THE GROWTH OF NITROGEN-FIXING *AZOTOBACTER CHROOCOCCUM* IN INTENSELY AGITATED CONTINUOUS CULTURE

BY

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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

AUGUST, 1975

The Growth of Nitrogen-Fixing Azotobacter Chroococcum in INtensely Agitated Continuous Culture.

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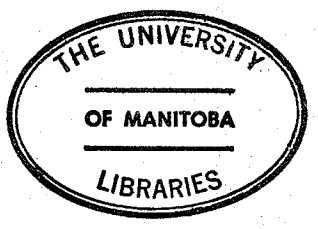

ACKNOWLEDGEMENTS

The author wishes to express his most sincere gratitude towards Dr. H. Lees, Professor of the Department of Microbiology at the University of Manitoba for his invaluable guidance and helpful encouragement throughout the course of this investigation and for his assistance in the preparation of this dissertation.

Special thanks are extended to the staff and graduate students of the Department of Microbiology at the University of Manitoba for their help in many ways.

TO MY MOTHER AND IN MEMORY OF MY LATE FATHER

ABSTRACT

Azotobacter chroococcum was grown and studied under N_2-fixing conditions in intensely agitated continuous culture (ie. at a stirring rate of 1750 r.p.m.) at various dilution rates and under varying 0_2 partial pressures in the atmosphere above the culture.

With this high agitation rate, extremely high growth yields, which reached a maximum of about 230 mg cell growth/ℓ culture/h and which were higher than any previously attained, were realized at the higher 0_2 partial pressures at the higher dilution rates. These cultures were also found to be very efficient at fixing nitrogen. Moreover, although the ambient dissolved oxygen concentration in the culture was zero or near zero (ie. $< 3\mu M$) under all conditions in which the cells were actively growing, the resident cells of these so-called 0_2-limited cultures had, in general, macromolecular compositions characteristic of 0_2-sufficient cultures.

Increasing the dilution rate or 0_2 partial pressure in the gas phase too much resulted in an increase in dissolved 0_2 concentration and a subsequent "washing-out" of the culture.

Attempts were made using the chemical sulfite oxidation method and a physical oxygen electrode method to assess the rate of oxygen uptake by the rapidly stirred cultures, but the biological measurement of the rate of CO_2 evolution was found to give a better indication of this.

A discussion of the term "O_2-limitation" on the basis of the growth yield value is presented.

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INTRODUCTION

INTRODUCTION

Aeration and agitation in cultures, (batch, continuous, or other) serve to supply the resident microorganisms with oxygen as well as to mix the cultures so that the microorganisms are uniformly distributed therein and the mass transfer rate of substances into and out of the cells is, as much as possible, maximized (1). Mechanical agitation of cultures to introduce and disintegrate air bubbles and to intensify the turbulence of the liquid can be achieved by a variety of methods. In this study a magnetic stirring bar - magnetic stirrer combination was used to agitate 240 ml cultures in a 500 ml continuous culture vessel. The supply of oxygen from air bubbles to the liquid and the demand for oxygen by the microbes are the two main aspects from which aeration and agitation can be examined; the former being a purely physical operation while a sequence of enzymatic reactions underlies the latter (1). Moreover, the rationale for the management of aeration and agitation is to achieve a rate of oxygen supply that can meet the oxygen demand of the microorganisms.

It is known that in a normally stirred culture (400-1000 r.p.m. using the previously described method of agitation), aerobic microorganisms are surrounded by a sheath of water molecules through which 0_2 must diffuse in order to reach the cell surface where it is reduced and where the concentration of dissolved oxygen is assumed to be zero (1). Thus, it can be reasoned that the presence of a water skin barrier at these relatively low stirring rates limits the rate of transfer of oxygen from the gas bubble to the surfaces of the bacteria and is therefore indirectly capable of limiting the rate of respiration of respiring aerobes. Thus, the observed values for the tolerances of different organisms for dissolved 0_2 ranging from 10-25 μM for Azotobacter chroococcum and up to 50 μM for other organisms may simply be rule-of-thumb values applicable to a given range of stirring rates and will inevitably be higher than the actual concentrations of 0_2 the cells will tolerate at their surface (2, 3, 4, 5).

In order to minimize the thickness and consequently the effects of this water sheath, the highly aerobic Azotobacter chroococcum was grown in intensely agitated continuous culture (at a "vortex" stirring rate of about 1750 r.p.m.). Under these conditions, with the water barrier apparently almost wholly removed by the intense "shredding" induced by the high stirring rate,

O_2-electrode measurements of oxygen tension in the culture should more closely approximate to the actual O_2 tension at the cell surface thereby giving a better estimate of the amount of oxygen that these cells will actually tolerate. Under these conditions of intense agitation, the oxygen percentage in the atmosphere above the culture was varied at different dilution rates and the important corresponding behavioral and physiological changes were monitored so that the effects of the degree of oxygenation on Azotobacter cultures could be seen. Attempts were also made to ascertain the respiration rates of the cultures using biological, physical and chemical methods of which the biological procedure of measuring the rate of CO_2 evolution by the cultures under different conditions was found to give the closest approximation to the theorectical respiration rate and represented the least amount of oxygen that could be used by the organisms to produce the observed cultural characteristics under those conditions. A physical method using an oxygen electrode to monitor the rate of oxygen's dissolving in a non-respiring culture determined the lowest rate of respiration required to keep the oxygen tension in a culture at zero. The chemical sulfite oxidation method measured the SOX value, the rate of reaction of oxygen from the atmosphere above the culture with sulfite in

solution in the culture vessel. This was taken to represent the maximum possible rate of oxygen consumption by a culture under the same conditions of aeration and agitation.

H I S T O R I C A L

HISTORICAL

The conversion of atmospheric nitrogen to chemical forms suitable for incorporation by plants and microorganisms is termed nitrogen (dinitrogen or N_2) fixation. Large quantities of fixed nitrogen are provided by both biological and chemical processes. However, in 1970, it was estimated that on a global scale biological nitrogen fixation, which is attributed mainly to the symbiotic and photosynthetic nitrogen-fixing systems and to a lesser degree to free-living organisms such as Clostridium spp. and Azotobacter spp., accounted for 9.1×10^{10}kg. fixed nitrogen/yr., while chemically fixed nitrogen from natural sources such as atmospheric reactions induced by lightning and artificial sources such as the fertilizer industry only contributed 2.2×10^{10}kg./yr (6). Thus, biological reactions appear to account for about 80 per cent of the earth's fixed nitrogen.

The process of biological nitrogen fixation has been recognized since 1862 (7). Since then, a number of nitrogen-fixing microorganisms have been isolated including Rhizobium spp. which fix when grown in symbiotic

association with certain legumes, as well as the free-living anaerobic Clostridium pasteurianum, and aerobic Azotobacter spp. (8,9,10). Certain species of blue-green algae can also fix nitrogen but, on the whole, nitrogen fixation has shown itself to be a property found exclusively among the procaryotes (11). Postgate recently provided a summary of the various nitrogen-fixing systems which have been recognized to date (12).

Biological nitrogen fixation involves the enzymatic reduction of atmospheric nitrogen to ammonia. Since it is such a broad subject, approachable from enzymological, chemical, physiological or ecological angles for example, the amount of literature on this process is enormous. Most of our present day understanding of its mechanism has been provided by research done within the past fifteen years. Studies on nitrogen fixation were restricted to whole cells until 1960 when suitably reinforced crude extracts of Clostridium pasteurianum were made to fix nitrogen (13,14). This achievement made possible an enzymological study of nitrogen fixation centering around nitrogenase, the enzyme complex responsible for the dinitrogen reduction.

Some of the more important information supplied by the investigations before 1960 can be summarized as

follows (11, 15, 16):

(i) Ammonia was the first stable product of the nitrogenase reaction.

(ii) Possible intermediates such as hydrazine, hydroxylamine, etc. were not reduced when supplied as substrates in place of dinitrogen.

(iii) The trace elements, iron, molybdenum, probably cobalt and in some cases calcium, were required by nitrogen-fixing organisms when grown on N_2 as a source of nitrogen.

(iv) Ammonia, when supplied in the medium as an alternate nitrogen source, repressed nitrogenase synthesis.

(v) Many other compounds such as oxygen, hydrogen, carbon monoxide and nitrous oxide, also inhibited nitrogen fixation.

(vi) A correlation existed between nitrogenase and hydrogenase activities.

The work of Carnahan et al. (1960), however, provided an active nitrogen-fixing crude extract of C. pasteurianum which was soluble, very oxygen-sensitive, and required large amounts of pyruvate. This extract was later separated into a hydrogen-donating pyruvic phosphoroclastic system and a

dinitrogen-activating system (17). The pyruvate, in this case, was later found to play a dual role. It served as a source of electrons while supplying ATP, an essential substrate in nitrogen fixation (18, 19). The electrons donated by pyruvate reduced ferredoxin, a non-haem iron-sulphur electron transport factor which, in turn, acted as a source of electrons for the dinitrogen reduction (20).

In 1964, the first extracts with reproducible activity of the aerobe Azotobacter vinelandii were obtained (21). In contrast to the clostridial extracts, these crude Azotobacter preparations were particulate in nature and relatively stable in air. Nitrogen-fixing activity was noticed when the extract was combined with a hydrogen-donating (hydrogenase-ferredoxin) preparation from Clostridium pasteurianum, and an ATP-generating system containing $MgCl_2$, creatine phosphate, creatine kinase, and a small amount of ATP. This ATP-generating system was required because it was found that inhibition of nitrogen fixation resulted from an ADP buildup when high concentrations of ATP were added to nitrogen-fixing extracts (14,21, 22). The same group of workers later discovered that they could replace the C. pasteurianum extract with the sodium dithionite which is an electron donor and a substrate

for the enzyme hydrogenase (23). Thus the Azotobacter extracts themselves possessed hydrogenase activity but its expression depended upon the presence of an ATP-generating system. Therefore when these preparations were supplied with dithionite under argon or helium, they produced considerable amounts of hydrogen gas. Less hydrogen was evolved, however, when dinitrogen was also provided as a substrate. This discrepancy could be explained by the fact that hydrogen was required for the reduction of nitrogen to ammonia. It was also deduced after examination of the ATP consumption during N_2 fixation that about fifteen moles of ATP were consumed per mole of nitrogen reduced (15).

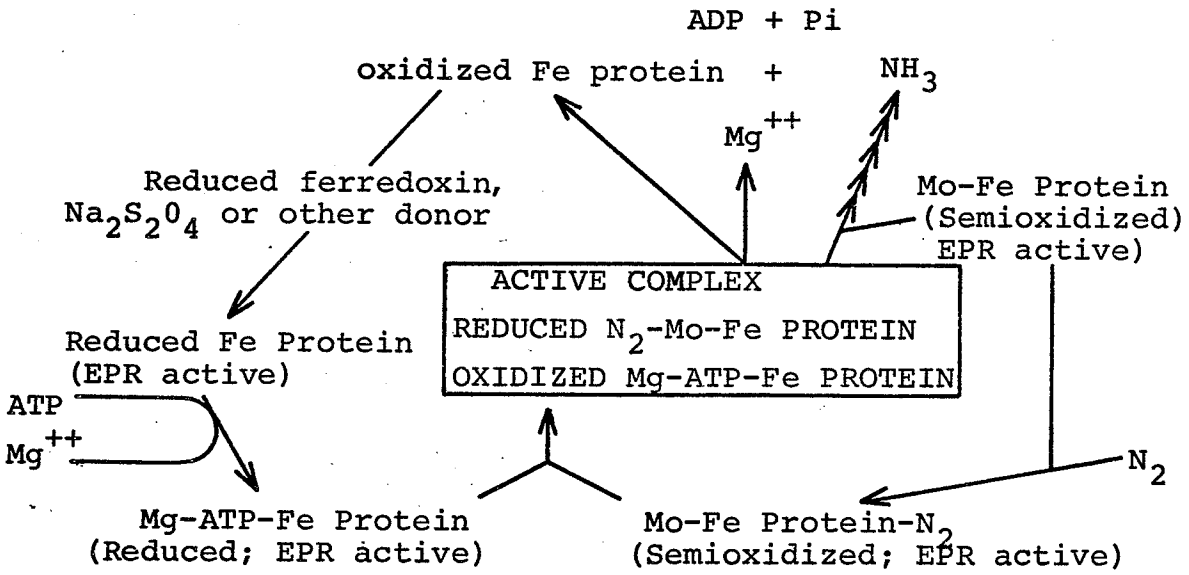
Shortly after this an ATP-dependent hydrogen evolution was demonstrated in extracts from other nitrogen-fixing bacteria. It was shown to occur in Clostridium pasteurianum after it had been realized that in extracts of this organism the large amounts of conventional hydrogenase present, which evolved hydrogen without requiring ATP, acted to mask the presence of the ATP-dependent hydrogenase system (24). Since the ATP-activated hydrogenase was not inhibited by CO, as was the conventional hydrogenase system, the ATP-dependent hydrogen evolution was distinguished by observing the clostridial preparation under an atmosphere

of carbon monoxide. It should also be noted here that ATP-ase activity accompanying the hydrogen-evolving reaction requires a reductant, i.e. the nitrogenase exhibits reductant-dependent ATP-ase activity so that ATP hydrolysis requires sodium dithionite or another hydrogen-donating system (25).

As suggested before, crude extracts of nitrogenase from A. vinelandii and A. chroococcum, in contrast to crude clostridial extracts, are generally particulate and relatively insensitive to air (20, 23, 26). However, like the extracts of C. pasteurianum, the azotobacter extracts can be separated into two, very-oxygen sensitive, soluble components, both of which are necessary for nitrogen fixation (27, 28, 29). These two components are found to be similar for the nitrogenases of all nitrogen-fixing organisms. One component contains molybdenum, acid-labile sulphur, and non-haem iron and has a molecular weight between 200,000 and 300,000 daltons while the other component has a molecular weight of 50,000 - 60,000 daltons and contains labile sulphur, some iron, but no molybdenum (15, 25). In all cases both components are sensitive to oxygen when separated; the smaller protein is always the more sensitive.

In general then the MoFe protein, the Fe protein, $Mg^{++}ATP$, and a reductant are all required in order to reduce N_2 to ammonia enzymatically *in vitro*. Biological electron donors like ferrodoxins or the flavodoxin of *Azotobacter* or low potential artificial donors like sodium dithionite or reduced viologen dye, may serve as the reductants for these *in vitro* systems (19, 30, 31). The ATP may be supplied by any ATP generating system that does not accumulate ADP. The Mg^{++} is essential not only for ATP generation but for the nitrogenase enzyme itself (22).

A simplified schematic representation of the possible events of nitrogen reduction was given by Postgate (1974) as follows (32):



An invaluable research tool for the study of nitrogen-fixing bacteria over the years has been continuous culture. The theory of the growth of microorganisms in chemostat culture, as it stands today, evolved from the basic principles put forth in 1950 by Monod and by Novick and Szilard (33, 34). A continuous culture starts its existence as a batch culture which can be characterized by its growth cycle; the sequence of changes occurring in such a culture beginning with a lag phase, and proceeding through an exponential phase to a stationary phase (35). The dynamics of the exponential growth phase can be described by the following equation:

$$(1) \quad \frac{1}{x}\frac{dx}{dt} = \mu = \frac{d\ (\ell n\ X)}{dt} = \frac{\ell n\ 2}{t_d}$$

where

x is the initial concentration of organisms in mg/cc.,

μ is the specific growth rate constant in h^{-1}

and t_d is the culture generation time (35, 36).

$$(2) \quad \mu = \mu_{max} \left(\frac{S}{K_s + S} \right)$$

where

μ_{max} is the maximum value of μ (i.e. when the value of μ is not limited by S),

K_s is the saturation constant (equal to S at 0.5 μ_{max})

and S is the substrate concentration (33, 37).

