrogen atmosphere for 3 mins., washed in distilled water and allowed to dry before they were viewed and photographed in an AEl model EM 6B electron microscope.

Staining of growing cells by silver nitrate

A 2.0 ml sample of 10^{-2}M $AgNO_3$ was aseptically added directly to the continuous culture. After a period of 30 minutes a sample of the culture was removed and prepared for electron microscopy as previously described.

Staining of growing cells by potassium tellurite

Medium containing 2.0 ppm potassium tellurite was supplied to a nitrogen-fixing culture grown in the presence of 20% oxygen. The culture was allowed to stabilize itself (3 days) before a sample was removed and prepared for electron microscopy as previously described.

Cytochemical localization of catalase

Fixation and incubation procedure

a) The cells were collected by centrifugation and suspended in 3% glutaraldehyde in 0.05M potassium phosphate buffer at pH 6.8. Fixation was at room temperature for 1 hour.

b) The suspension was then centrifuged and washed at least 3 times in 0.05M potassium phosphate buffer, pH 6.8, at room temperature in intervals of 15-20 mins.

c) The incubation method followed was basically that of Novikoff and Goldfischer (1968) as described by Beard and Novikoff (1969). The cells were collected by centrifugation and suspended in the DAB (3,3'-diaminobenzidine, Sigma Chemical Co.) incubation mixture. The standard incubation medium was prepared just prior to use and consisted of:

DAB	10 mg.
0.05M propanediol buffer (2-amino-2-methyl-1,3-propane-diol, Sigma Chemical Co.)	5 ml.
0.3% H_2O_2	0.1 ml.

This solution was filtered through a millipore filter to remove a reddish precipitate. The suspension was added to the incubation mix and incubated in the dark at 37°C for 2 hours while being gently agitated.

To determine if the observed activity was due to catalase several controls were necessary.

1) incubation in the standard DAB medium minus H_2O_2

ii) preincubation for 20 mins. in propanediol buffer containing 0.02M aminotriazole (3-amino-1,2,4-triazole, Sigma Chemical Co.) followed by incubation in standard DAB medium containing 0.02M AT

iii) preincubation for 20 mins. in propanediol buffer containing 0.01M KCN, followed by incubation in standard DAB medium containing 0.01M KCN

iv) incubation in the propanediol buffer only

(Frederick and Newcomb, 1969)

d) Wash 4 times, 15 mins. each in 0.05M phosphate buffer

e) The cells were then suspended in 3% glutaraldehyde in 0.05M potassium phosphate buffer, pH 6.8, for 3 hours in the cold.

f) Wash twice, 15 mins. each in 0.05M phosphate buffer, pH 6.8

g) The suspension was centrifuged and the pellet mixed with warm agar. The agar suspension was then spread onto a glass slide, allowed to harden and cut into tiny cubes.

h) Wash twice, 15 mins. each in 0.05M phosphate buffer, pH 6.8

i) Fix for 2 hours in 1% OsO_4 in 0.05M phosphate buffer, pH 6.8, in the cold.

j) Wash 4 times, 15 mins. each in 0.05. phosphate buffer, pH 6.8.

k) Place in 0.5% uranyl acetate overnight.

l) Wash once in distilled water for 5 mins.

The method of dehydration, embedding, polymerization and post-staining was the same as that described previously.

RESULTS

RESULTS

Growth of Azotobacter chroococcum

Azotobacter chroococcum was grown in a chemostat under nitrogen-fixing conditions or in the presence of various ammonium concentrations in the medium while being subjected to a gas phase containing 5%, 20% or 30% oxygen.

The results are subsumed under various ammonium concentrations and various percentages of oxygen in the gas supply according to the cultural conditions used.

The main difference in the electron micrographic appearance of cells grown under the different cultural conditions noted above lay in the absence or presence of small structures lying just under the cytoplasmic membranes of the cells. These small structures are referred to as "vesicles".

Nitrogen-fixing (no NH_4^+)

Regardless of the concentration of oxygen in the gas phase, the vesicles were always associated with the periphery of the cell. Cells grown under 5% oxygen contained a few randomly spaced vesicles whereas under 20% oxygen the vesicles were more numerous and more evenly placed. At 30% oxygen, the number of vesicles was greatest and their distribution was the most regular. (Figure 1 A-C)

FIGURE 1

A. chroococcum grown under nitrogen-fixing conditions and in a gas phase of

A. 5% oxygen

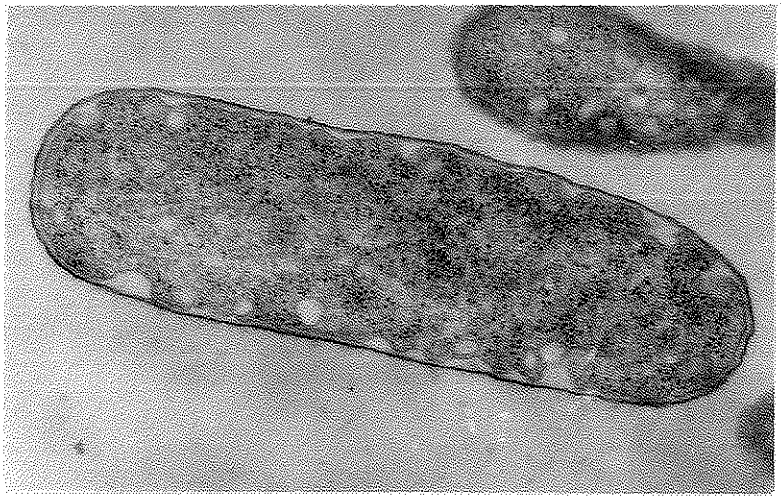
x 40,000

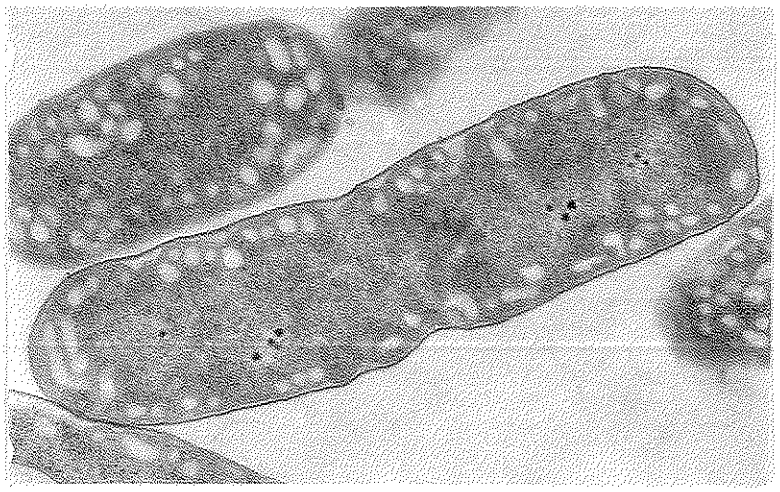
B. 20% oxygen

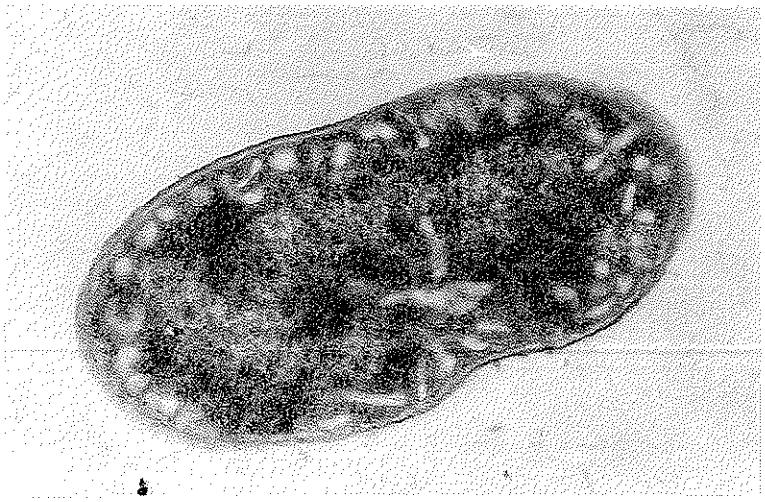
x 32,000

C. 30% oxygen

x 40,500







1 mM NH_4^+

The cells grown under 5% oxygen and 20% oxygen both contained vesicles around the periphery of the cell. The distribution of the vesicles within the 20% oxygen grown cells was very regular and dense. Cells grown under 5% oxygen contained fewer, more loosely spaced vesicles. (Figure 2 A&B)