(3) $\frac{dx}{dt} = -Y \frac{ds}{dt}$

where

Y = yield factor = $\frac{\text{weight of bacteria formed}}{\text{weight of substrate consumed}}$

over a time period (37).

Now, if DV cc. of fresh medium is added per hour to a culture of V cc. while DV cc. of culture is removed per hour, (D is the "dilution rate" expressed as h^{-1}), then within the culture:

(4) $\frac{dx}{dt} = \mu x - DX = X(\mu - D)$

When the culture reaches a steady state, $\frac{dx}{dt} = 0$

and (5) $D = \mu = \mu_{max} \left(\frac{S}{K_s + S}\right) = \frac{\ell n\ Z}{(t_d)}$ (35).

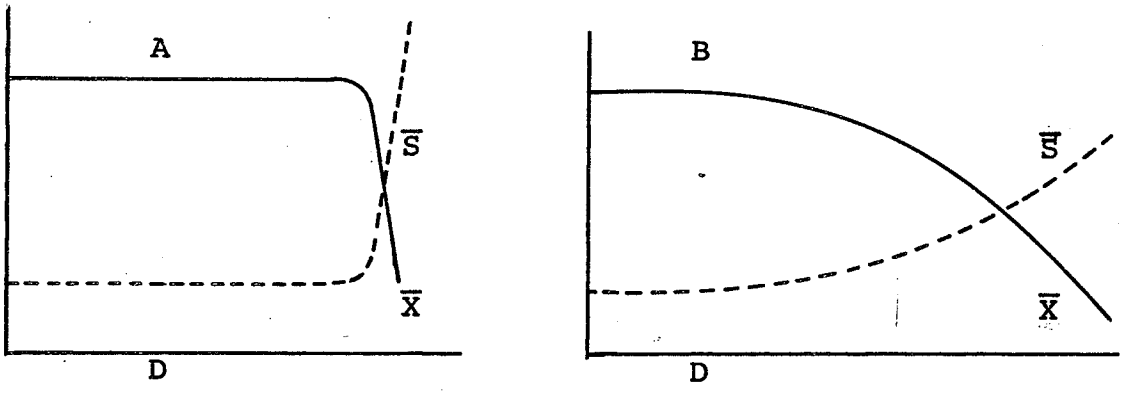
If we call the steady state value of S, "$\bar{S}$", then a rearrangement of the last equation shows that;

(6) $\bar{S} = K_s \left(\frac{D}{\mu\ max - D}\right)$ (38).

If substrate is supplied to the culture from a reservoir at a concentration S_r and metabolism within the culture reduces the concentration to $\bar{S}$, then the concentration of organisms, $\bar{X}$, is given by:

(7) $\bar{X} = Y(S_r - \bar{S}) - Y\left[S_r - K_s \left(\frac{D}{\mu\ max - D}\right)\right]$ (38).

These equations yield curves of type A when K_s is small compared with S_r and curves of type B when K_s is large compared with S_r. It should be noted that the above derivations are not rigid. They are illustrative only and contain several unexpressed assumptions.



Now, the macromolecular composition and metabolic activity, like the microbial and growth-limiting substrate concentrations, are relatively constant in a steady state chemostat culture, but they vary appreciably with growth condition or dilution rate (39, 40, 41, 43). The yield value, Y, is also dependent on growth rate (35). The cellular content of a storage type compound like poly-β-hydroxybutyrate, glycogen, or polyphosphate, and, subsequently, the yield, will also vary with growth rate (43, 44). Moreover, since the carbon source provides both materials for assimilation into cell substances and oxidizable compound required to provide energy for biosynthesis (the ratio of which seems to vary with cell

synthesis or growth rate), there is also a variation in yield with growth rate in some carbon-limited cultures. This has been taken to reflect a cellular "maintenance energy" requirement which is sometimes and sometimes not dependent on growth rate (41, 45, 46, 47, 48).

Hence the equation:

$$(8) \quad Y = Y_G \left(\frac{\mu}{\mu + \mu_e}\right)$$

where

μ_e is the maintenance energy,

and Y is the observed growth yield,

Y_G is the true growth yield (49).

One can more easily study the changes in the behavior and physiology of microorganisms as their position in their growth curve changes by employing continuous culture than by using batch culturing methods. Continuous culture has been important in studies of the highly aerobic azotobacters, the most studied of the nitrogen-fixing bacteria, since Málek (1952) and Macura and Kotkava (1953) made use of it to study morphological changes of Azotobacter chroococcum with varying growth rates (50, 51). Chemostat culture investigations on these bacteria carried out subsequently have centered specifically around their nitrogen-fixing

ability and the effects of varying the partial pressure of oxygen above the cultures on nitrogen fixation and cellular physiology.

Ierusalimskii et al. (1961) were of the first to investigate the physiology of Azotobacter in flow culture (52). They noted that the biomass variation curve in flow culture of A. vinelandii resembled a typical chemostatic curve in that the size of the biomass remained constant over several increasing dilution rates and then decreased at higher dilution rates; moreover the chemical composition of the cells varied with the dilution rate. In particular, the amount of RNA per cell increased significantly with increasing dilution rate. The DNA content, however, remained nearly constant and was independent of the dilution rate.

Lisenkova and Khmel (1967) examined the effect of cultural conditions on cytochrome content of A. vinelandii cells in continuous culture (53). They showed that cytochrome synthesis was optimal at a particular aeration rate and that at higher or lower rates the concentrations of cytochromes b and c in the cells fell. Also, the cytochrome content decreased in cells grown in iron-deficient medium as would be expected since cytochromes contain iron. As the rate of growth of cells increased so did their cytochrome content. Nitrogen-fixing cells contain

more cytochrome than cells grown in a medium containing ammonia (i.e. a non nitrogen-fixing situation). This seems reasonable since nitrogen-fixing Azotobacter cells utilize more carbon per unit of biomass than cells not fixing nitrogen; an effect probably due to the fact that extra reducing power is needed to reduce dinitrogen to ammonia.

Probably the first studies relating to the effect of oxygen on nitrogen fixation by Azotobacter in continuous culture were done by Khmel and Ierusalimskii in 1967 (54). In general, they discussed the effects of changing growth conditions in continuous culture on the composition of A. vinelandii. For some time prior to this, it had been known that Azotobacter spp. were inhibited by high pO_2 (55). The above researchers confirmed this in continuous culture and showed that excessive aeration could "poison" such cultures. Moreover they noted that the economy factor, the ratio of biomass to the amount of carbon source consumed, was highest when the culture was under oxygen-deficient conditions at all flow rates. The aeration requirement increased with dilution rate. The growth-limiting factors; the carbon source and the oxygen, "switched" during the continuous culture of Azotobacter at different dilution rates. All these observations were discussed in detail, later, in a review by Hill et al.

(1972) (56).

Aiba et al. (1967) reported low yields in continuous cultures of A. vinelandii limited by the carbon-energy source (57, 58). This was interpreted as indicating a high maintenance coefficient. The consumption of glucose, the carbon source in this case, was assumed to be a sum of that principally due to cellular growth and that due to cellular maintenance which involves the hypothetical phenomenon of respiratory protection to be discussed later.

Khmel and Andreeva (1967) used A. vinelandii cells in continuous culture grown with ammonium sulphate as the fixed nitrogen source and found that the highest rate of growth obtainable was much higher than under conditions of nitrogen fixation (59). The economic coefficient increases with an increase in the value of the dilution rate from 0.1 to 0.4 h^{-1}; with a further increase in dilution rate, however, its value falls. They also studied nucleic acid content of the cells under different conditions and saw that the RNA content in cells growing in ammonia was much higher than in cells of a nitrogen-fixing culture with the same growth rates. They also observed a linear relation between the growth rate of the cells and the RNA content while the DNA varies only slightly with dilution rate.

Dalton and Postgate (1969) examined the growth and physiology of A. chroococcum in continuous culture as well as the effect of oxygen on growth (2, 60). It was seen that due to the hypersensitivity of Azotobacter to oxygen, less intense aeration was required for the initiation of growth the less dense the starting inoculum. Ammonia-grown cultures did not show any appreciable sensitivity to oxygen and it seemed that the lower the pO_2, the more efficient was nitrogen fixation, while growth was inhibited at high pO_2's no matter how dense the culture was. Carbon - and phosphate-limited cultures were even more oxygen sensitive; excess oxygen being lethal in the case of phosphate-limitation. To explain their results, Dalton and Postgate suggested that two mechanisms existed in the cell to protect the oxygen-sensitive components of nitrogenase. One was the augmented respiration to scavenge excess oxygen and the other was a conformational change of the nitrogenase state that prevented the enzyme from being damaged by oxygen. Also, A. chroococcum, grown in continuous culture without fixed nitrogen had a chemical composition characteristic of so-called nitrogen-limited populations at varying dilution rates in suitable media. Carbon - and phosphate-limited populations had different compositions. Carbon-limited populations

using ammonia under argon were not oxygen sensitive for the most part. Nitrogen fixation showed a maintenance coefficient of about twice that seen with ammonia assimilation, apparently because nitrogenase is so oxygen sensitive that excess oxygen near the nitrogenase site must be "burned off"; hence, the high maintenance coefficient. The authors proposed that carbon-limited cultures were hypersensitive to oxygen because they did not have the necessary raw material to employ in burning off the excess oxygen around the nitrogenase site. When cultures were phosphate-starved, the resulting ATP/ADP ratio alteration exerted a restraint on respiration which antagonized the respiratory protection. This suggestion assumed that respiration in Azotobacter is always coupled to ATP synthesis. It was also hypothesized that when all soluble components of the medium like the carbon, phosphate and sulphate sources, as well as oxygen, are in excess then the culture must be nitrogen-limited by the process of elimination. However, since solubility considerations made it appear unlikely that the physical availability of dissolved nitrogen was limiting, the organisms must be regulating their fixation rate themselves. Thus, the populations were subject to an intrinsic nutrient (nitrogen) limitation (56).

Andreeva and Khmel (1970) continued work on the kinetics of oxygen consumption of A. vinelandii in batch and continuous cultures (61). They used oxygen-limited cultures of A. vinelandii to study metabolic activity and chemical composition and also to obtain a value for the K_s of oxygen (K_s = 6.9 x 10^{-6} moles 0_2/ liter).

The results of much more research done between 1969 and 1972 was summarized in a review by Hill, Drozd and Postgate (1972) who discuss how studies with A. chroococcum and other azotobacters have clarified several aspects of the physiology of nitrogen fixation by these obligate aerobes (56). Also the work done by Hill et al. has helped to elucidate much of the "hodge-podge" of information collected over the years in terms of nitrogenase and its oxygen sensitivity. Their work has revealed that respiration in azoto-bacters apparently plays the novel physiological role of protecting oxygen-sensitive enzymes from oxygen damage. It has also indicated that ordinary batch cultures begin their growth as nitrogen-limited pop-ulations whose growth is limited endocellularly by the rate of nitrogen uptake. Finally it has led to the discovery of an unusual short-term control process which involves a rapid, reversible, "switch-on" and

"switch-off" of nitrogenase activity in responce to oxygen.

Since 1972, Drozd et al. have done chemostat studies on fixed-nitrogen source effects on nitrogen fixation, membranes, and free amino acids in A. chroococcum (62). They showed most importantly that free ammonium ions as well as nitrate ions were capable of repressing nitrogenase activity when added to a medium of a nitrogen-fixing continuous culture of A. chroococcum.

Recently, Lees and Postgate (1973) examined the nutritional status and behavior of A. chroococcum in oxygen-limited chemostatic culture; the first time oxygen-limitation had been looked at specifically (4). Among other things, this study showed an inverse relationship between biomass and dilution rate. This was accounted for largely by increased polysaccharide and poly-β-hydroxybutyrate content (i.e. the storage materials) at the lower dilution rates: the percentage of this storage material being linearly related to the residence time of the organisms in the culture. This work also confirmed the hypersensitivity of phosphate-limited cultures to excess oxygen.

Thus, continuous culture has proven to be a very useful tool in studies of the changes in behavior and

physiology in Azotobacter spp. which are directly correlated to their nitrogen fixing properties. It has helped enormously in resolving properties and functions of nitrogenase, while conversely Azotobacter spp. have been useful organisms for studying and enlarging the theory behind continuous culture.

MATERIALS AND METHODS

MATERIALS AND METHODS

ORGANISM

Azotobacter chroococcum ATCC 7493 was used throughout the course of this investigation.

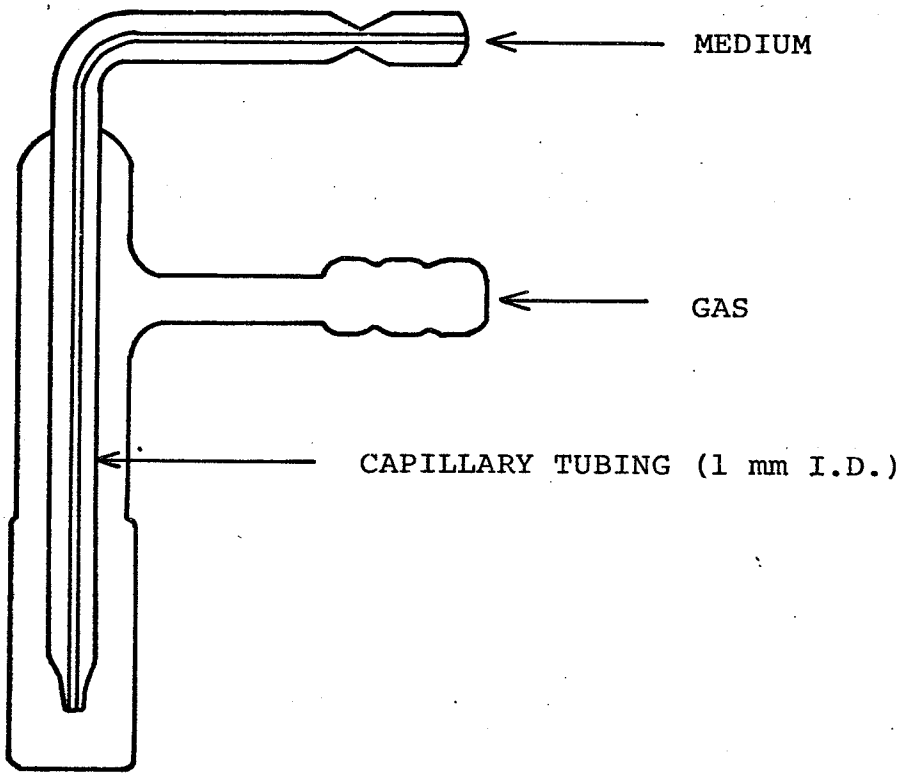
GROWTH AND MAINTENANCE OF CULTURES

The medium used for batch and continuous culturing was modified nitrogen-free Burk's medium ("mannitol B_6") as described by Dalton and Postgate (1969) (2, 60).

1) *Batch cultures* - Lyophilized cultures were revived according to the procedure outlined in the instruction sheet accompanying the ATCC *Azotobacter* preparations and subculturing was carried out aseptically every month in 125 ml flasks containing about 50 ml medium. The incubation temperature was 28°C. Initially, after inoculation, flask cultures were shaken gently at about 120 r.p.m. on shakers to prevent inhibition of initiation of growth by overaeration that might have occurred if the shaking had been more vigorous (see

Dalton and Postgate, 1969) (2). After the cultures had grown to a density great enough to allow for more intense aeration by agitation (ie. after about 24-48 hrs. depending upon the size, and the position in the growth phase, of the inoculum), the cultures were transferred to faster shakers where they were allowed to grow for another day or two. The stock cultures were then removed from the shakers and stored in a cold room maintained at 5-10^{o}C until they were needed as inocula for continuous cultures or subcultures.

2) Continuous cultures - The continuous culture apparatus based on the design of Baker (1968) (63) was used and maintained at 30^{o}C and was essentially the same as that employed by Lees and Postgate (4) and described by Dalton and Postgate (1969) (2, 60) with two exceptions. One was that the magnetic stirrer used to agitate the culture was a transistorised instrument capable of rotating the 1.5 inch teflon-coated stirring bar at fixed speeds of up to 1750 r.p.m. The second exception was that the medium inlet tubing of the glass medium and gas inlet port, illustrated below, was made from capillary tubing with an internal diameter of 1 mm rather than 5 mm I.D. ordinary glass tubing. This modification helped to discourage growback by allowing



a higher flow rate (about 1 m/min at a dilution rate (D) of 0.25) of sterile medium into the culture along the last 5½" of the path from the medium reservoir to the culture vessel. Growback along the medium feed line is normally encountered when tubing of wider I.D. is used and this is undesirable since, under this condition, the culture is being continually freshly inoculated with organisms in the incoming medium instead of being supplied with sterile medium. Moreover, the medium and gas inlet port is structured such that the

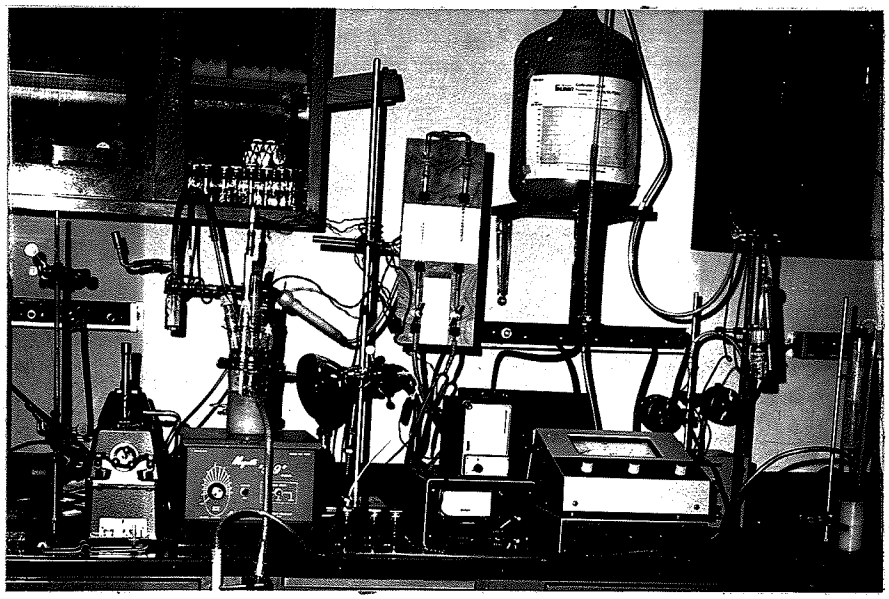
gas flows over the medium, thus deflecting microbial aerosol and reducing the risk of back contamination to the medium reservoir (Baker, 1968) (63). The continuous culture apparatus is pictured in Fig. (1 a).

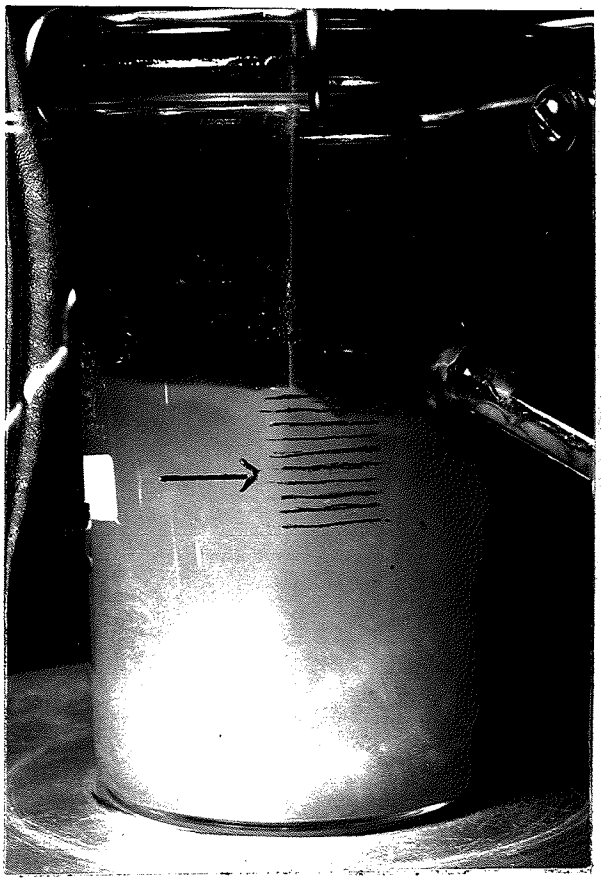
As with the batch cultures, care was taken not to overaerate a freshly inoculated continuous culture. An approximate 50 ml stock culture was used to inoculate aseptically the continuous culture vessel containing about 200 ml of medium and the culture was allowed to grow up overnight as a batch culture with a stirring rate of 300-600 r.p.m. and a gas phase of air. The following day, the dilution rate was set to 0.10 h^{-1} and the stirrer was switched to 1750 r.p.m. whereupon the whole culture was forced into a highly aerated "vortex" resembling the conditions in a domestic homogeniser (about 20% of the solution volume was occupied by air bubbles) as is pictured in Fig. (1 b). (The arrow marks the static level of the culture corresponding to a culture volume of about 240 ml). As a result, the dissolved oxygen, as measured by a submerged galavanic electrode, rapidly rose from 0 μM to well over 100 μM. The air above the culture was then diluted with N_2 until the dissolved 0_2 was about 20 μM from which value it sank in about 30 minutes to zero as the organisms adapted

Fig. 1. The continuous culture of Azotobacter chroococcum.

(a) The continuous culture apparatus.

(b) The intense agitation of the culture in the vessel. Arrow indicates rest level of culture.





to a higher 0_2 consumption. The N_2 supply was then decreased until the dissolved 0_2 was again about 20 μM from which value it again sank to zero. After less than 24 hrs of manipulation of the N_2 supply the culture was able to maintain a dissolved 0_2 concentration of zero when the gas phase above the culture was simply air. The air could then be supplemented with 0_2 in one-day steps of 10% v/v up to, in some cases, a final volume of 50% 0_2 without any detectable (or just barely detectable) dissolved 0_2 remaining in solution. (see Results)

MEASUREMENT OF DISSOLVED OXYGEN CONCENTRATION IN THE CULTURE

Measurement of the dissolved oxygen concentration in the culture was done using a steam sterilizable galvanic or self-polarising type of oxygen electrode connected to an oxygen meter; both of which were obtained from L.H. Engineering Co. Ltd., Bells Hill, Stoke Poges, Bucks, England. The meter in turn was connected to a Rustrak recorder (Rustrak Instrument Div., Gulton Industries, Inc., Manchester, N.H., U.S.A.) whose chart paper was arbitarily set to move at 1"/hr. The electrode was calibrated before use by setting the

needles of both the meter and recorder to read zero on scale when the electrode was immersed in a nitrogen-saturated medium and by setting same needles to read 100 when the electrode was immersed in a medium containing about 100 μMO_2. An electrode already in use could be tested from time to time for continuing sensitivity by aseptically lifting it out of the culture and observing if it responded properly to exposure to the known oxygen concentration in the gas phase above the culture. Moreover, the zero oxygen reading on the scale could be substantiated at any time by simply "flushing out" the culture with N_2 and observing whether the meter needle still read zero. If, after extended use, the polypropylene membrane of the electrode became coated with a bacterial film or was broken or the sensitivity of the electrode was otherwise lost, it was replaced by a new sterile electrode. Damaged membranes on electrodes were easily replaced.

MICROSCOPICAL CHECKS AND TESTS FOR PURITY

Samples from the continuous culture were examined under the microscope from time to time to check for possible cell lysis. The purity of the continuous culture was checked once a week by aseptically streaking

a loopful of the contents of the culture onto a trypticase soy agar plate which was incubated at 28°C for a few days and then examined for colony types to ensure that only Azotobacter chroococcum colonies were present.

SAMPLING PROCEDURE AND STORAGE OF SAMPLES

Duplicate 10-12 ml samples from the continuous culture vessel were taken aseptically into 20 ml storage bottles when required (Baker, 1968) (63). The pH of the samples was then determined. When immediate analysis of the samples for dry weight, for instance, was not convenient, about 0.05 ml concentrated H_2SO_4 was added to each sample to arrest metabolism. The bottles were then tightly capped and stored at 5-10°C.

CHEMICALS

Reagent-grade chemicals obtained from standard chemical companies were used for all media and analyses throughout this investigation.

ANALYTICAL PROCEDURES

1) BIOMASS - The dry weight of a culture sample was determined by centrifuging a 10 ml volume

of the sample in a 12 ml conical centrifuge tube, washing the cells twice in glass distilled water and then transferring them in distilled water to a pre-dried and weighed aluminum foil weighing dish which was subsequently placed in a drying oven until the following day when it was transferred to a desiccator and then reweighed.

2) $\underline{CO_2}$ - The measurement of the CO_2 output of the culture was carried out as described in Lees and Postgate (1973) (4) and was always performed immediately before culture samples were taken.

3) MANNITOL -
4) PROTEIN -
5) POLYSACCHARIDE -
6) POLY-β-HYDROXYBUTYRATE- (PHB)

The methods for these determinations were described in Dalton and Postgate (1969) (2, 60).

7) RNA - The method used for this analysis was as described in Dalton and Postgate (1969) (60) except that ribose was used as a standard instead of yeast RNA.

8) NITROGEN CONTENT - The nitrogen content of the cells was determined by a micro-Kjeldall technique followed by analysis for ammonia by microdistillation

into 1.0% boric acid containing a methyl red/brom cresol green indicator (64).

OXYGEN SOLUTION RATE ESTIMATIONS

1) SULFITE OXIDATION METHOD - The sulfite oxidation method of Cooper, Fernstrom, and Miller (1944) (65) was used to estimate the oxygen solution rate (thus expressed as the SOX value) into a volume of water in the continuous culture vessel which was approximately equal to that of the culture itself. The stirring rate was 1750 r.p.m.; the normal rate of agitation used throughout the course of this study. The gas phase was air.

2) OXYGEN ELECTRODE ("kz") METHOD - The rate of oxygen supply to the culture was also assessed using an oxygen electrode method whereby an oxygen electrode-meter-recorder combination was used to monitor the rate of oxygen solution into an azide-killed Azotobacter culture. A 1.5 M azide solution was prepared in an Azotobacter culture which had a dry weight of 1.30-1.40 mg/ml. About 250 ml of this was added to the continuous culture vessel in which an 0_2 electrode was immersed. While the suspension was being stirred at the vortex rate of 1750 r.p.m., air was fed into the

system at about 200 cc/min. When the solution was saturated with air, the needles of the oxygen meter and recorder were set to read 100 on their scales. The air supply was then replaced by a nitrogen gas supply which was fed into the dead culture until it was nitrogen saturated. The meter and recorder needles were then set to read zero. At this point, the stirrer was turned off and air was again fed into the system for about 5 mins in order to flush out the nitrogen gas phase. The stirring rate was then suddenly turned back up to 1750 r.p.m. At the same time, the chart paper of the recorder was moving at 12"/min. Thus, the rate of solution of oxygen into the culture was monitored by the recorder as the air displaced the nitrogen from the solution. The same experiment was repeated using both pure water and a 1.5 M aqueous azide solution in place of the azide-killed culture.

It was reasoned that the rate of 0_2 solution here could be represented by the equation;

$$\frac{-\,dz}{dt} = k.z$$

where k is a constant, dependent on many factors, including interface resistance, which determines the rate of 0_2 solution independently of concentration gradients, and where z represents the difference between

the dissolved oxygen concentration at time "t" the maximum possible O_2 concentration (ie. the saturation concentration which, since air was the solute, was taken to be 260 μM). Thus, when the equation is integrated, it yields:

$$t_1 - t_2 = \frac{1}{k} \ell n \left(\frac{Z_2}{Z_1}\right)$$

so that a plot of ℓnZ vs t should yield a straight line with slope k. Different Z values at different times were read from the chart paper which monitored the increase in concentration of oxygen with time.
ie.

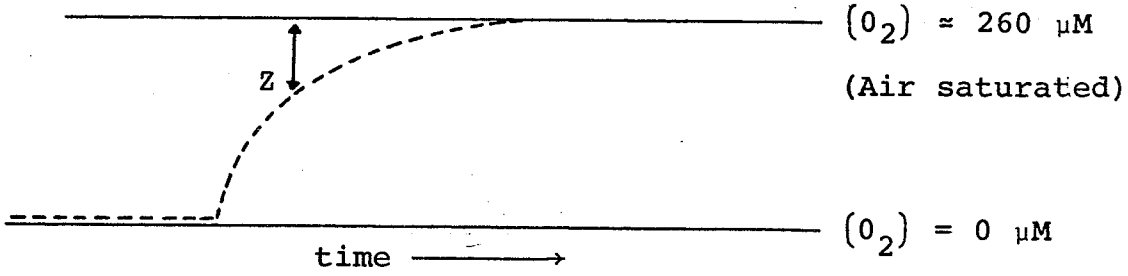


CHART PAPER MOVES AT 12"/min

It should be mentioned that oxygen uptake experiments using an oxygraph were performed to determine the concentration of azide needed to stop immediately all oxygen uptake by the Azotobacter culture. This concentration was found to be about 1.0 - 1.5 M. It was decided

to poison the cells with azide rather than to kill them by addition of other toxic compounds or by heating, for instance, because azide addition apparently did not cause any overt change in the appearance or physical properties of the cultures. Its addition did not seem to affect the viscosity of the culture, cause a precipitate to form, or otherwise alter the culture in any way as to change its solvent characteristics with respect to oxygen. On the other hand, killing the cells by heating would cause protein denaturation and cell lysis while mercury poisoning, for example, would produce a precipitate.

RESULTS

RESULTS

GENERAL GROWTH CONDITIONS AND RESPONSES

Continuous cultures of Azotobacter chroococcum were grown at various dilution rates and under varying atmospheric oxygen partial pressures. The incubation temperatures was 30°C and the normal stirring rate was 1750 r.p.m. thus providing intense agitation of the culture. The purity of the cultures under all conditions was confirmed (see Materials and Methods). The dissolved oxygen tension was found to be zero (or nearly so) in cultures grown under the following conditions:

Dilution rate (D)	% Oxygen in Atmosphere above culture
~ .10	20
~ .10	30
~ .15	20
~ .15	30
~ .15	40

Dilution rate (D)	% Oxygen in Atmosphere above culture
~ .22	20
~ .22	30
~ .22	40
~ .22	50
~ .29	20
~ .29	30
~ .29	40

The oxygen tension was also found to be zero in continuous cultures grown at below vortex stirring rates of 300 r.p.m. at a dilution rate of about 0.10 under atmospheres of air and 30% 0_2. This condition of mild agitation was imposed on the culture at this one dilution rate in order to observe if and how there would be differences in the growth and behavior between the slowly stirred cultures and the intensely agitated cultures (see Fig. 3 and Table I).

It was found necessary to raise the mannitol concentration in the incoming fresh medium to 20 g/ℓ from the normal 10 g/ℓ when growing cultures at D ≈ 0.10 under a gas phase of 30% 0_2 after it was discovered

that carbon-(mannitol)-limitation of growth would occur if only 10 g/ℓ was supplied.

Cultures grown at a D ≃ 0.15 under 50% 0_2 were also found to be carbon-limited. The oxygen tension in these cultures rose well above zero and "wash-out" began (as is represented in Fig. 3 by a dry weight decrease).