FIGURE 2

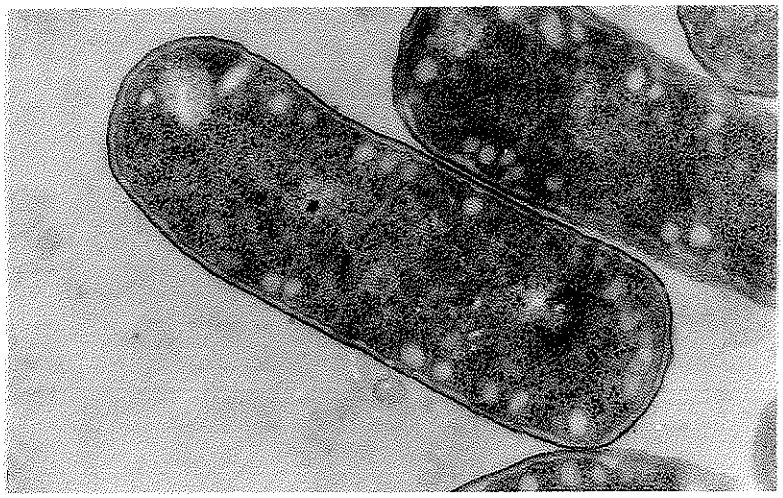
A. chroococcum grown in the presence of 1 mM NH_4^+ and under a gas phase of

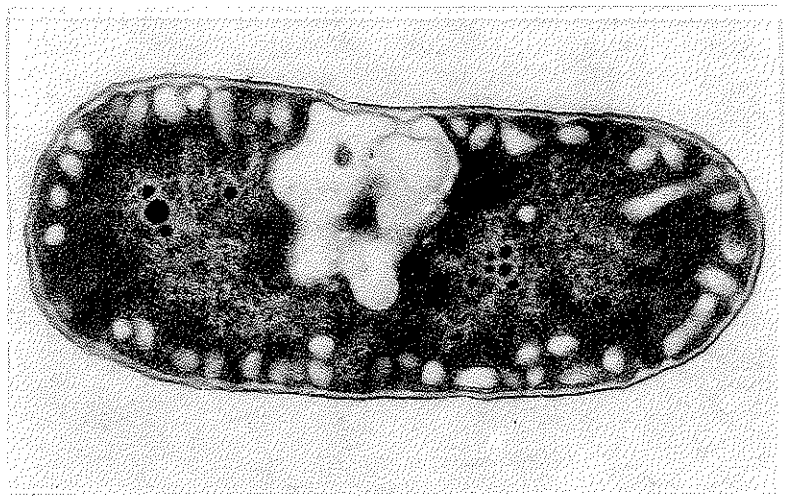
A. 5% oxygen

x 42,000

B. 20% oxygen

x 42,000





4 mM NH_4^+

Regularly spaced vesicles around the periphery of the cells could be noted in the cells grown under both 20% and 30% oxygen. The vesicles appeared to be a little more numerous than they were in cells grown in the presence of 1 mM NH_4^+. (Figure 3 A&B)

FIGURE 3

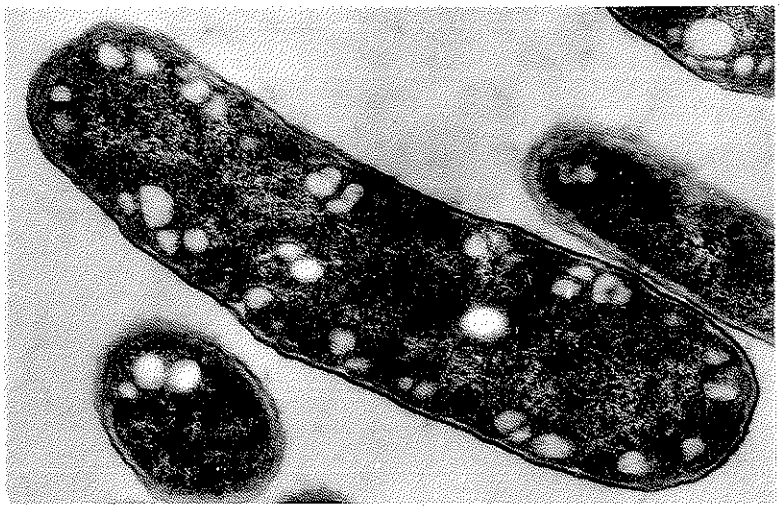
A. chroococcum grown in the presence of 4 mM NH_4^+ and under a gas phase of

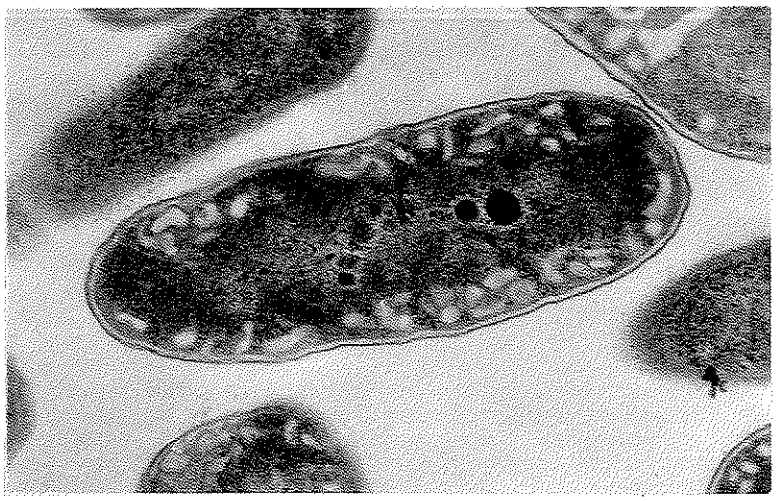
A. 20% oxygen

x 40,000

B. 30% oxygen

x 39,000





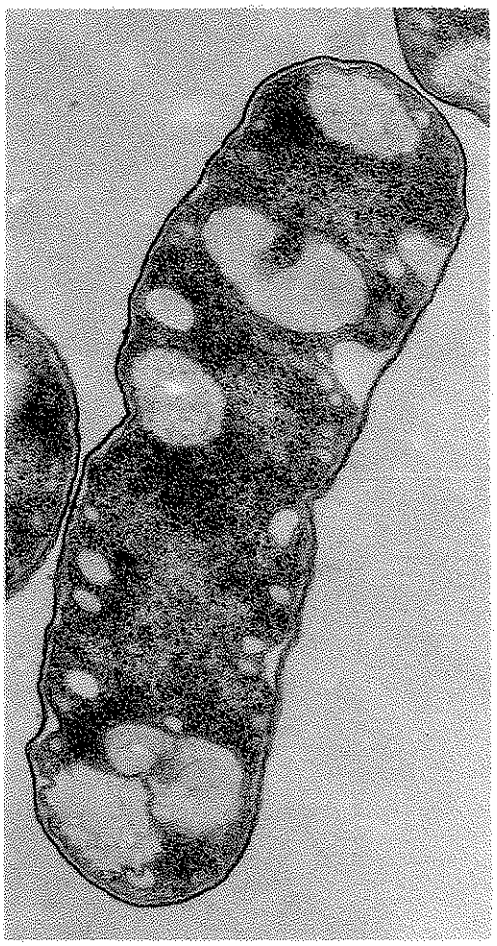
20 mM NH_4^+

Vesicle formation was present around the cell's periphery but the extent to which vesicle formation was found varied considerably from cell to cell. The cells were beginning to assume abnormal shapes, with areas of the cytoplasm pulling away or shrinking in from the cell wall. In some cases the cell wall was assuming a wave-like configuration. Definite, clearer areas were appearing within the cytoplasm. (Figure 4 A).

FIGURE 4

A. chroococcum grown in the presence of 20 mM NH_4^+ and under a gas phase of 20% oxygen

x 40,000



30 mM NH_4^+

The cells grown under 5% oxygen showed a wide variation in their size and shape. There was a similarly wide variation in the amount of vesicle formation which was not necessarily confined to the periphery of these cells. In cells grown in the presence of 20% oxygen, one noted the absence of or a small amount of vesicle formation which was restricted to the periphery of the cell. (Figure 5 A&B).

FIGURE 5

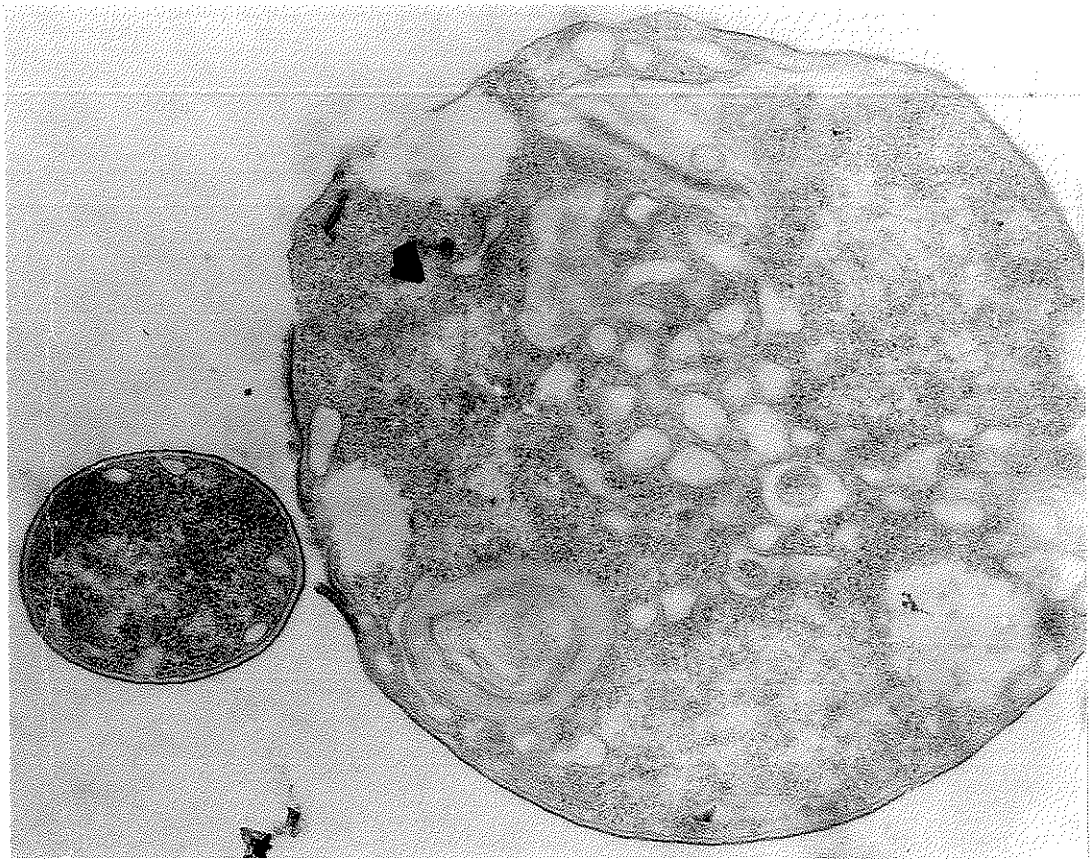
A. chroococcum grown in the presence of 30 mM NH_4^+ and under a gas phase of

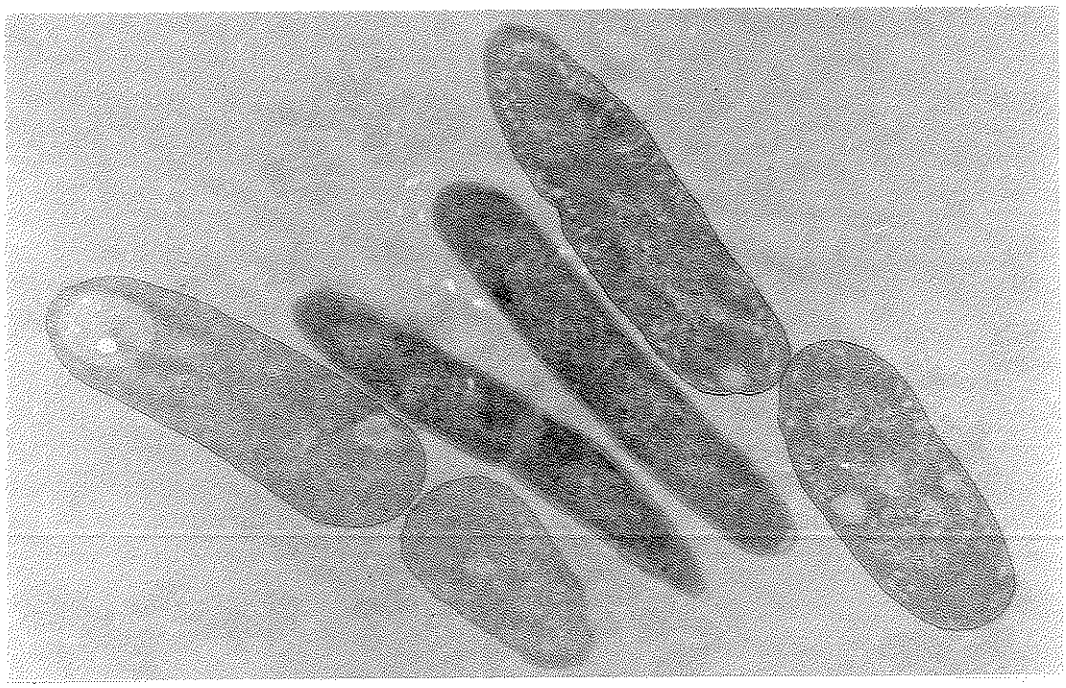
A. 5% oxygen

x 42,000

B. 20% oxygen

x 21,000





5% oxygen

The cells grown in an nitrogen-fixing environment and those grown in the presence of 1 mM NH_4^+ demonstrated a constancy in appearance and in the extent of vesicle formation which was not evident in those cells grown in 30 mM NH_4^+. The 1 mM NH_4^+ grown cells contained a higher degree of vesicle formation than did the nitrogen-fixing cells. (Figure 6 A-C).