Cultures grown at a D ≃ 0.29 under 50% 0_2 were also found to "wash-out" as the oxygen concentration in the culture rose significantly above zero.

It should be noted here that although cultures were also grown at a D ≃ 0.05, meaningful data comparable to that obtained at higher dilution rates could not be obtained at this low dilution rate since both carbon-limitation and much cell lysis occurred when the average residence time of the organisms in the culture was so long.

Moreover, microscopic checks revealed that a small but noticeable amount of cell lysis also appeared in cultures grown at D ≃ 0.10 while a slightly lesser quantity occurred at D ≃ 0.15.

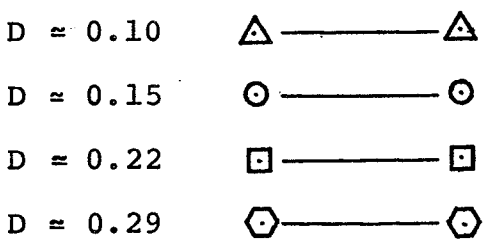
VARIATIONS IN pH WITH CHANGING CULTURAL CONDITIONS

The variation at different dilution rates in the pH of continuous cultures of Azotobacter chroococcum as a function of oxygen concentration in the gas phase above the culture is plotted in Fig. 2. It appears that, on the average, the pH in the culture tends to drop with increasing oxygen partial pressure in the atmosphere. The dashed lines on the graph showing pH increases represent special situations where cultures are in different stages of "wash-out".

VARIATIONS IN BIOMASS WITH CHANGING CULTURAL CONDITIONS

The variation at different dilution rates in the biomass of continuous cultures of Azotobacter chroococcum as a function of 0_2 concentration in the atmosphere above the culture is plotted in Fig. 3. It appears that, at higher dilution rates anyway, the biomass increases with increasing oxygen percentage in the gas phase. Again, the dashed lines on the graph showing decreases in biomass represent special situations where the culture is washing out. It is also obvious from this figure that the lower stirring rates of 300 r.p.m. at $D \simeq 0.10$ resulted in much lower

Fig. 2. Variation in pH of continuous cultures of Azotobacter chroococcum.



Dashed lines represent transitions to "unsteady" or "wash-out" states.

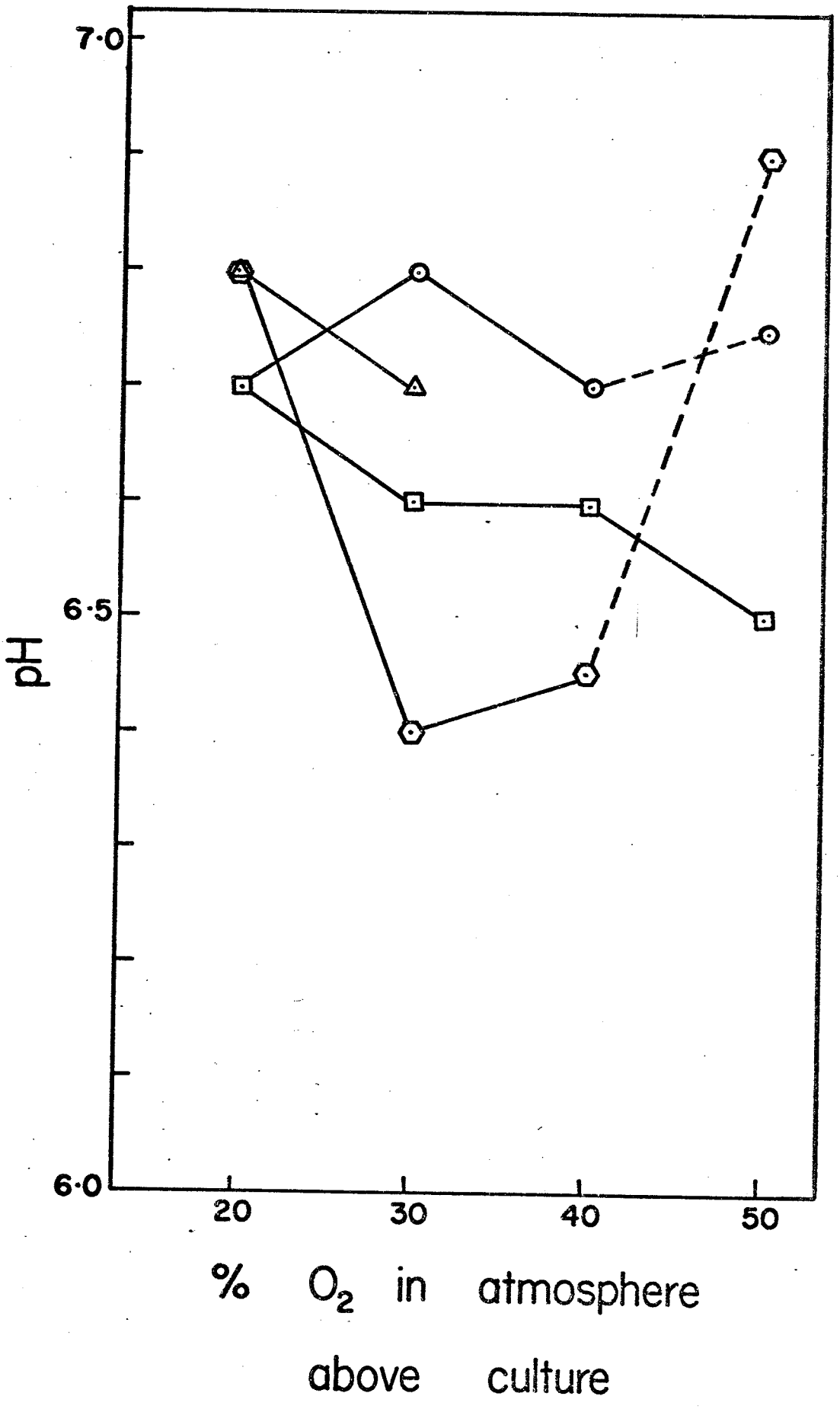
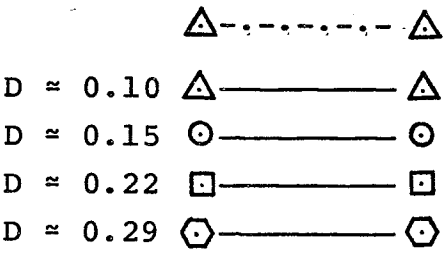
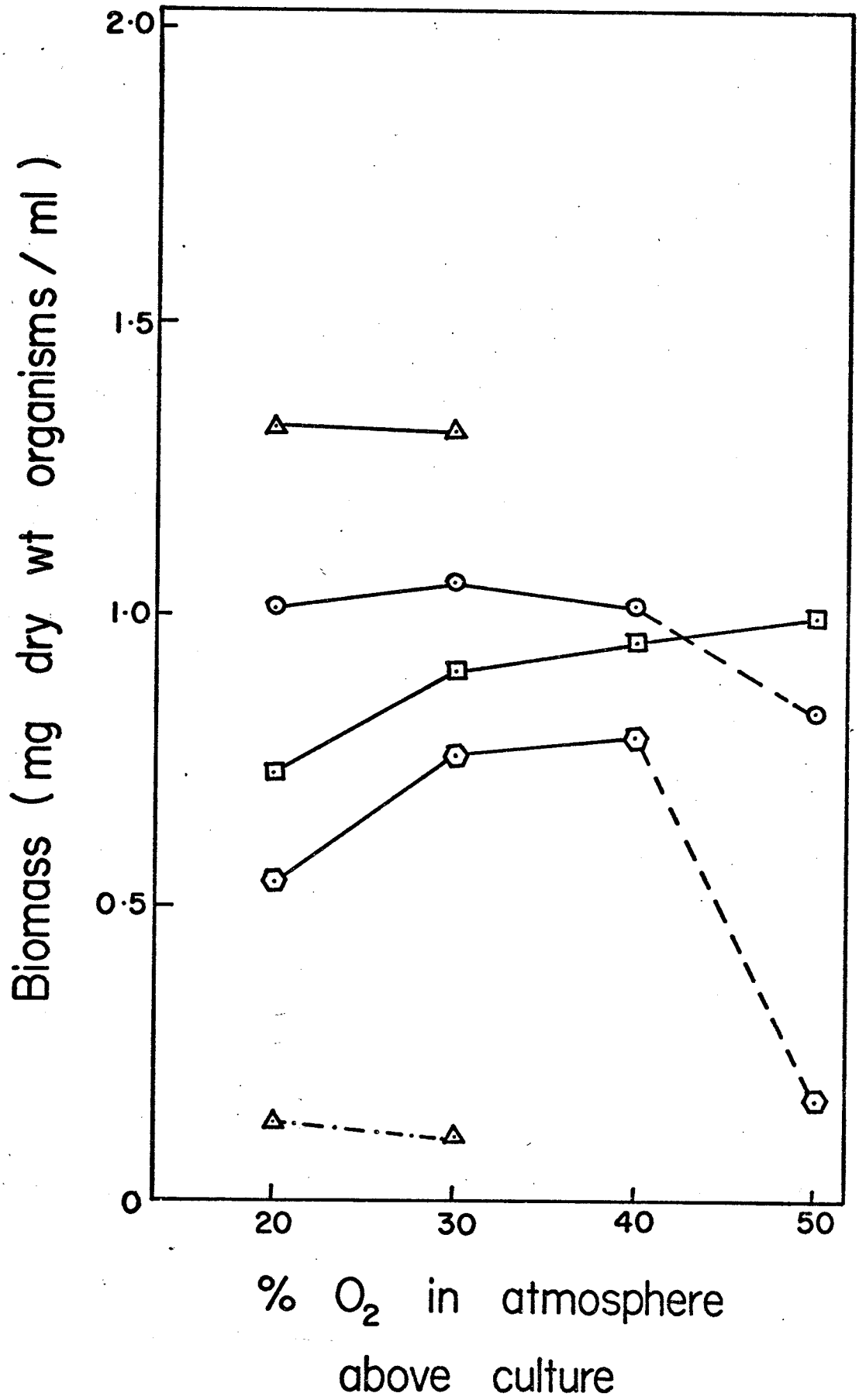

7·0
6·5
6·0
pH
20
30
40
50
% O_2 in atmosphere above culture

Fig. 3. Variation in biomass of continuous cultures of Azotobacter chroococcum (a).



Dashed lines represent transitions to "unsteady" or "wash-out" states.


2·0
1·5
1·0
0·5
0
Biomass (mg dry wt organisms / ml)
20
30
40
50
% O_2 in atmosphere above culture

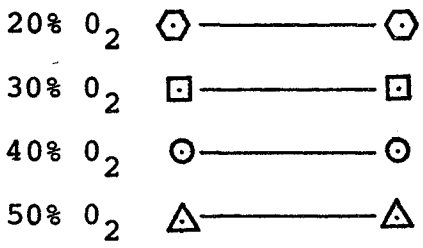
biomass values than those obtained at the normal stirring rate of 1750 r.p.m. at the same dilution rate.

The variation at different atmospheric oxygen concentrations in the biomass as a function of dilution rates is plotted in Fig. 4. In general, the biomass of Azotobacter continuous cultures seems to decrease steadily and quite sharply with increasing dilution rate, especially when grown under 20% 0_2. However, this rate of decrease is somewhat lower at the higher oxygen partial pressures of 30% and 40%. Again, the dashed lines represent cultures in "unsteady states" of "wash-out".

VARIATIONS IN CO_2 EVOLUTION RATE WITH CHANGING CULTURAL CONDITIONS

The variation at different dilution rates in the CO_2 evolution rate of continuous cultures of Azotobacter chroococcum as a function of oxygen concentration in the atmosphere above the culture is illustrated in Fig. 5. It is obvious that the rate of evolution of CO_2 by the culture increases markedly and linearly with increasing atmospheric oxygen concentration at all dilution rates. The dashed lines represent cultures in different stages of "wash-out".

Fig. 4. Variation in biomass of continuous cultures of Azotobacter chroococcum (b).



Dashed lines represent transitions to or from "unsteady" or "wash'out" states.

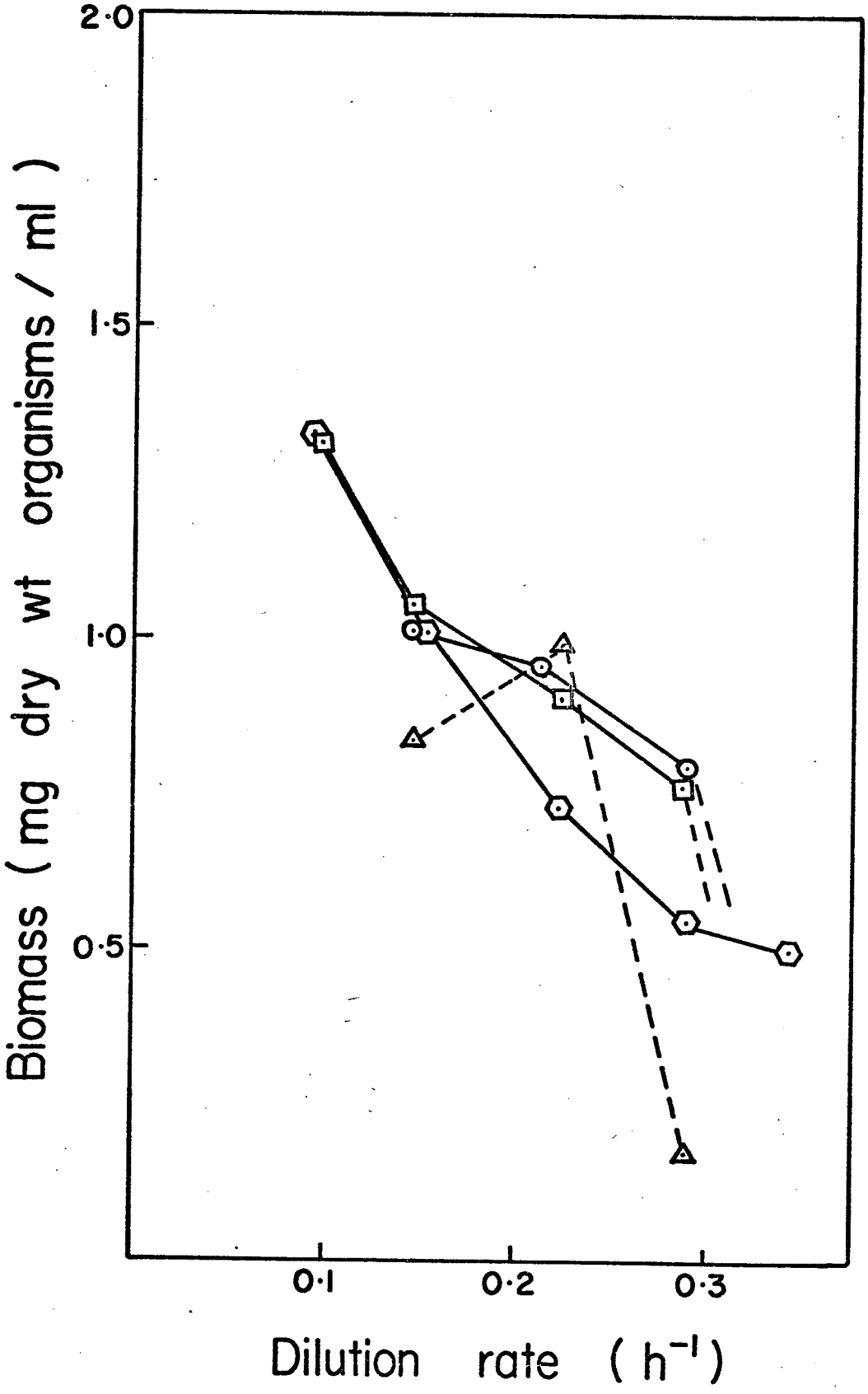
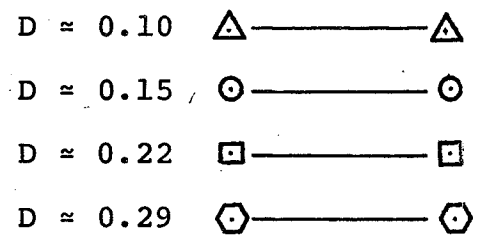

2·0
1·5
1·0
0·5
Biomass (mg dry wt organisms / ml)
0·1
0·2
0·3
Dilution rate (h⁻¹)

Fig. 5. Variation in CO_2 evolution rates per volume of continuous cultures of Azotobacter chroococcum (a).