FIGURE 6

A. chroococcum grown under a gas phase of 5% oxygen and in the presence of

A. nitrogen-fixing conditions

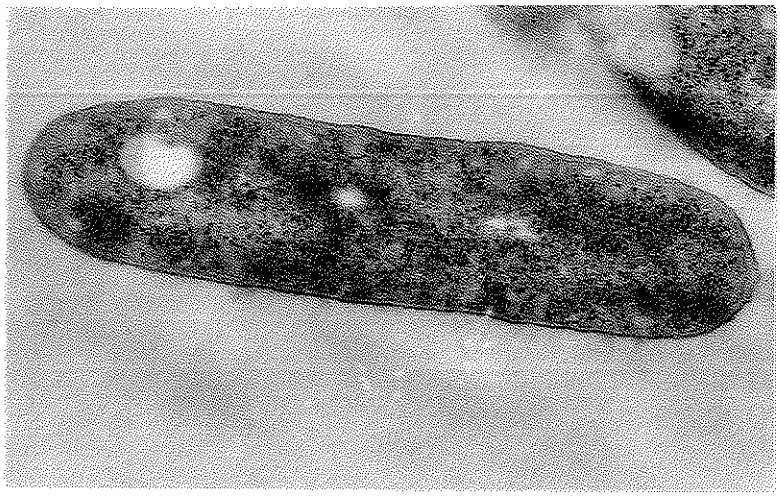
x 42,000

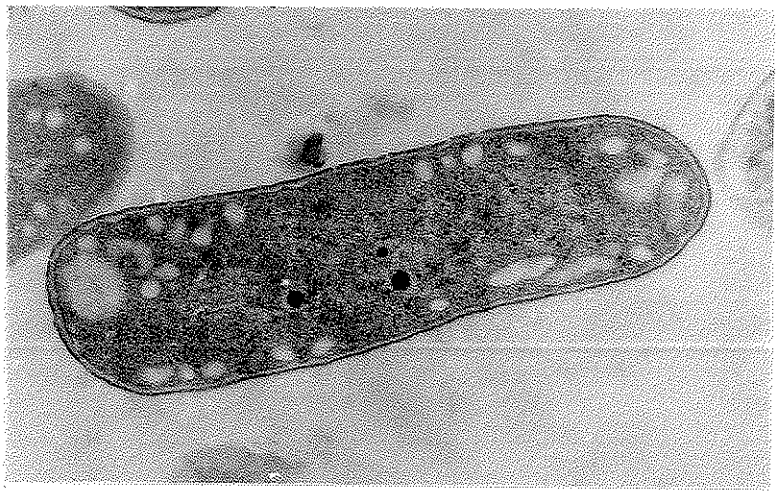
B. 1 mM NH_4^+

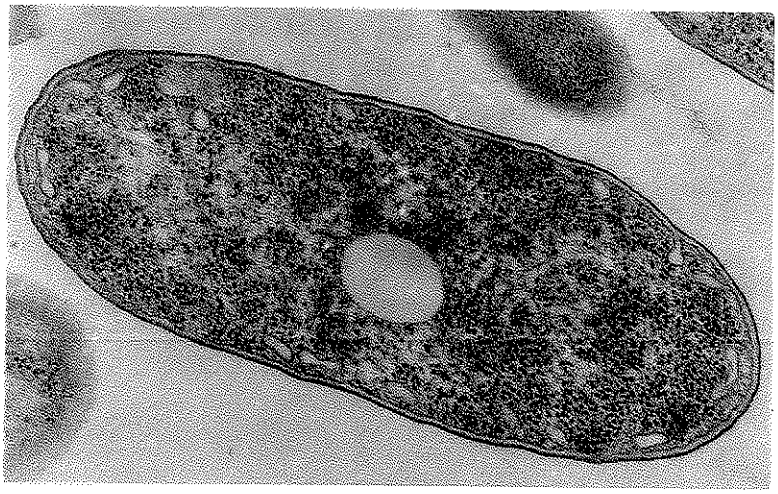
x 43,000

C. 30 mM NH_4^+

x 43,000







20% oxygen

The nitrogen-fixing, the 1 mM NH_4^+ and the 4 mM NH_4^+ grown cells appeared to contain a constant and comparable amount of vesicle formation. The 20 mM NH_4^+ grown cells contained vesicles but the cells have begun to become distorted as the cytoplasm seemed to be pulling away from the cell wall. The cell wall has taken on a wave-like configuration. Cells grown in the presence of 30 mM NH_4^+ were no longer consistent in appearance. The particular cell photographed showed a reduction in vesicle number and size compared compared to cells grown in the presence of 1 mM NH_4^+ or those grown in nitrogen-fixing conditions. (Figure 7 A-E).

FIGURE 7

A. chroococcum grown under a gas phase of 20% oxygen and in the presence of

A. nitrogen-fixing conditions

x 49,000

B. 1 mM NH_4^+

x 40,000

C. 4 mM NH_4^+

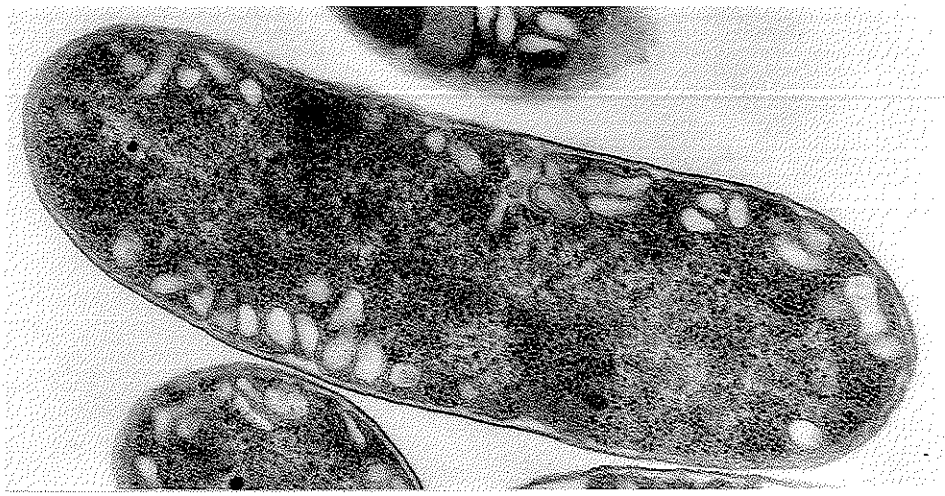
x 40,000

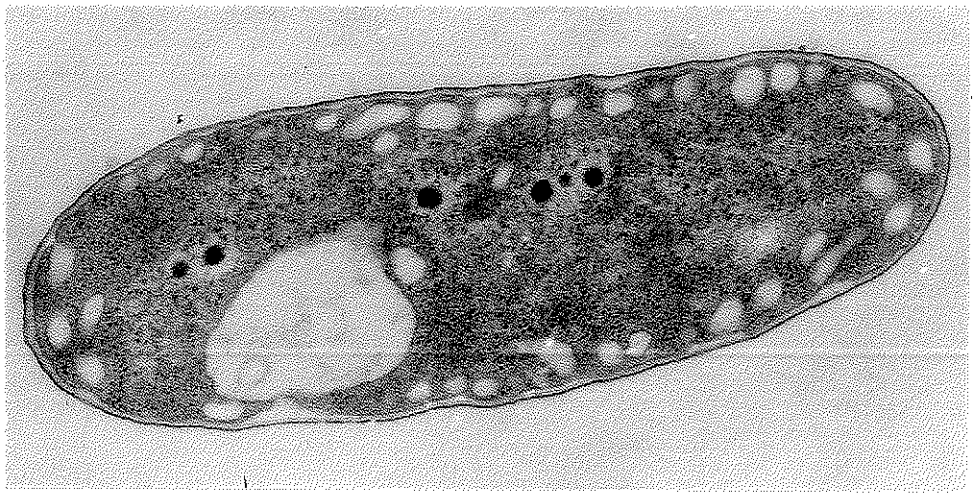
D. 20 mM NH_4^+

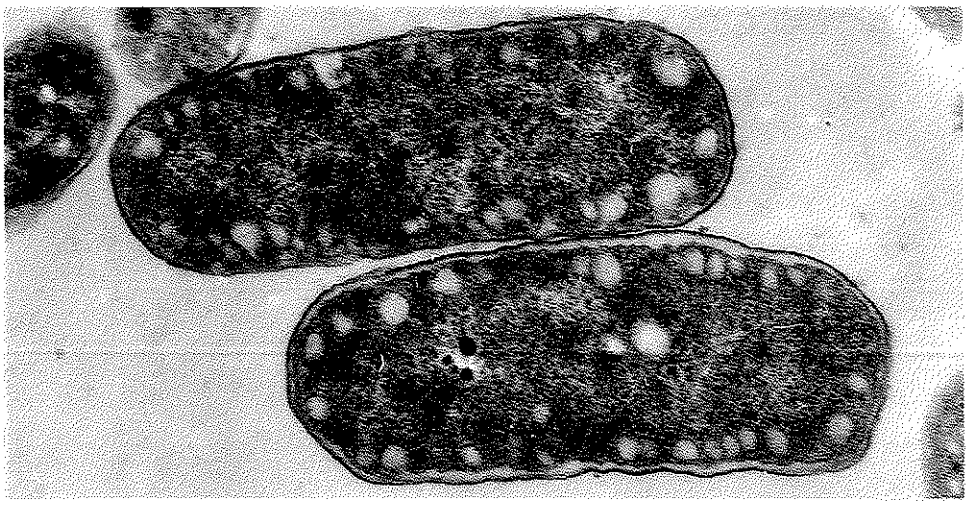
x 40,000

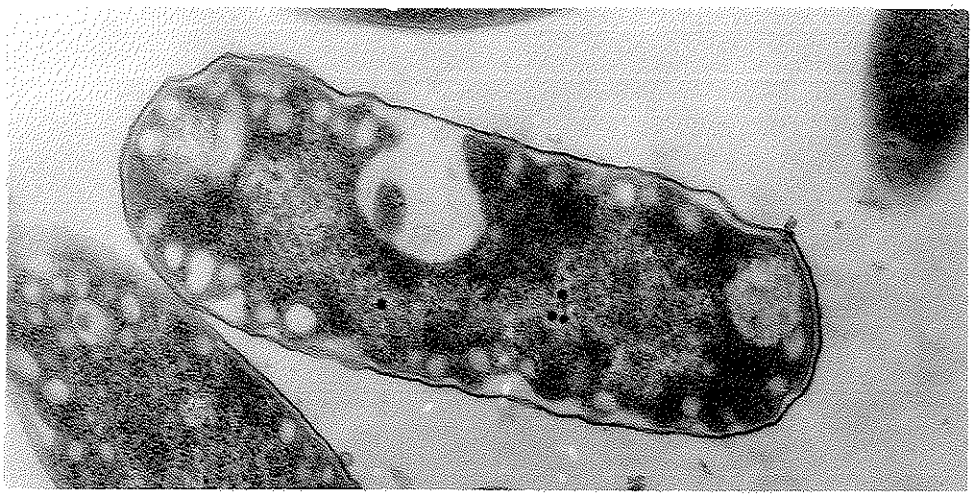
E. 30 mM NH_4^+

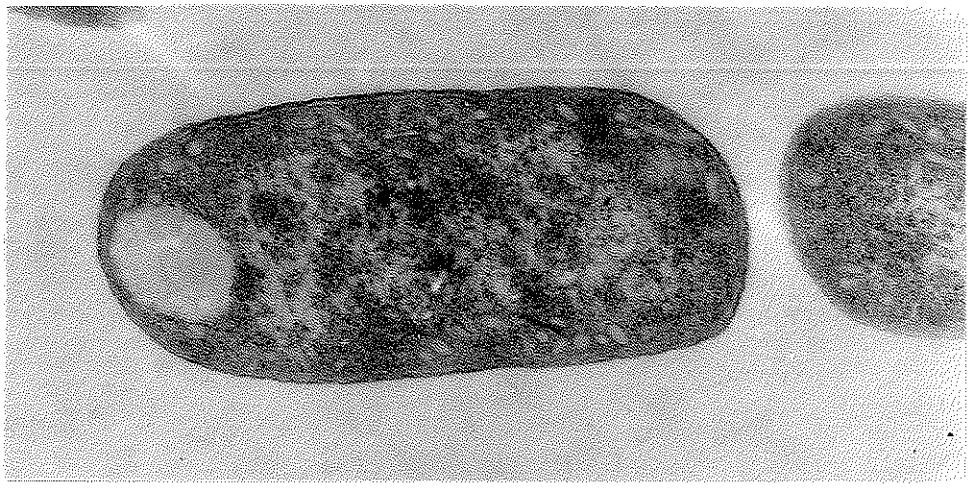
x 41,000











30% oxygen

Both cell types (nitrogen-fixing and 4 mM NH_4^+) contained a high degree of vesicle formation tightly packed around the periphery of the cell. (Figure 8 A&B).

FIGURE 8

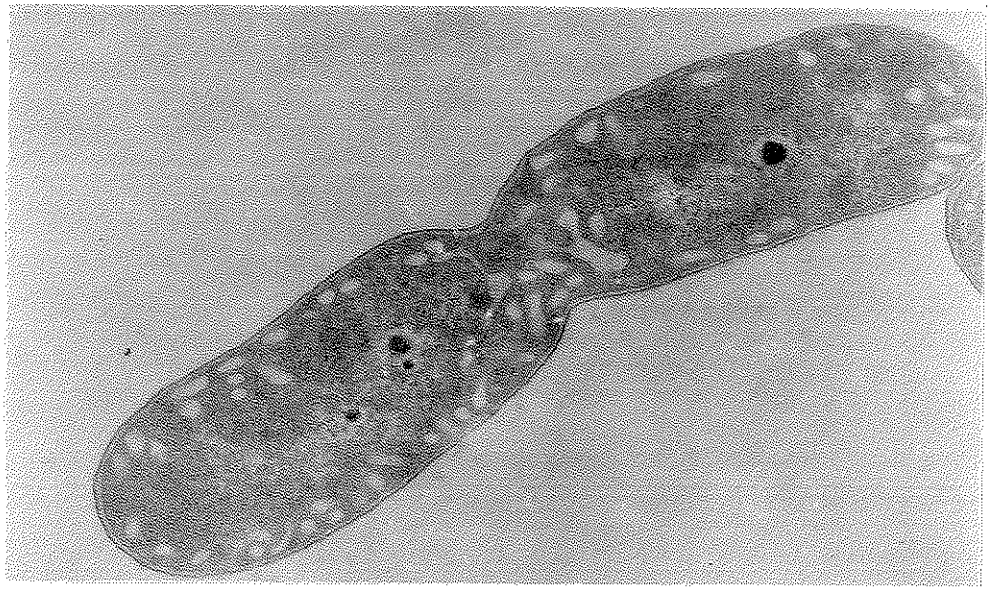
A. chroococcum grown under a gas phase of 30% oxygen and in the presence of

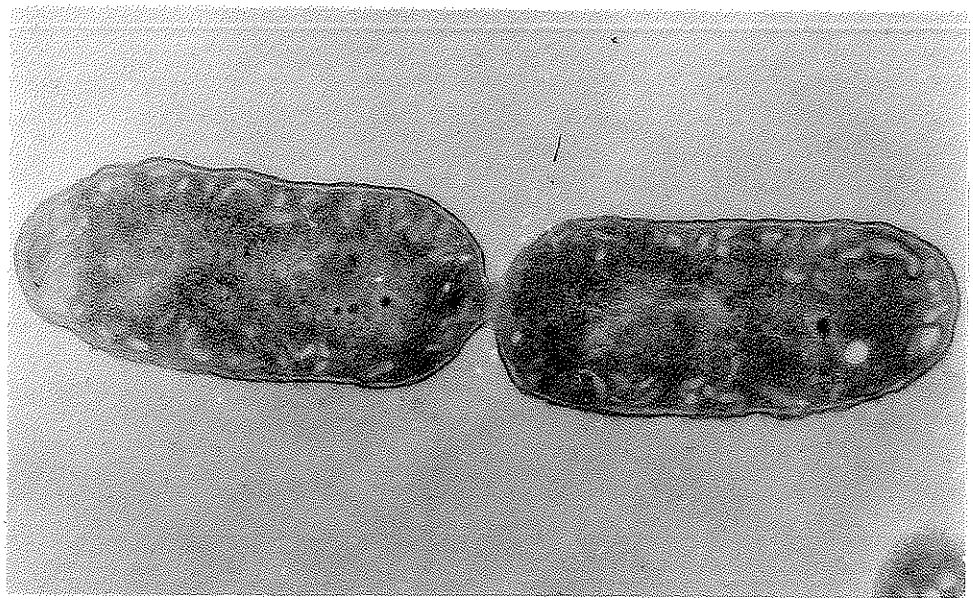
A. nitrogen-fixing conditions

x 35,000

B. 4 mM NH_4^+

x 34,000





Silver nitrate stain (10^{-4}M)

The silver nitrate stain marked a definite type of structure (marked A in Figure 9B) within the cytoplasm of the cell. When grown under an atmosphere of 20% oxygen, the cells have a much greater tendency to contain this type of structure labelled by the silver nitrate than do the cells grown in the presence of 5% oxygen. The addition of the Ag^{+} ions to the continuous culture caused an immediate drop in the oxygen consumption as indicated by the rise of the dissolved oxygen tension in the culture and by the decrease in the CO_2 output of the culture. Silver nitrate is usually considered to be a poison however the addition of the substance to the culture did not completely kill the cells but only knocked out the respiratory system for a time. Once the $AgNO_3$ was washed out of the culture vessel the cells recovered. (Figure 9 A&B).

FIGURE 9

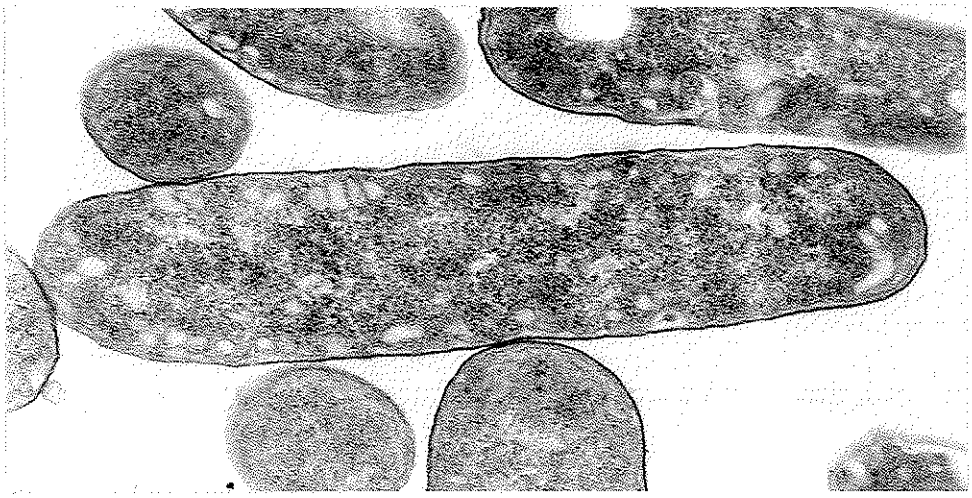
A. chroococcum grown under nitrogen-fixing conditions, in the presence of $AgNO_3$ (10^{-4}M) and in a gas phase of