Dashed lines represent transitions to "unsteady" or "wash-out" states.

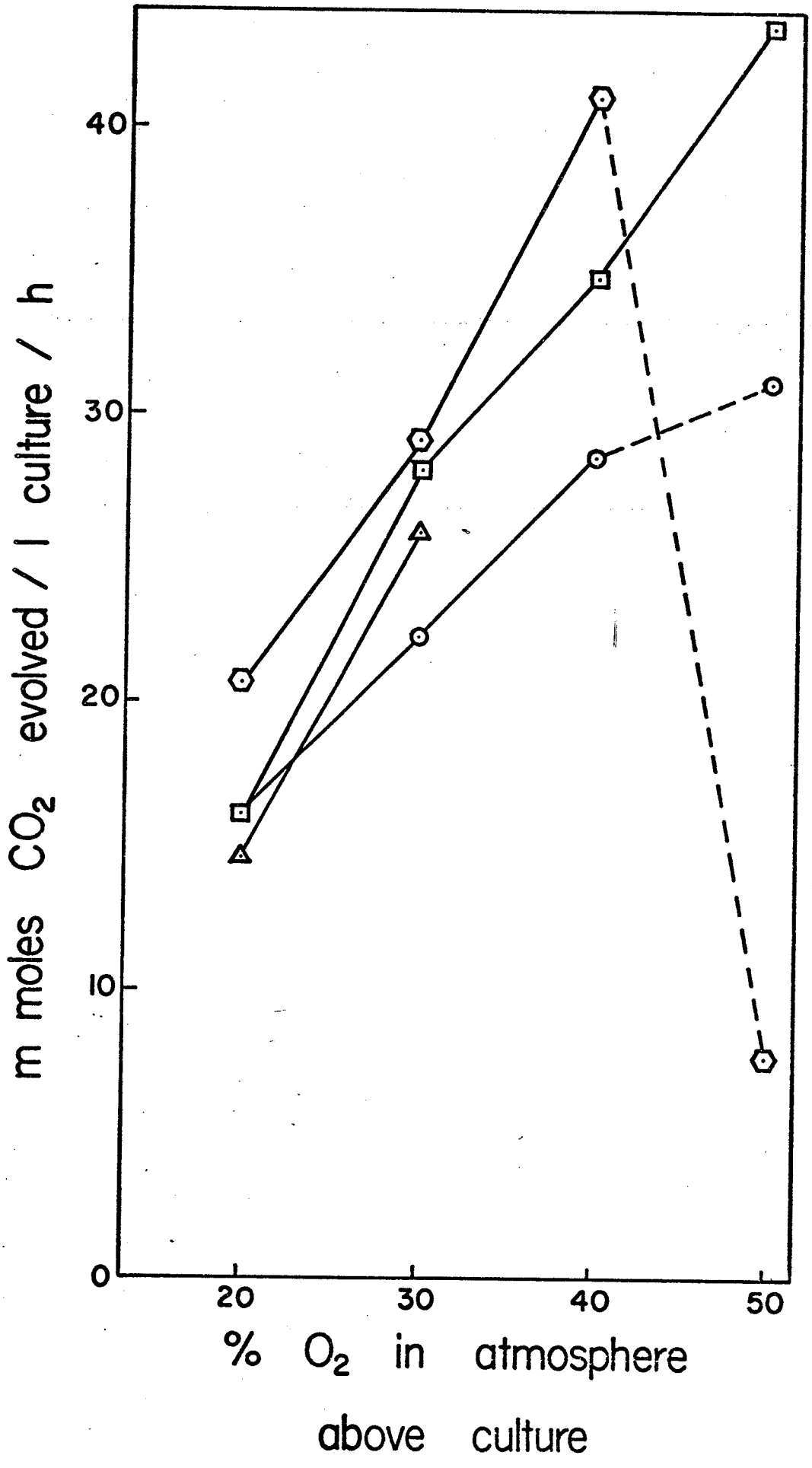
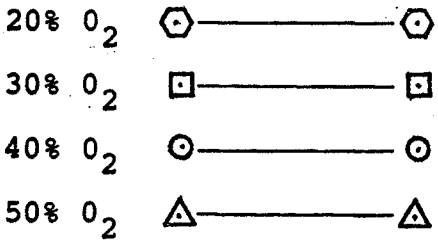

40
30
20
10
0
m moles CO_2 evolved / l culture / h
20
30
40
50
% O_2 in atmosphere above culture

Fig. 6 depicts the pattern of change in rate of CO_2 evolution with changing dilution rate at different atmospheric oxygen concentrations. In general, the CO_2 evolution rates increase with increasing dilution rates at all oxygen concentrations. The point on the graph indicating the CO_2 value at $D \simeq 0.10$ and 30% O_2 in the gas phase and which seems to be slightly "out", represents another special cultural condition where extra mannitol was added to the medium. The dashed lines denote conditions of culture "washout".

The variation at different dilution rates in the CO_2 evolution rate of a unit of biomass of continuous cultures of Azotobacter chroococcum as a function of O_2 concentration in the gas phase above the culture is shown in Fig. 7. The pattern is similar to that shown in Fig. 5; the CO_2 evolution rate of a milligram of biomass increases almost linearly with increasing O_2 partial pressure at all dilution rates. It is interesting to note that although the cultures are washing out at both $D \simeq 0.15$; 50% O_2 and $D \simeq 0.29$; 50% O_2, the rate of CO_2 production by each unit of biomass under these conditions does not decrease significantly if at all.

Fig. 6. Variation in CO_2 evolution rates per volume of continuous cultures of Azotobacter chroococcum (b).



Dashed lines represent transitions to or from "unsteady" or "wash-out" states.

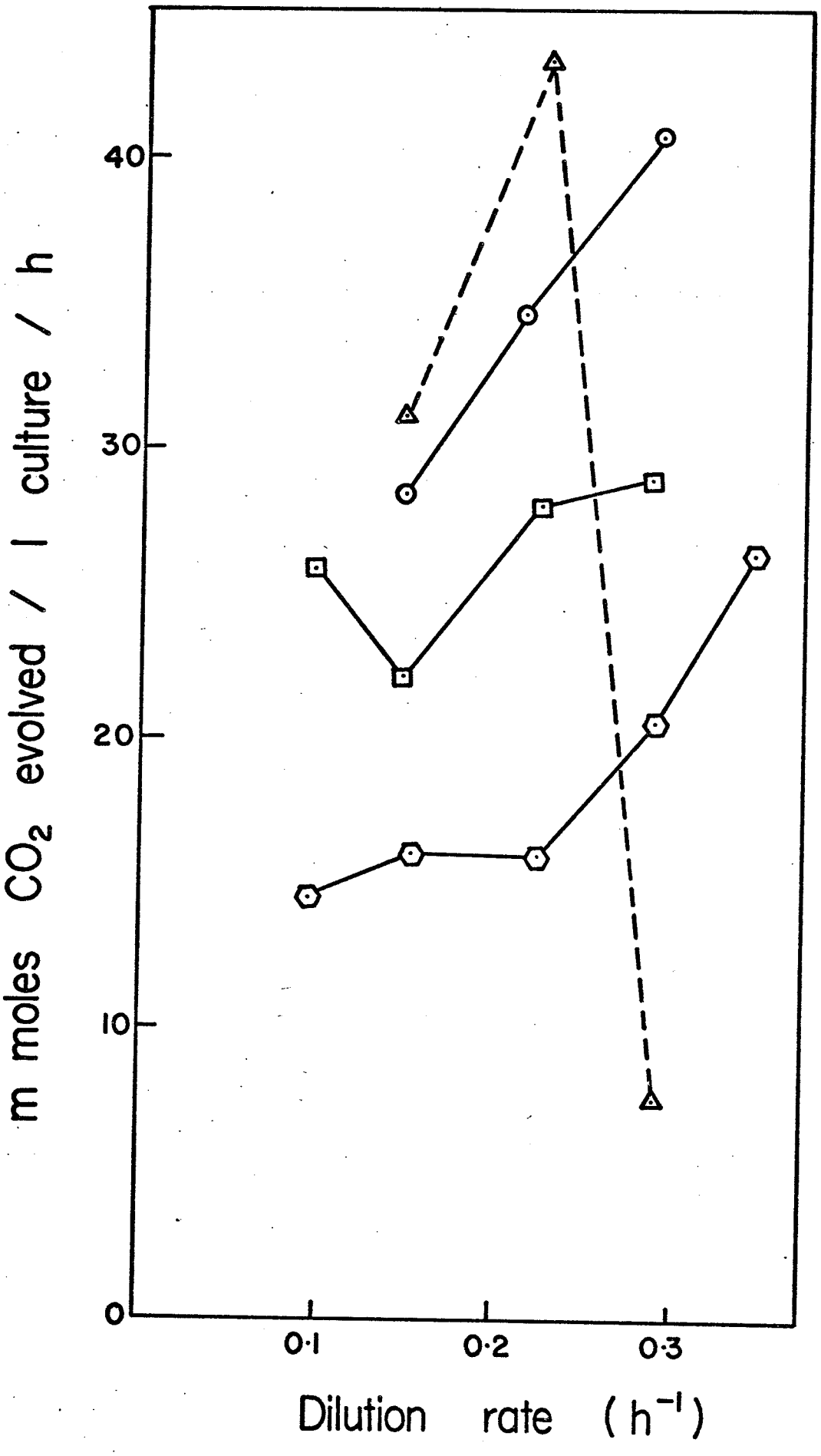
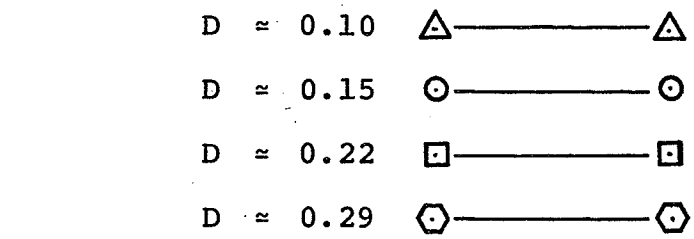

m moles CO_2 evolved / l culture / h
40
30
20
10
0
0·1
0·2
0·3
Dilution rate (h^{-1})

Fig. 7. Variation in CO_2 evolution rates per unit biomass of continuous cultures of Azotobacter chroococcum (a).



Dashed lines represent transitions to "unsteady" or "wash-out" states.

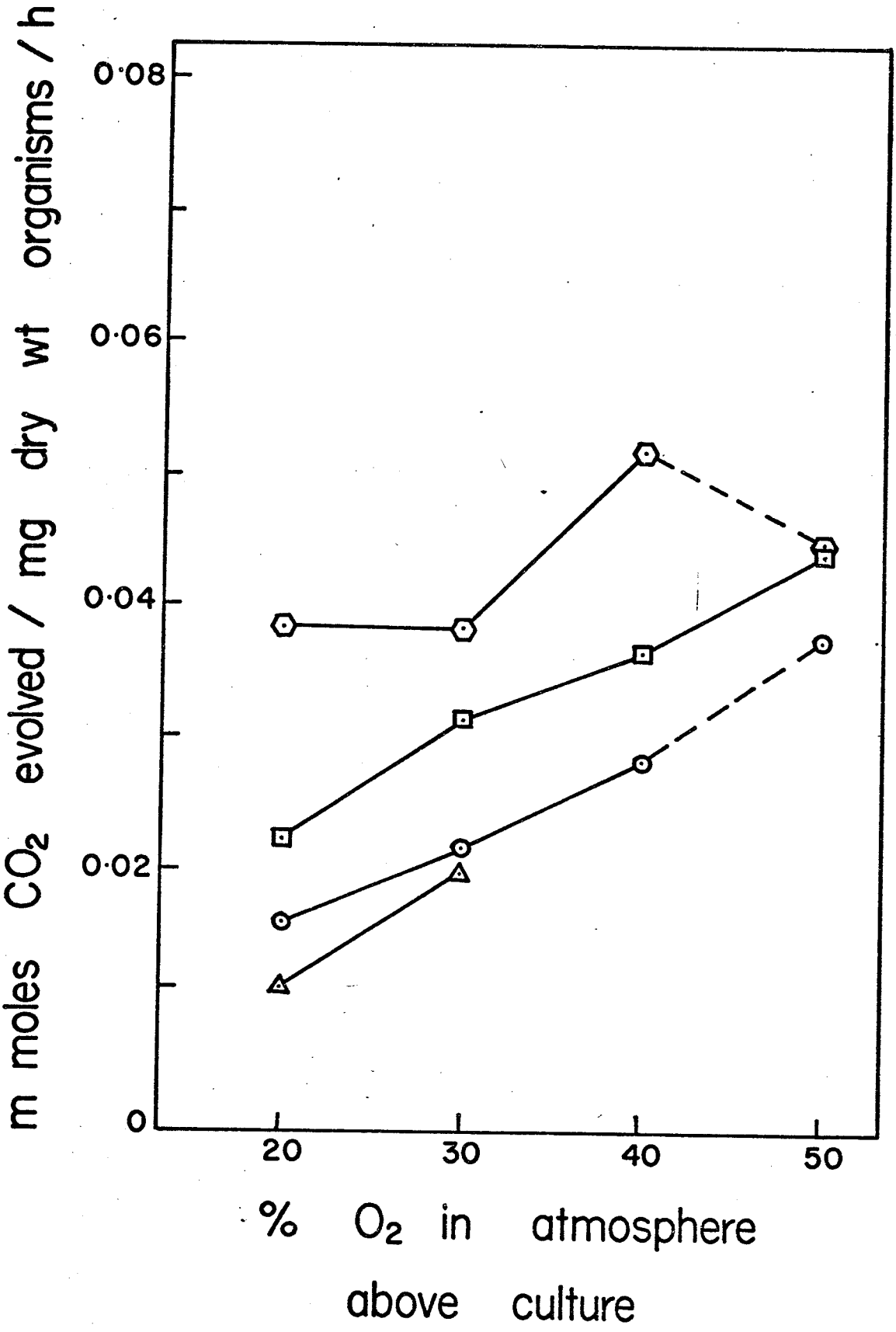

m moles CO_2 evolved / mg dry wt organisms / h
0·08
0·06
0·04
0·02
0
20
30
40
50
% O_2 in atmosphere above culture

Fig. 8 shows the change, at various atmospheric O_2 concentrations, in rate of CO_2 evolution by a unit of biomass with changing dilution rate. This rate increases almost linearly with increasing dilution rate at all concentrations of oxygen. Again, although the dashed lines symbolize unsteady "wash-out" states of the cultures, the corresponding rates of CO_2 evolution per unit biomass do not seem to be drastically affected.