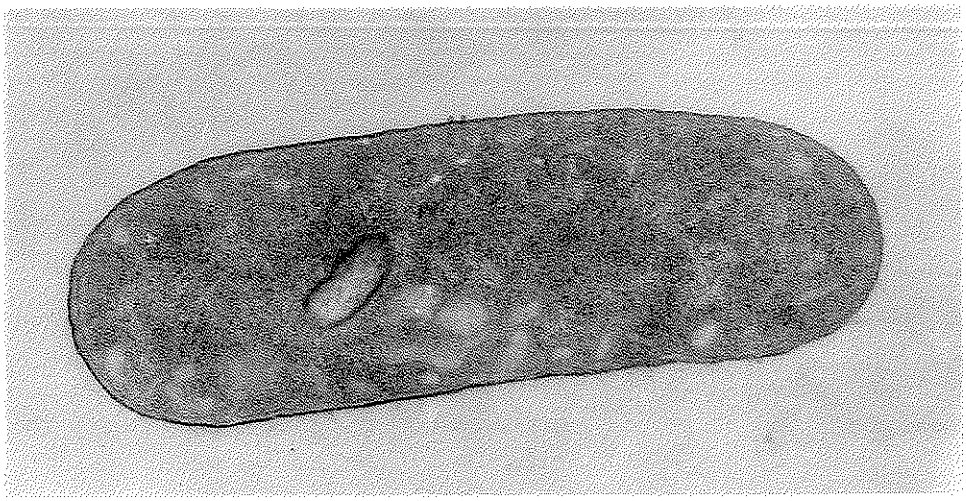
A. 5% oxygen

x 41,000

B. 20% oxygen

x 40,000





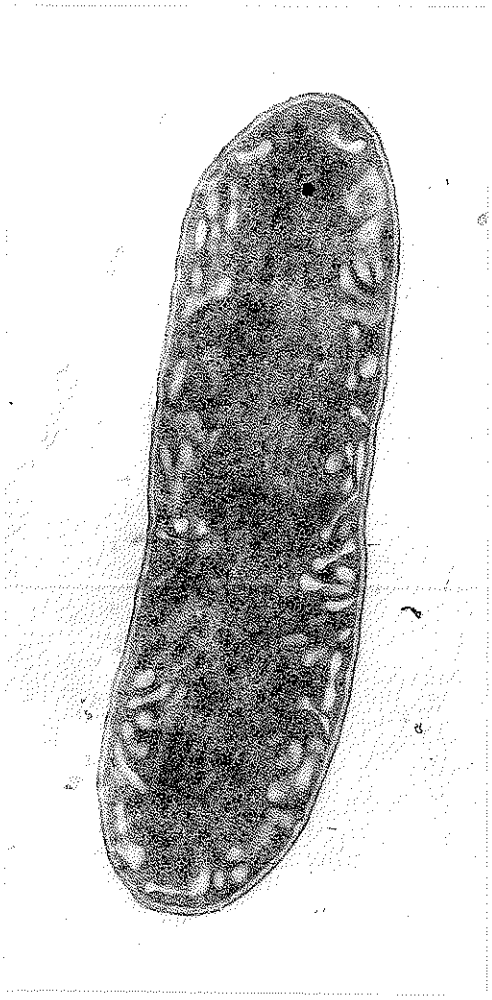
Potassium tellurite stain (2.0 ppm)

The vesicles about the periphery of the cells grown in the presence of 2.0 ppm potassium tellurite appeared to be more distinct than the vesicles in the cells grown in the absence of potassium tellurite. When the culture was fed medium containing potassium tellurite the strong smell of H_2Te similar to H_2S was given off. (Figure 10 A).

FIGURE 10

A. chroococcum grown under nitrogen-fixing conditions in a gas phase of 20% oxygen and in the presence of 2.0 ppm potassium tellurite.

x 41,000



Localization of catalase by 3,3'-diaminobenzidine (DAB)

The DAB stain was localized in the periplasmic space and within certain vesicle-like structures along the periphery of the cytoplasm. Definite granular deposits could be noted. Cells incubated in the propanediol buffer did not show any labelling and showed a reduced number of vesicles compared to the control cells. Cells pre-incubated in buffer and 0.01m KCN and then incubated in 0.01M KCN plus the complete incubation mix gave no evidence of staining nor was staining by DAB indicated in the cells which were pre-incubated in 0.02M AT followed by incubation in the complete incubation mix plus 0.02M AT. The vesicles seem to have disappeared in the cells incubated in the presence of KCN whereas they can be seen in the cells which have been exposed to AT. (Figure 11 A-E).

FIGURE 11

A. chroococcum grown under nitrogen-fixing conditions in the presence of a gas phase consisting of 20% oxygen and incubated in

A. propanediol buffer only

x 41,000

B. 0.01M KCN plus the complete incubation mix (pre-incubated in 0.01M KCN and buffer)

x 41,000

C. 0.02M AT plus the complete incubation mix (pre-incubated in 0.02M AT and buffer)

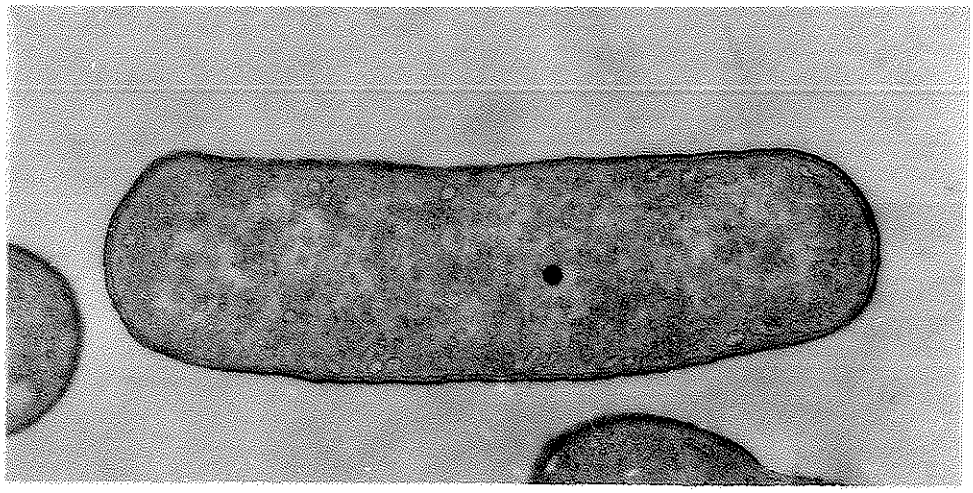
x 40,000

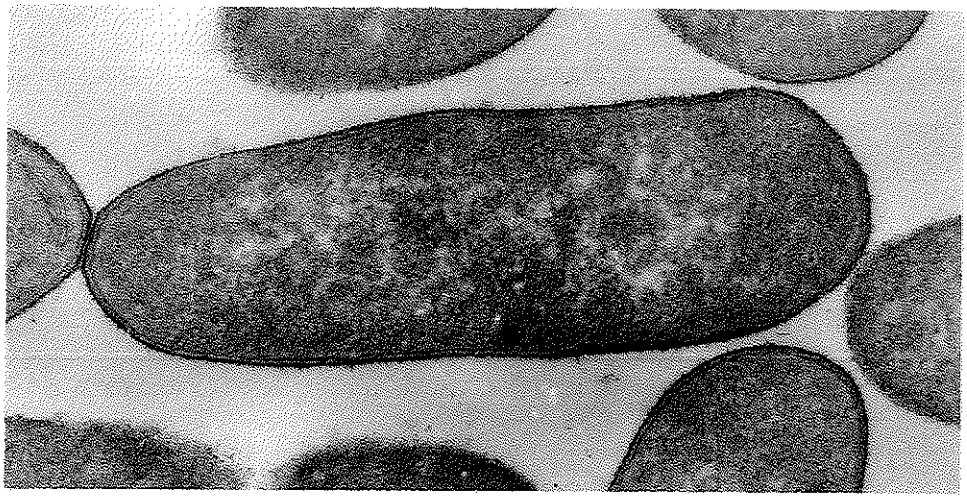
D. complete DAB incubation mix

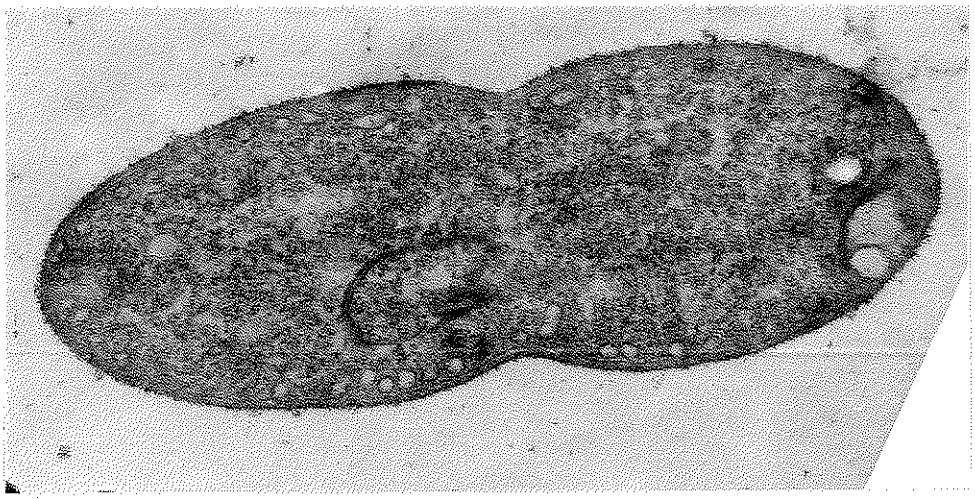
x 45,000

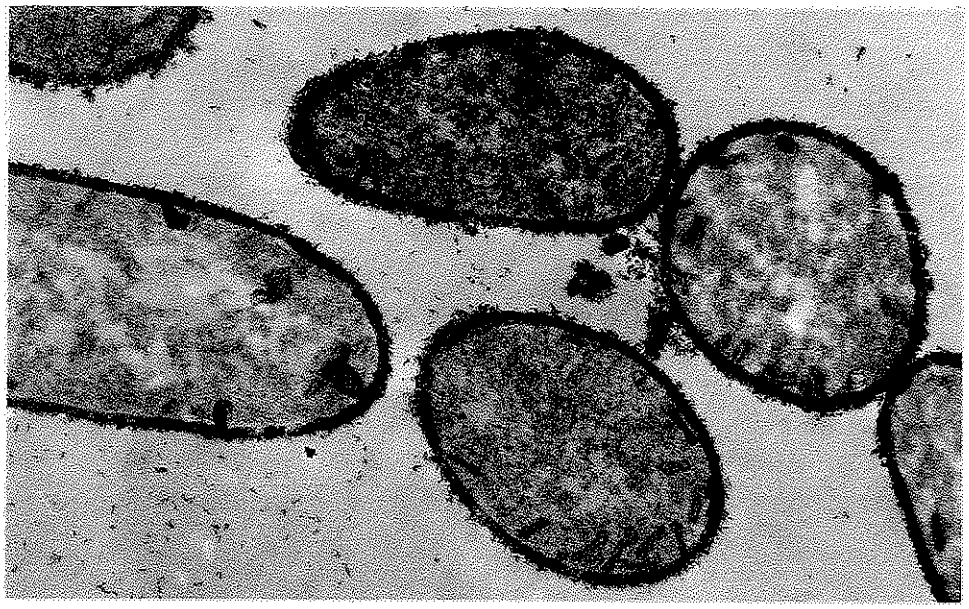
E. complete DAB incubation mix

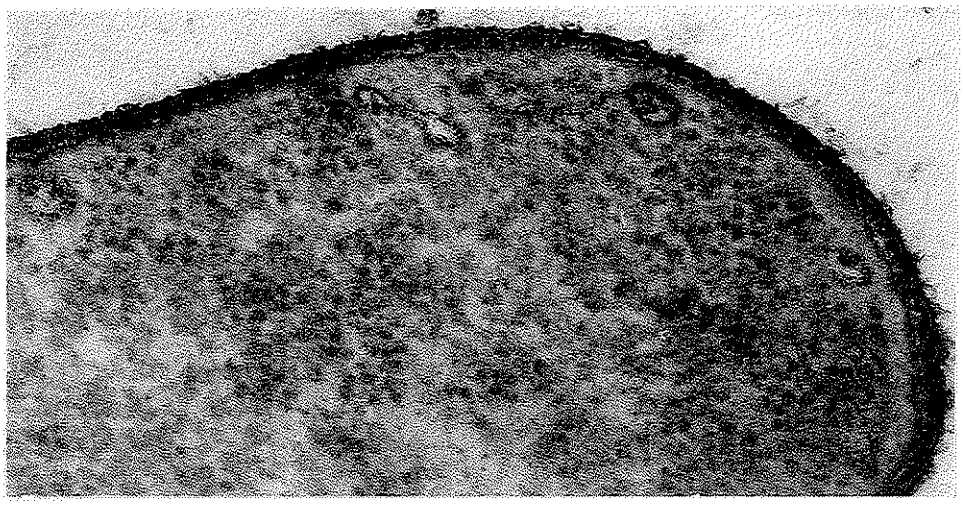
x 120,000











DISCUSSION

DISCUSSION

Ultrastructure studies on Azotobacter cells have previously been conducted with cells grown either in batch culture (Oppenheim and Marcus, 1970; Pate et al., 1973) or in continuous culture (Hill et al., 1972). Although different provisions were made to increase the aeration within these cultures, the aeration and agitation used did not compare with the high (vortex) aeration and agitation rates used in the cultures for this study. When this vortex stirring is used, cell growth can be twice as fast as it is under 'normal' conditions (Hine and Lees, 1976). The purpose of this vortex aeration (agitation) was to ensure that the cultures were not subjected to any oxygen limitation. In order for the oxygen in the gas phase to reach the cell surface, it must cross a gas-liquid interface and a liquid-cell interface. Both these interfaces can hinder the passage of oxygen to the cell surface but the vortex stirring of the liquid phase appears to reduce the resistance of the cell-liquid interface, thus allowing an apparently adequate supply of oxygen to reach the cells (Hine and Lees, 1976). Tsim (1976) has shown that the bacteria did not experience any oxygen-limitation when grown in a gas phase of air, but were possibly under a slight oxygen limitation when fixing nitrogen at 10% oxygen in the gas phase.

Contrary to previous reports by Oppenheim and Marcus (1970) and Hill et al. (1972) the addition of fixed nitrogen (NH_4^+) to nitrogen-fixing cultures grown under vortex conditions did not decrease the amount of vesicles present as compared to those present in the nitrogen-fixing cells. The concentration of oxygen that the nitrogen-fixing cells were exposed to appeared to determine the extent of the vesicle formation

and the regularity of their spacing. The cells grown in a gas phase of 30% oxygen contained markedly more vesicles than cells grown in a gas phase of 5% oxygen (Figure 1 A-C).

Cells subjected to low concentrations of NH_4^+ (1 and 4 mM, Figure 2) in fact responded by increasing the number of vesicles per cell for a given concentration of oxygen as compared to the nitrogen-fixing cells (Figure 1). The size and shape of the cells supplied with ammonia were not noticeably different from the nitrogen-fixing cells and one still noted the tendency towards tighter packing of the vesicles as the oxygen concentration increased.

Cells supplied with high concentrations of NH_4^+ (20 and 30 mM, Figures 4&5) in the medium responded in a variety of ways. A "typical" cell no longer existed. The size and shape of these cells was no longer constant. The cell wall was either rigid as in the nitrogen-fixing or low NH_4^+ grown cells or it could assume a wave-like configuration. Vesicle formation ranged from nothing to extremely extensive. It is known in fact (Tsim, 1976) that cells grown under these concentrations of NH_4^+ are not "normal".

While these results are not comparable to those of Oppenheim and Marcus (1970) and Hill et al. (1972) neither are they comparable to the results of Pate et al. (1973). This last group reported that there was no difference in the extent of vesicle formation between the nitrogen-fixing cells and the cells which were provided with fixed nitrogen. It was noted that cells harvested during the late exponential phase contained more internal membranes than cells harvested in the early exponential phase. Growth in a continuous culture system does not allow for such a comparison but with a dilution rate of D = 0.22 (i.e. a re-

placement time of approximately 8 hours) most of the cells in a continuous culture might be expected to be well into the exponential phase.

Recently Tsim (1976) completed a series of biochemical studies on A. chroococcum grown in continuous culture under vortex; it is therefore interesting to compare the present cytological (ultrastructural) pictures with his biochemical results.