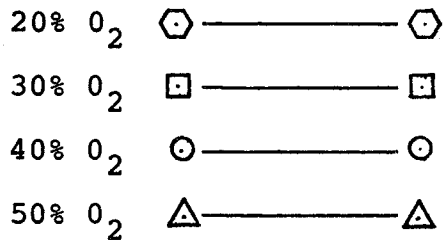
VARIATIONS IN AMOUNT OF RESIDUAL MANNITOL WITH CHANGING CULTURAL CONDITIONS

The variation, at different dilution rates, in the amount of mannitol ramaining in continuous cultures of Azotobacter chroococcum as a function of the % O_2 in the gas phase above the culture is plotted in Fig. 9. At all dilution rates, the amount of mannitol remaining in the culture decreases more or less linearly with increasing O_2 concentration. However, as the culture washs out at $D \simeq 0.29$ under 50% O_2, the residual mannitol increases, while at $D \simeq 0.15$ under 50% O_2, the culture is approaching both mannitol limitation and wash-out.

TOTAL CARBON INFLOW VS. TOTAL CARBON OUTFLOW

The rates of inflow of carbon to continuous cultures of Azotobacter chroococcum were compared to

Fig. 8. Variation in CO_2 evolution rates per unit biomass of continuous cultures of Azotobacter chroococcum (b).



Dashed lines represent transitions to and from "unsteady" or "wash-out" states.

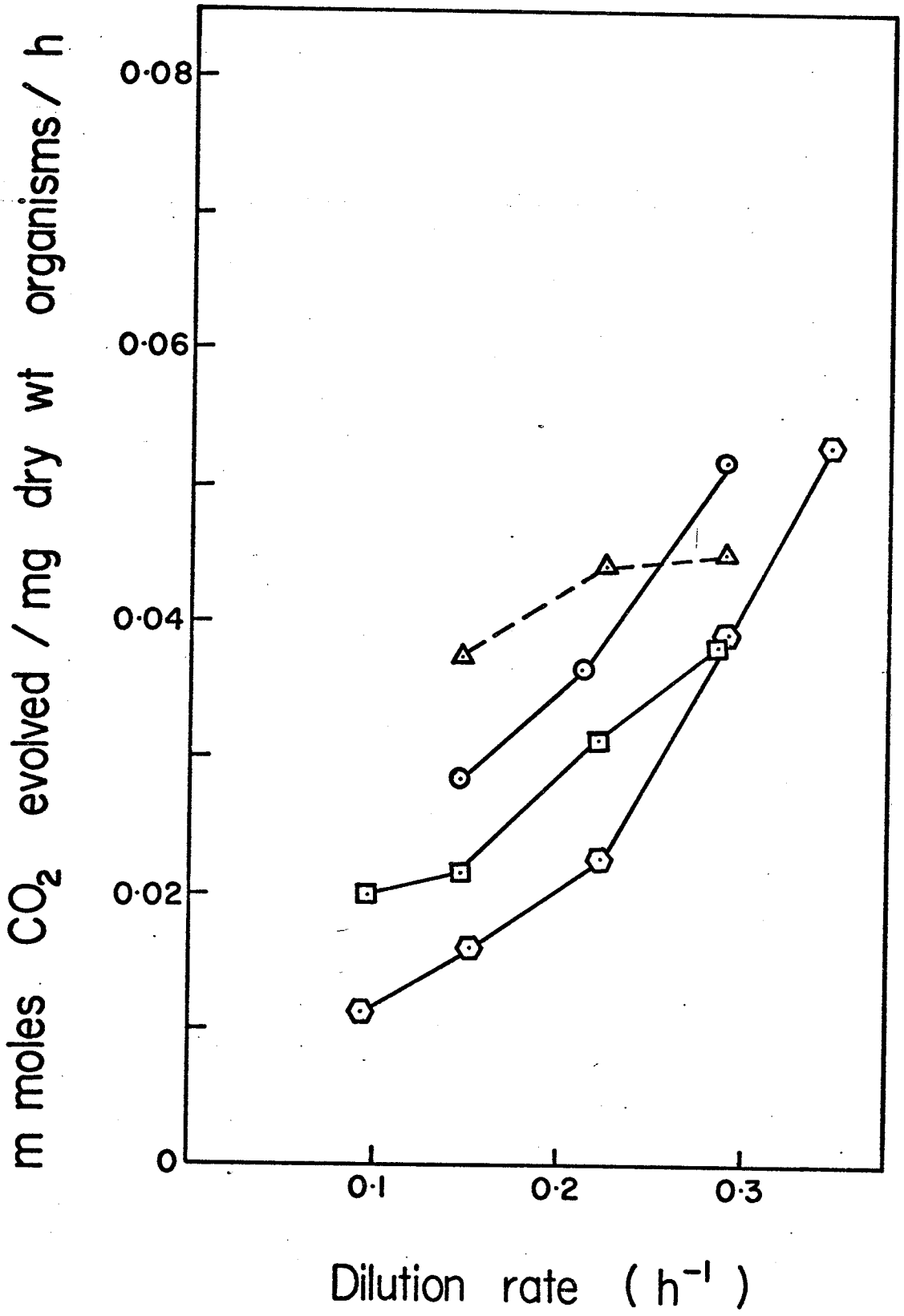
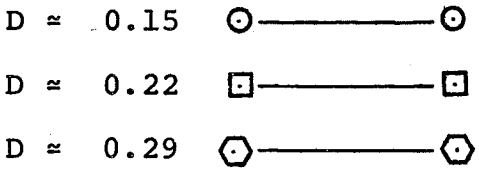
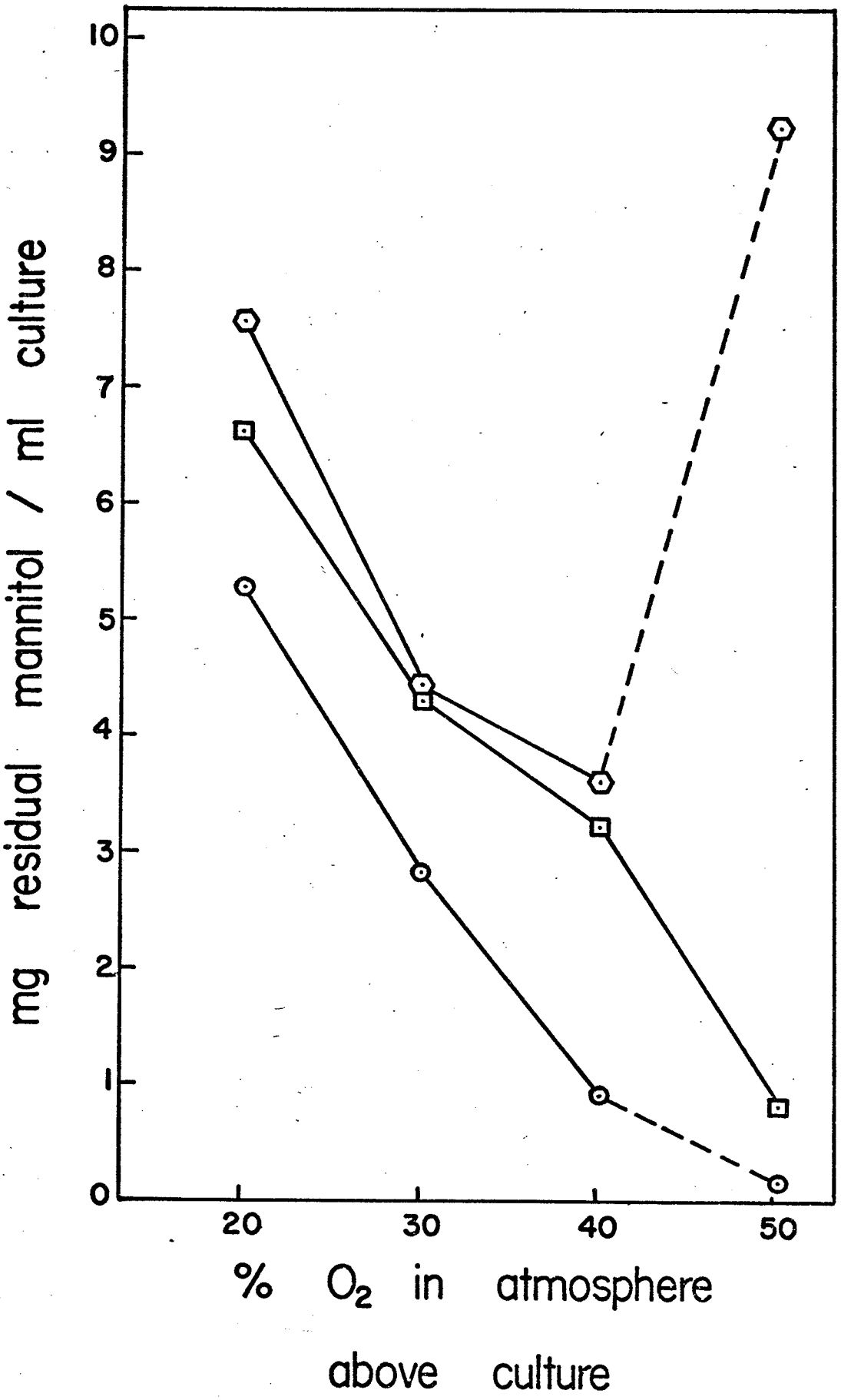

m moles CO_2 evolved / mg dry wt organisms / h
0·08
0·06
0·04
0·02
0
0·1
0·2
0·3
Dilution rate (h^{-1})

Fig. 9. Variation in residual mannitol concentration in continuous cultures of Azotobacter chroococcum.



Dashed lines represent transitions to "unsteady" or "wash-out" states.

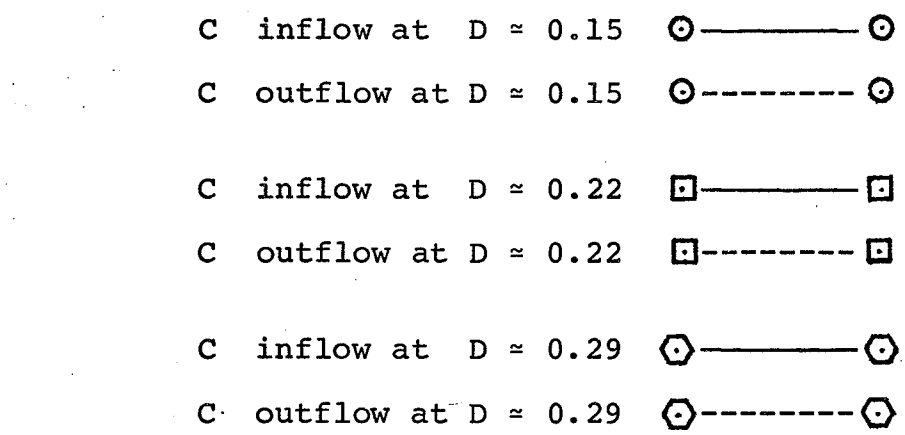

mg residual mannitol / ml culture
0
1
2
3
4
5
6
7
8
9
10
20
30
40
50
% O_2 in atmosphere above culture

the rates of outflow of carbon from the cultures at different atmospheric oxygen concentrations for each dilution rate (as shown in Fig. 10). The following explanatory table shows how these total carbon values were calculated.

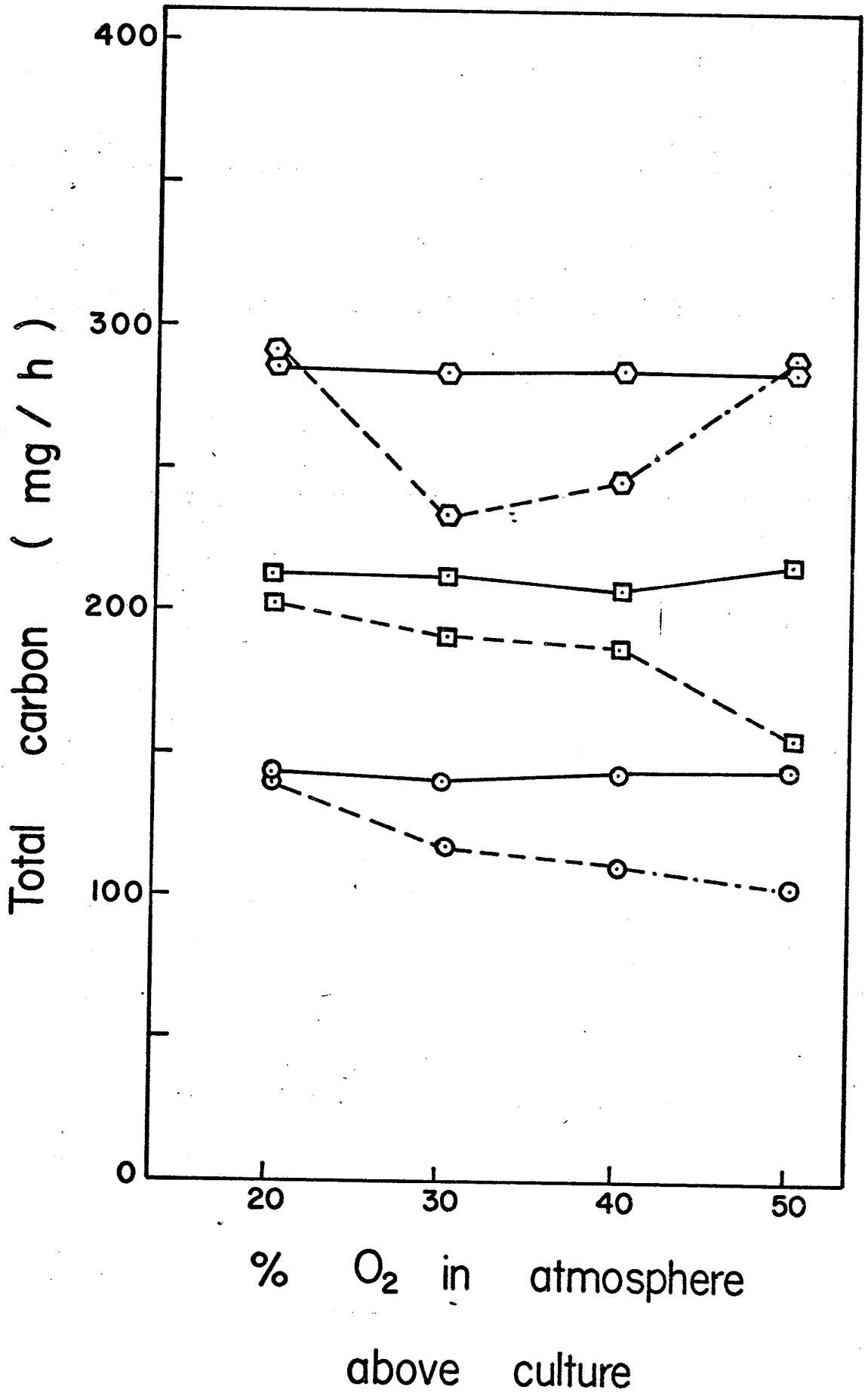
	CARBON SOURCE	RATE OF INFLOW OR OUTFLOW OF CARBON (mg C/h)
Inflow	MANNITOL	Rate at which mannitol carbon is going into the culture where mannitol is taken to be 40% carbon by weight
Outflow	MANNITOL	Rate at which residuel mannitol carbon is coming out of the culture
	CO_2	Rate at which CO_2-carbon is being evolved by the culture where 1 meq CO_2 ≡ 6 mg C
	CELLS	Rate at which bacterial carbon is coming out of the culture where cells are taken to be 40% carbon by weight
	TOTAL	

Although cultures growing under 20% 0_2 at all dilution rates show close carbon balances between rates of total incoming and total outflowing carbon, the difference between these two values tends to increase with in-

Fig. 10. Total carbon inflow to and outflow from continuous cultures of Azotobacter chroococcum.



Dashed-Dotted lines represent transitions to "unsteady" or "wash-out" states.


400
300
200
100
0
Total carbon (mg / h)
20
30
40
50
% O2 in atmosphere above culture

creasing oxygen partial pressures at all dilution rates. The dashed-dotted lines indicate those special situations where the cultures are in different stages of "wash-out" and it should be noted that in "washed-out" cultures at $D \simeq 0.29$ under 50% O_2, the rates of inflowing and outflowing carbon again show a close balance.