Figure 11 in Tsim (1976) indicated that cultures fixing nitrogen or growing in low concentrations (1 and 4 mM) of NH_4^+ were able to change their respiratory index (R.I.) rapidly with the change in the concentration of oxygen, while cells growing in high concentrations (20 and 30 mM) of NH_4^+ were not able to do so. Tsim noted that the activities of some enzymes (e.g. nitrogenase and glutamate dehydrogenase) were very different in the cells grown in 20 mM NH_4^+ (Fig. 19 of Tsim, 1976) from those cells grown under nitrogen-fixing conditions or in the presence of low concentrations (1 and 4 mM) of NH_4^+ and that the pattern of enzyme activities in the cells grown in 30 mM NH_4^+ (Figure 20 of Tsim, 1976) had become quite bizarre. These findings correlate well with the ultrastructural studies (Figure 5). The appearance of the nitrogen-fixing cells (Figure 1) and the cells grown in the presence of low NH_4^+ (1 and 4 mM, Figures 2&3) was very similar, the only difference being reflected in the degree of vesicle formation. Cells grown in the presence of high concentrations of NH_4^+ (20 and 30 mM, Figures 4&5) no longer maintained a constant appearance. There was a wide variation in the degree of vesicle formation, in the size and shape of the organisms and in the configuration assumed by the cell wall.

Tsim (1976) has shown in his Figures 16-20 the activity of nitrogenase at various levels of NH_4^+ in the medium and under different con-

centrations of oxygen. As the concentration of oxygen in the gas phase increased, the specific activity of the nitrogenase decreased in the nitrogen-fixing cells; and in those cells grown in the presence of fixed ammonia the nitrogenase decreased logarithmically as the ammonia concentration was increased.

Since the extent of vesicle formation within the cells increased as the concentration of oxygen was increased in the gas phase, it seems as if these structures may be in some way concerned with keeping oxygen out of the cell to protect the nitrogenase from inactivation or possibly to protect the entire cell from the effects of oxygen (Figures 7&8, in the present thesis).

The study of the localization of catalase (Figure 11) has indicated that this enzyme is associated predominantly with the periplasmic space. The lack of staining within the cells when either AT or KCN was added to the pre-incubation and incubation mixes suggested that the staining in Figure 11 was due to catalase. AT is an effective inhibitor of catalase (Heim et al., 1956) while KCN inhibits staining due to peroxidase and catalase (Novikoff and Goldfischer, 1969). Since catalase is one of the enzymes responsible for the conversion of the toxic intermediates formed in the reduction of oxygen to water in aerobic respiration (Fridovich, 1975) it appears as if a barrier has been set up to prevent the passage of oxygen or its products into the cell. If the cells are furnished with an ample supply of NH_4^+, the requirement for energy to drive the nitrogen-fixing reaction no longer exists, the CO_2 evolution decreases, and the R.I. falls (Tsim, 1976). Therefore the cells have lost a method of limiting the amount of oxygen available to the cytoplasm. Perhaps the increase in the vesicle formation is an attempt to provide a more effect-

ive barrier to block the entrance of oxygen or its reduction products into the cell.

It had been hoped that through the use of silver nitrate (Figure 9) and potassium tellurite (Figure 10) one would have been able to detect the nitrogenase within the cells. The nitrogenase complex, having the highest reducing power in the cell, should have been able to reduce the silver ion to silver metal and the tellurite ion to tellurium. These products would leave an electron dense deposit at the site of the nitrogenase complex which would be discernable in electron micrographs. In fact, the silver nitrate stain caused a specific structure within the cells to be accented. Its occurence was more common in the nitrogen-fixing cells grown under a gas phase of 20% oxygen (Figure 9B) then in the cells grown under 5% oxygen (Figure 9A). The potassium tellurite did not produce localized areas of stain but the cytoplasm of the cells did seem to take on a darker more homogeneous quality which contrasted with the lightness of the vesicles. Unfortunately the location of the nitrogenase within the cell could not be marked by either of these methods.

On the other hand Stasny et al. (1973), using a ferritin-conjugate of anti-Mo-Fe protein IgG were able to label the nitrogenase in A. vinelandii. Eighty percent of the ferritin conjugated antibody label was found in the periphery of the cell cytoplasm while twenty percent was located within the cytoplasm. Therefore, it would appear as if the nitrogenase is situated in approximately the same area of the cell as are the vesicles. However, this by no means indicated that the areas of nitrogenase marked in the cell were comparable to the vesicles seen in our micrographs.

The findings of Stasny et al. (1973) would agree with the hypothesis

of Oppenheim and Marcus (1970) and Hill et al. (1972) that the membranes they saw were serving to protect the nitrogenase from oxygen (conformational protection) either by surrounding the enzyme complex or by being intimately associated with it. Moreover this hypothesis was substantiated by the appearance of the vesicles in nitrogen-fixing conditions and their disappearance in cells which have been supplied with NH_4^+. Our studies have shown that the presence of NH_4^+ in the medium did not decrease the extent of the vesicle formation but if anything increased it (Figures 2-5).

Under nitrogen-fixing conditions, the cells consume oxygen (and consequently produce CO_2) in at least three ways: 1) oxygen used for growth and maintenance, O_{2_g} 2) oxygen used to generate energy for nitrogen fixation, O_{2_n} 3) oxygen wasted to keep the cell's interior free from oxygen and its reduction products (such as O_2^-, H_2O_2) O_{2_w}. Since in the presence of NH_4^+, O_{2_n} will be reduced or eliminated, O_{2_w} must necessarily increase if the growth rate and oxygen supply remain the same. If the vesicles shown in the present electron micrographs are in fact connected with the waste-disposal of excess oxygen their number and disposition immediately fall into place.

It is clear that as the concentration of oxygen supplied to the nitrogen-fixing cultures and the cultures grown in the presence of low concentrations (1 and 4 mM) of NH_4^+ was increased there was a corresponding increase in the extent of the vesicle formation (Figures 1-3). Similarly, as the concentration of NH_4^+ supplied to the culture was increased for a given oxygen concentration, the degree of vesicle formation present in the cell increased (Figures 6-8). Tsim (1976) found that cultures supplied with NH_4^+ were more sensitive to the dissolved oxygen

than were cultures fixing nitrogen. As the concentration of oxygen supplied to the nitrogen-fixing cultures and the cultures grown in the presence of low concentrations (1 and 4mM) of NH_4^+ increased, the R.I. values increased (Figure 11 in Tsim, 1976) but there was a decrease in the level of nitrogenase (Figures 16-18 in Tsim, 1976). The R.I. values (Figure 11 in Tsim, 1976) also showed that as the concentration of NH_4^+ in the medium was increased the abilty of the culture to produce CO_2 decreased. These data indicated that respiratory protection is not totally effective in protecting nitrogenase against oxygen inhibition nor is any other sort of protection effective.

If in fact the vesicles are connected with the waste-disposal of excess oxygen, this disposal is not directed solely towards nitrogenase but towards the entire cell. The synthesis of vesicles was a result of an attempt by the cell to respire excess oxygen and to prevent it and its reduction products from entering the cell's interior. Nitrogen-fixing cultures require energy to drive the nitrogen-fixing reaction. As increasing amounts of NH_4^+ are supplied to the culture, the requirement for oxygen is reduced. Therefore the cells have a need to get rid of even more oxygen than the nitrogen-fixing cells and the synthesis of vesicles to increase the area available to respiratory enzymes appears to be the way in which the cells try to accomplish this. It would seem that as the oxygen concentration increased in nitrogen-fixing cells, or as the concentration of NH_4^+ as well as the concentration of oxygen available to the NH_4^+ grown cells increased, they are less able to prevent the passage of oxygen or its toxic intermediates (O_2^-, H_2O_2, $OH^\cdot$ and $^1O_2$, singlet oxygen) into the interior of the cell. It may be that the decrease in the specific activity of nitrogenase is a consequence

of this since the enzyme is especially sensitive to oxygen (Burns and Hardy, 1975; Dalton and Mortenson, 1972; Postgate, 1971).

It would appear that nitrogen-fixing cells are able to cope adequately with increased concentrations of oxygen while cells subjected to increasingly higher concentrations of NH_4^+ become extremely oxygen sensitive. The cells exposed to the 20 and 30 mM NH_4^+ concentrations were being given far too much fixed nitrogen. This was apparent in Figures 4&5 since these cells were distorted, in many cases being much larger than usual, and there was great variation in the extent of vesicle formation from cell to cell.

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