MACROMOLECULAR COMPOSITION

Macromolecular composition determinations were done on samples taken from continuous cultures at various dilution rates and at various atmospheric oxygen concentrations. The results are presented in Table 1. Because all values are in percent dry weight of organisms and due to the fact that lysis occurred in the culture at the lower dilution rates, these analyses were performed on washed suspensions of the cells from the whole samples taken at $D \simeq 0.10$, instead of on the whole samples themselves. In this case, whole samples contained much lysed cell material which was not accounted for by the dry weight values because the biomass determination method only weighs intact cells. By the same token, macromolecular analyses were done on the cell-free supernatants of the samples taken at $D \simeq 0.15$ (as well as on the whole samples, themselves)

TABLE I

THE MACROMOLECULAR COMPOSITION OF NITROGEN-FIXING AZOTOBACTER CHROOCOCCUM IN CONTINUOUS CULTURE

All Values as % Dry Weight of Organisms Except Nitrogen Content[a]; Cultures at 30°C

Dilution Rate (h^{-1})	% oxygen in atmosphere above culture	Protein	RNA	Polysaccharide	PHB	SUM	Nitrogen Content
0.10	20[b]	27.1	5.8	4.8	41.6	79.3	—[d]
0.10	30[b]	33.3	8.0	5.3	30.1	76.7	—[d]
0.10	20	47.5	16.3	3.2	15.0	82.0	97.0
0.10[c]	30	55.9	15.4	3.0	5.4	79.7	97.4
0.15	20	66.5	9.2	3.0	3.8	82.5	97.9
0.15	30	74.8	8.9	3.0	1.4	88.1	97.7
0.15	40	75.7	8.3	3.2	1.3	88.5	102.0
0.22	20	76.9	17.3	11.8	0.8	106.8	98.0
0.22	30	81.4	11.5	10.1	0.6	103.6	97.7
0.22	40	83.7	14.3	10.9	—[d]	108.9	98.8
0.22	50	91.9	13.9	14.0	0.5	120.3	98.4
0.29	20	79.4	15.9	10.4	1.1	106.8	100.5
0.29	30	78.3	15.4	9.9	0.6	104.2	98.6

Table I. Continued.

0.29	40	78.8	15.0	11.6	——[d]	105.4	95.0
0.34	20	84.5	15.5	7.8	1.1	108.9	101.8

[a]Cellular nitrogen content expressed as:

$$\frac{\text{Actual (Kjeldahl) cellular N content (mg/ml)}}{\text{Theoretical cellular N content (mg/ml)}} \times 100$$

where Theoretical cellular N = (Protein content (mg/ml) x 16% (the estimated % of protein that is N) + RNA content x 25% (the estimated % of RNA that is N))

[b]Stirring rate in culture was about 300 r.p.m.

[c]Extra 10 g/ℓ MANNITOL ADDED to medium here to prevent carbon-limitation (see Text).

[d]Not done

Note: Stirring rate throughout was about 1750 r.p.m. except where indicated.

The oxygen tension in the culture was zero or nearly so under all conditions (ie. < 3 μM O_2 registering in the culture).

At D ≃ 0.10, macromolecular composition determinations were done on washed suspensions of the original samples (see Text).

At D ≃ 0.15, macromolecular composition figures were obtained by substracting supernatant composition figures from unwashed sample composition figures (See Text).

and these supernatant values thus obtained were subtracted from the corresponding whole sample values. This method, too, would therefore correct for lysed cell material.

The general pattern in Table 1 shows an increase in protein content at the lower D's with both increasing dilution rate and increasing oxygen percentage in the atmosphere above the culture, until at higher dilution rates, it levels out at a maximum average value. Poly-β-hydroxybutyrate (PHB), on the other hand, shows the exact opposite relationship in that its content decreases with both increasing dilution rate and increasing 0_2 concentration until, at the higher dilution rates, it disappears almost entirely. The RNA content seems to stay relatively constant throughout the whole range of dilution rates and atmospheric 0_2 concentrations as does the polysaccharide content throughout the higher dilution rates and their corresponding 0_2 percentages. It is interesting to note the affect of the stirring rate on the macromolecular composition at $D \simeq 0.10$. At this dilution rate and at the lower stirring rate of 300 r.p.m., the percentage protein is much lower and the PHB percentage is much higher than the corresponding values at the higher stirring rate. As can be deduced from the table, at the lower dilution rates of 0.10 and 0.15, the sums of the macromolecular composition values do not

agree well with the corresponding dry weight values, whereas, at higher dilution rates, there is much better agreement in this respect.

OXYGEN SOLUTION RATE ESTIMATIONS

1) Sulfite oxidation method - The oxygen solution rate as measured by the sulfite oxidation method was found to be 45.65 m moles/ℓ culture/h as shown in Table 2.

2) Oxygen electrode ("kz") method - The values for the oxygen solution rates into the azide-killed Azotobacter culture, the 1.5 M aqueous azide solution and the water, as calculated by the "kz" method are presented in Table 2. The corresponding ℓn vs t plots which yielded the different k values (in min^{-1}) for these different liquids are illustrated in Fig. 11. The k values were obtained by taking the slope of each line. The oxygen solution rates were then computed using the equation,

$$-\frac{dz}{dt} = k.z.$$

Thus, the rate at which 0_2 is taken up from air by water, for instance, when the water is saturated with N_2 (ie. when it is dissolved oxygen-free) is:

(Continued on page 77)

TABLE II

OXYGEN SOLUTION RATES

(as estimated by oxygen electrode ("kz") method, sulfite oxidation (SOX) method and CO_2 evolution rates)

Values expressed in m moles/ℓ culture/h

Stirring rate = 1750 r.p.m.

CO_2 evolution rates			O_2 solution rates [b]	
	Conditions	Values	A) As measured by sulfite oxidation (SOX) method:	B) As measured by oxygen electrode ("kz") method:
D(h^{-1})	% O_2 in Gas Phase Above Culture			
0.10	20	14.65	45.65	i) kz (azide) = 11.23
0.10	30	25.94		
0.15	20	16.17		
0.15	30	22.27		
0.15	40	28.49		ii) $kz(H_2O)$ = 8.24
0.15	50	31.08[a]		
0.22	20	16.09		
0.22	30	28.09		
0.22	40	34.67		iii) $kz\left(^{\text{culture}}_{+\text{azide}}\right)$ = 4.60
0.22	50	43.43		
0.29	20	20.65		
0.29	30	29.11		
0.29	40	40.88		
0.29	50	7.63[a]		

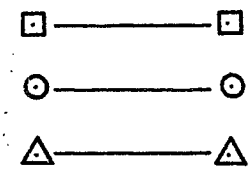
Table II. Continued.

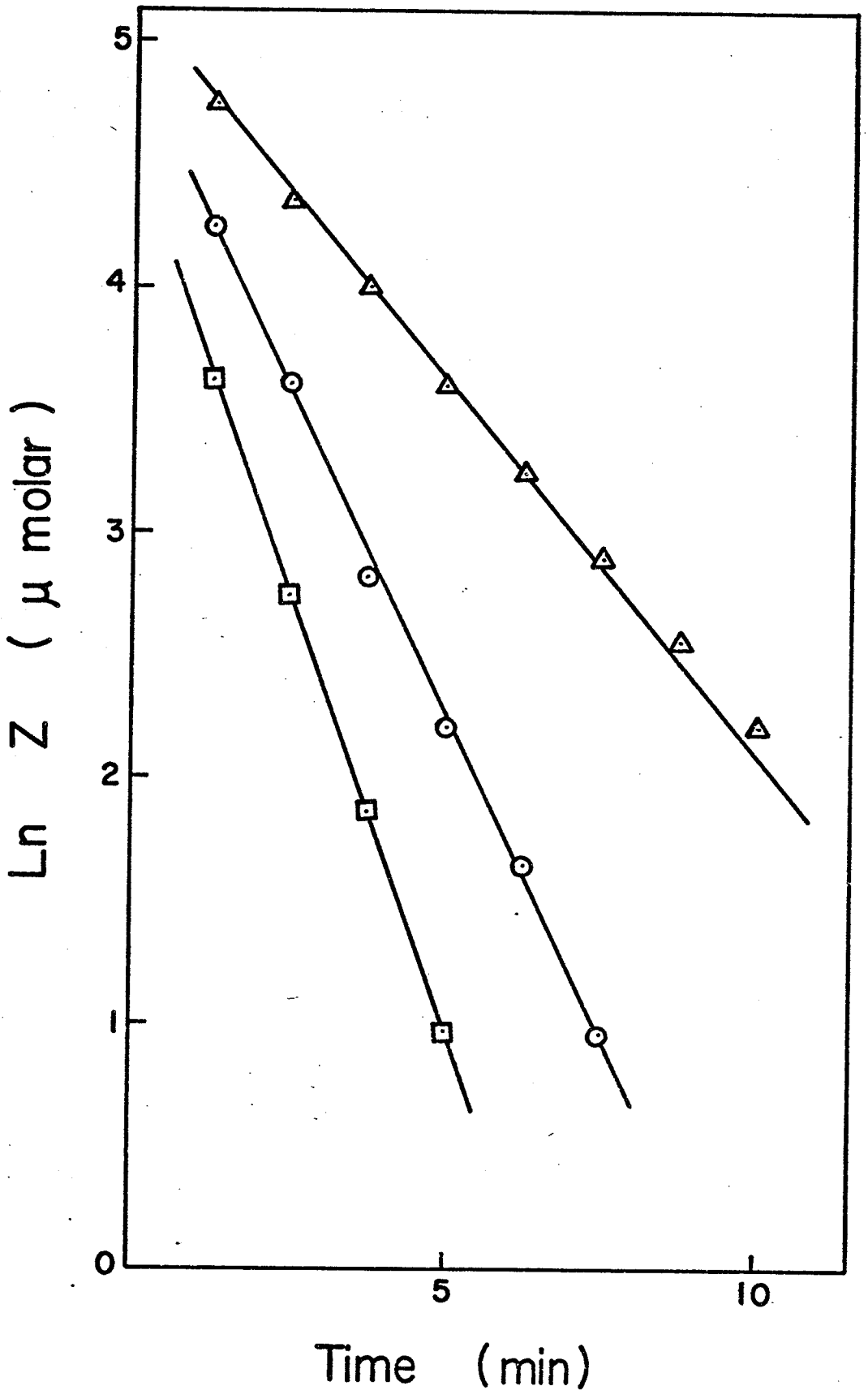
0.34	20	26.42

[a] Culture in "unsteady" ("wash-out") state (See Text).

[b] 20% oxygen in GAS PHASE (See Materials and Methods).

Fig. 11. Oxygen solution rates as measured by an oxygen electrode ("kz") method.




5
4
3
2
1
0
Ln Z (μ molar)
5
10
Time (min)

$$\frac{dz}{dt}(H_2O) = k \text{ (water)} \times Z \times 60 \text{ min}$$
$$= .528 \text{ min}^{-1} \times 260 \text{ } \mu M \times 60 \text{ min}$$
$$= 8.24 \text{ m mole}/\ell/h$$

It was found that the aqueous azide solution dissolved 0_2 faster than did the water. The water, in turn, took up oxygen faster than did the dead culture.

VARIATIONS IN CELL GROWTH AND RESPIRATORY INDEX WITH CHANGING CULTURAL CONDITIONS

The calculated rates of cell growth (in mg/ℓ culture/h), as presented in Table 3, were found, in general, to increase with both increasing dilution rate and increasing 0_2 concentration in the gas phase above the culture except at 20% 0_2 where the values stayed essentially constant as the dilution rate increased and also at D ≈ 0.15 where the values remained relatively constant over the range of oxygen concentrations. A maximum cell growth value of 229 was reached at D ≈ 0.29; 40% 0_2.

The respiratory index values (in m moles CO_2 output/mg cell growth) were calculated as described in Table 4. Since these values were found to stay relatively constant over the range of dilution rates at each oxygen concentration, the figures were averaged as shown in

TABLE III

CELL GROWTH

(in mg/ℓ culture/h)[a]

BRACKETED VALUES REPRESENT CORRESPONDING CO_2 VALUES EXPRESSED IN M MOLES/ℓ CULTURE/h

% O_2 in Atmosphere Above Culture	Dilution Rate 0.15	0.22	0.29	0.34
20	152 (16)	160 (16)	157 (20)	170 (26)
30	158 (22)	198 (28)	220 (29)	———
40	152 (28)	209 (35)	229 (41)	———
50	125 (31)[b]	218 (43)	———[b]	———

[a] determined by multiplying dry wt. (mg/cc) x Dilution rate (h^{-1}) x 1000

[b] culture in "unsteady state" ("wash-out") (See Text)

TABLE IV

RESPIRATORY INDEX

(m moles CO_2 output/mg cell growth) x 10[a]

% O_2 in atmosphere above culture	Dilution rate 0.5	0.22	0.29	0.34	Average
20	1.05	1.00	1.27	1.53	1.21
30	1.39	1.41	1.32	——	1.37
40	1.84	1.67	1.79	——	1.77
50	2.48[b]	1.97	——[b]	——	2.23

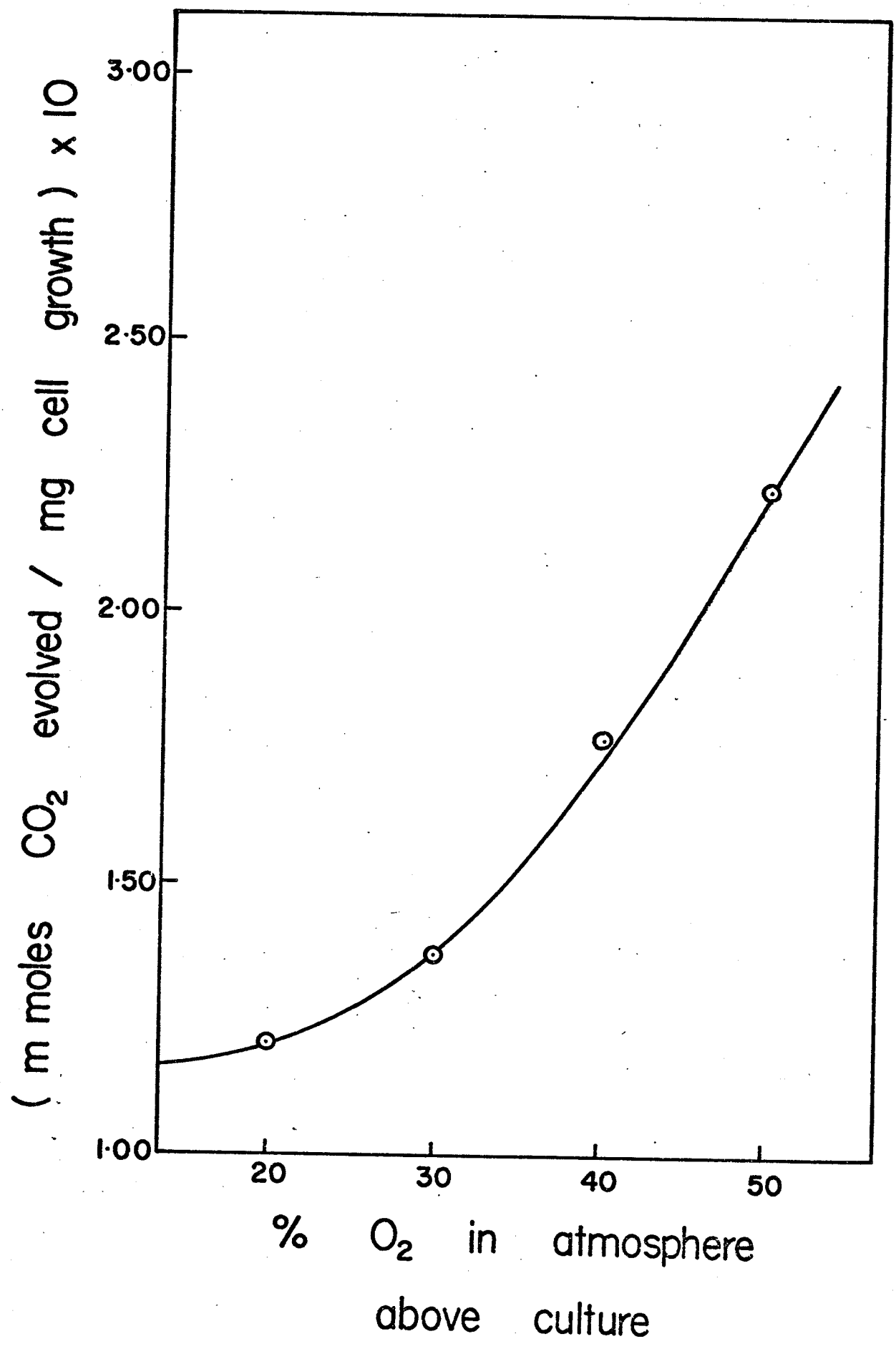
[a] given by $\left(\frac{\text{m moles } CO_2/\ell \text{ culture/h}}{\text{mg cell growth}/\ell \text{ culture/h}}\right) \times 10$

[b] culture in "unsteady state" (wash-out) (See Text)

Note: Values of m moles CO_2/mg cell growth were multiplied by 10 in order to raise them to the level of whole numbers for convenience.

Table 4. These average values were subsequently plotted against oxygen concentration in the atmosphere above the culture as shown in Fig. 12 and a curve which levelled out at a respiratory index value of about 0.115 m moles CO_2 evolved/mg cell growth was obtained.

Fig. 12. Respiratory index plot.


3·00
2·50
2·00
1·50
1·00
(m moles CO2 evolved / mg cell growth) x 10
20
30
40
50
% O2 in atmosphere above culture

DISCUSSION

DISCUSSION

Azotobacter chroococcum was grown under N_2-fixing conditions in normally intensely agitated continuous culture (ie. at a culture stirring rate of 1750 r.p.m.) at different dilution rates (D's) with a gas supply that varied from 20% 0_2 (air) to 50% 0_2. Previous similar studies have used much milder agitation. Apparently as a result of employing this higher stirring rate, two obvious differences from the data of previous investigations arose in these present results. One difference was that, in this study, at most dilution rates and at gas phase oxygen concentrations as high as 50%, the ambient oxygen tension in the culture, as measured by an 0_2 electrode, was zero or nearly zero, whereas, in studies in the past, 0_2 concentrations ranging from 20-45 μM have been recorded in cultures growing at the same dilution rates under the same gas phase 0_2 concentrations but with milder agitation (2, 60) (See Results). The other difference was that higher cell yields (Figs. 3 and 4 and TableIII)than what had been obtained in earlier experiments were achieved in this study at the higher gas phase 0_2 con

centrations at the higher dilution rates (2, 4, 60).

It should be stressed that just because the dissolved O_2 tension in the culture was zero under most conditions in this present study, this does not mean that the cells were oxygen-limited; indeed their gross composition (Table I) was typical of cells grown with a more than adequate supply of oxygen (2, 4, 60) in that the cells contained a high percentage of protein and RNA which is characteristic of an actively metabolizing condition and a very low percentage of storage materials like polysaccharide and poly-β-hydroxybutyrate (PHB). A high content of PHB is a characteristic of cells (when grown under O_2-limited conditions) which store unmetabolized carbon in such forms that will serve for future use in an environment more conducive to active growth (ie. a O_2 - sufficient environment) (4). The rate at which O_2 is supplied from the gas phase to the cells in an aerated culture is governed by complex factors not fully understood (66), but what is understood is that oxygen passing from the gas phase to the cell surface (where the O_2 concentration is normally assumed to be zero (1)) must traverse a gas/liquid interface and a liquid/cell interface, both of which interfaces can offer resistance to O_2 passage. If the resistance of the liquid/cell interface is reduced, as it seems to be by intense agitation of the liquid phase,

the dissolved 0_2 concentration of the liquid may be low despite an adequate 0_2 supply to the cells. On the other hand if the resistance of the liquid/cell interface is high as is apparently the case in slowly-stirred cultures, an appreciable dissolved 0_2 concentration is no guarantee of an adequate 0_2 supply to the organisms. It is assumed in this study that the intense agitation used did reduce the resistance of liquid/cell interface, presumably by "shredding" the liquid over the cell surface in the manner of a standard microbial reactor, because no other explanation of an adequate 0_2 supply to the cells coupled with a low concentration of dissolved 0_2 seems reasonable.

We have used two non-biological methods of measuring the rate at which 0_2 could be supplied to the culture: (i) the SOX value (ii) the "kz" value (See Materials and Methods). The first of these methods measures the rate at which 0_2 within a gas bubble reacts with $SO_3^=$ in the surrounding solution (ie. it measures the maximum possible rate at which 0_2 can dissolve in the solution). The second method measures the rate at which oxygen diffuses into a suspension of dead bacteria and it gives full weight to the impedence to this 0_2 diffusion offered by the physical presence of cells and polysaccharide films at the gas/liquid interface but does not allow for 0_2-uptake by the living bacteria of a normal culture (ie. it measures

the minimum rate of 0_2-diffusion into a culture). Thus, although it might seem that such intense aeration as we have described would inevitably result in excess aeration with a consequent positive value of dissolved 0_2, these "*kz*" experiments have shown that even dead cells present a barrier to 0_2 diffusing from bubbles into the solution which is quite sufficient to limit dissolved 0_2 concentrations to zero when an organism with such a high Q_{0_2} as Azotobacter is being studied. The actual "*kz*" value for the dead culture without the azide ion present can be estimated by considering the degree of facilitating effect that azide has on the oxygen solution rate (Table II). As can be seen from Fig. 11 and Table II, the two values, SOX and "*kz*" for the dead culture, straddle the observed CO_2 outputs of the metabolizing cultures (Figs. 5 and 6). Since the CO_2 outputs are a reasonable measure of 0_2 uptakes (Fig.10), this is a reasonably satisfactory result. However, our experiments lead us to conclude that neither the SOX value nor the "*kz*" value, nor the measurement of dissolved 0_2 concentration is a satisfactory absolute measure of the 0_2 supply to a culture of respiring bacteria. The only methods seem to be measurement of CO_2 output (with some very slight reservations based on the implications of Fig. 10 as will be discussed later), direct measurement of 0_2 consumption (67), or measurement of cell growth (Table III);

in all of these cases, moreover, it is essential that the culture be well agitated to ensure, as far as possible, maximally easy passage of 0_2 from the liquid to the cell surface and maximally easy passage of metabolic end-products in the opposite direction.

The reactions of the continuous culture with respect to biomass, CO_2 evolution rate and residual mannitol concentration change when the D and the % 0_2 in the gas phase are increased as shown in the results section. The residual mannitol pattern is the exact opposite of the CO_2 evolution rate pattern, as is obviously to be expected. Since the amount of oxygen that the cells must burn off to maintain a zero ambient 0_2 tension in the culture increases with both increasing oxygen concentration in the gas phase and increasing dilution rate (since fresh medium brings fresh oxygen into the culture), the CO_2 evolution rate increases as described. Also, it should be noted that there is no clear tendency for the biomass to show a plateau between two values of D as would be the case if some component in the growth medium were limiting (60).

Returning to a consideration of the increased cell yields compared to earlier experiments at the higher dilution rates and oxygen concentrations in the gas phase, Fig. 4 and Table III demonstrate that the very term,

"oxygen-limitation" is rather vague. As seen from Table III, since there is virtually no increase in the rate of biomass production at 20% 0_2 even when the D is doubled, the growth of the organisms even with the high agitation rate is 0_2-limited in air. Thus, while the gross composition of the organisms changes when 0_2-limitation becomes severe (4), the effect of 0_2-limitation on growth is apparent before there is an appreciable change in composition in that there is less growth, the more oxygen-limited the culture is. It is therefore suggested that the term, "0_2-limitation" be confined to 0_2-limitation of growth because growth-limitation is something that is simple to conceive and easy to measure. At higher 0_2 concentrations the growth increases with D until a maximum value of 230 mg/ℓ/h is attained. Above this point, the dissolved 0_2 begins to rise, the cells cease to grow, and the culture washes out. There is no increase in growth rate, no matter what 0_2 concentration is used, at D ≃ 0.15 presumably because a cell population of 1.0 - 1.5 mg/ml is about the maximum that can be achieved due to the occurrence of overcrowding, cell lysis, etc. at these lower D's. That at 20% 0_2 there is something approaching 0_2-limitation is also shown by the fact that when the CO_2 output curve at 20% 0_2 (Fig. 8) is extrapolated back to D=0, it yields a maintenance coefficient of 0.004 m moles CO_2/mg dry wt/h which approximates closely

to the maintenance coefficient of 0.0055 m moles CO_2/mg dry wt./h found by Nagai and Aiba (67) for A. vinelandii under O_2-limited conditions. From Table IV, it can be seen that the average values of the respiratory index at different O_2 concentrations plotted against O_2 concentration yields a smooth curve that apparently begins to flatten out as the % O_2 drops to 20 and a respiratory index of about .115 m moles CO_2 evolved/mg cell growth is reached. Points on the graph above some base that may be roughly set at about 15% O_2 concentration represent extra CO_2 produced by the organisms not accounted for by extra growth. In other words this corresponds to the respiratory protection of the cells' nitrogenase proposed for Azotobacter (56) where some of the carbon source (mannitol, in this case) is used to respire away excess O_2 in solution as CO_2. These are rough figures for respiratory protection because they imply that all O_2 taken up by the organisms is converted to CO_2. This is not quite true since Fig. 10 shows that there is an increasing discrepancy between mannitol disappearance and CO_2 output as O_2 concentration increases which seems to indicate an increasing accumulation of incompletely oxidized products; this is confirmed by small decreases in pH values of the culture (Fig. 2) that follow such an accumulation. The amount of these products is however, comparatively small. It should also be noted, however, that the close balance between total carbon inflow and total

carbon outflow at 20% 0_2 at all D's and again at 50% 0_2 at D ≃ 0.29 where "wash-out" occurred, indicates that the CO_2 accounts for virtually all of the 0_2 consumed. There appears to be an upper limit to cell growth which is about 230 mg cells/ℓ culture/h. Above this value, respiratory protection fails, the 0_2 in solution rapidly increases, and the cells wash out. Although the fact is not mentioned in the results section, we noted that cells that apparently had been hindered from growing (presumably by excess 0_2) when they had reached their growth limit, were not dead. Viability studies (68) showed that when transferred to normal solid media (B_6 Medium + 2% Agar), they grew normally with normal viability. Furthermore, as seen from Figs. 7 and 8, the rate of CO_2 production per unit of biomass of culture in this same condition of "wash-out" did not decrease significantly, if at all, indicating that the cells were not dead. It was found that respiratory protection also failed in cultures grown at a dilution rate of ~0.15 under an atmosphere of 50% 0_2 when the cultures became carbon-(mannitol)-limited. Again, the 0_2 in solution began to rise and the culture started to wash out due to 0_2-poisoning.

As alluded to previously, the macromolecular composition of organisms grown at the higher dilution rates and the higher gas phase oxygen concentrations (Table I) was

characteristic of cells from 0_2-sufficient cultures although the 0_2 tension in these cultures was zero. The fact that increased growth rates reflect higher metabolic activity which, in turn, reflects greater enzyme activity is one explanation for why the protein content of the cells increased with increasing dilution rate and increasing gas phase 0_2 concentration and then stabilized at a maximum percentage protein at the higher dilution rates. PHB content, however, decreased with increasing growth rate until it disappeared since it was neither needed nor wanted as storage material under these conditions because the oxygen supply to the culture was more than adequate. It should be mentioned that the total nitrogen determination confirmed the values obtained for quantities of protein and RNA in all cases as explained in Table I.

Continuous cultures of Azotobacter chroococcum were also grown at "below vortex" stirring rates of 300 r.p.m. at a D ≃ 0.10 under atmospheres of 20% and 30% oxygen. The steady state oxygen tension in this culture was also found to be zero although very little growth (Fig. 3) or mannitol degradation occurred. Therefore, this very mild agitation presumably allowed only a very slow oxygen solution rate. Furthermore, the macromolecular composition studies (Table I) of the cells grown under these conditions showed that

the cultures contained relatively high percentages of storage materials and relatively low percentages of protein which is characteristic of severe 0_2-limitation (4). The percent of storage materials decreased slightly and the percent of protein increased slightly as the gas phase 0_2 concentration was raised from 20% to 30%.

The slight amount of lysis that appeared in cultures grown at D's of ~ 0.10 and ~ 0.15 was accounted for by the fact that the relatively long average residence time of the organisms in the vessel at these low D's allowed for the death and subsequent lysis of a certain small percentage of the culture while still in the vessel. The estimated dry weight of the organisms did not account for this lysed cell material. This effect, however, was not great enough to cause a significant underestimate of the total carbon outflow value at D ≃ 0.15 since cellular carbon only contributes slightly to this figure. It appears that the unusually low polysaccharide figures (Table I) at D's of ~ 0.10 and ~ 0.15 could account, for the most part, for the discrepancy (as indicated in the SUM column of the table) between actual dry weight values and macromolecular composition totals. The low polysaccharide figures, at D ≃ 0.15 anyway, could possibly be accounted for, in turn, by the suggestion that the cellular polysaccharide at this low D may be, for the most part, a soluble capsular poly-

saccharide which would therefore appear in the supernatant of the sample rather than be accounted for by the dry weight estimation.

When the present results are compared to those of Dalton and Postgate (2, 60), which probably represent the most careful and exhaustive studies to date on the growth of Azotobacter chroococcum in chemostat culture, some interesting contrasts appear. Their maximum growth rates (calculated as in Table III) were about 120 mg cells/ℓ culture/h compared with growth rates of up to ~ 230 mg cells/ℓ culture/h obtained in these experiments. By deliberately cautious reasoning they reached the tentative conclusion that their cells were growing under N_2-limited conditions. In the light of the present work, which was carried out under exactly the same conditions except for the high agitation rate, this conclusion does not seem valid because in this study the best stable growth levels were attained at 30 and 40% O_2 where the N_2 concentrations in the gas supply were correspondingly less than the 80% found in air. Moreover, their dissolved O_2 concentrations with air as the gas phase, were about 20 µM which could be increased to 45 µM by more vigorous stirring or by increasing the O_2 concentration in the gas phase; when this was done, however, the cells ceased to grow. Since vigorous stirring at vortex rates and increasing the O_2 in the gas supply

increases the cell yield in these present experiments, it can only be assumed that in some way the vortex stirring increases the elimination of toxic products enabling the cells to grow more rapidly. Perhaps, however, the most significant difference between the present work and some of the previous studies is the efficiency of N_2 fixation. In batch culture Jensen (69) found 10-15 mg N fixed per g carbon source in chemostat culture with limited 0_2 supply. If we take the 0.115 m moles CO_2 produced for each mg of cell growth (Fig. 12), this can be calculated as 1.38 mg carbon or 3.45 mg carbon source per 0.15 mg N_2 fixed; ie. 43 mg N/g carbon source consumed. Since this is three to four times the value calculated by Jensen, it lends weight to the important suggestion made by Dalton and Postgate that N_2 fixation by free living non-symbiotic organisms such as Azotobacter may be more important in the world's nitrogen economy than has hitherto been realized.

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