THE UNIVERSITY OF MANITOBA

EFFECT OF HIGH OXYGEN CONCENTRATIONS ON THE GROWTH, STRUCTURE AND RESPIRATION OF AZOTOBACTER

by

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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To

Jadwiga and my Father

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ABSTRACT

Azotobacter vinelandii and A. chroococum were independently grown in highly stirred vortex continuous culture. In this culture mode, rates of CO_2 production and cell growth were lower for A. chroococum than for A. vinelandii. The latter was not inhibited by high oxygen tensions above the culture, rather, given sufficient nutrients its growth appeared to be oxygen-limited up to 40% oxygen. A comparison of various carbon sources for growth and respiration of A. vinelandii indicated that the oxygen solution rate, as judged by CO_2 production was greatly affected by the nature of the carbon source. A study of the effect of excess dissolved oxygen on A. vinelandii under vortex conditions and carbon-limitation revealed that this species was capable of both growth and N_2 fixation in the presence of as much as 45 μM O_2. In the presence of excess oxygen a greater proportion of carbon was oxidized for respiration, the amount of uncoupled respiration was dependent upon the concentration of dissolved oxygen. The levels of NADH oxidase, superoxide dismutase, catalase, isocitrate lyase, cytochromes and the rates of oxygen and mannitol uptake were examined in various degrees of mannitol-limitation. All showed marked effects dependent upon the degree of mannitol-limitation. Electron microscopy of cells grown in the presence of excess O_2 showed impressive structural differences. These included cell wall-cell membrane separation, vesicle formation and the formation of organelle-like structures apparently derived from electron-dense granular inclusion bodies in the cytoplasm. The carbon-limited chemostat cultures were examined for their oxygen uptake ability in the presence

of different substrates. Glucose oxidation by mannitol starved cells occurred at a constant rate, malate was oxidized slowly but the rate increased linearly with time. Citrate produced a complete cessation of oxygen uptake. From a carbon-limited state, the addition of mannitol or fructose resulted in a rapid increase in the rate of oxygen-uptake and excretion of pyruvic acid. Pyruvic acid accumulation was thought to be due to overflow metabolism rather than to the effect of oxygen on pyruvate dehydrogenase. Other Krebs cycle intermediates also showed a respiration inhibition effect similar to that caused by citrate.

The effect of KCN on the respiration of A. vinelandii was also examined. In a closed system with high KCN concentrations the rate of oxygen uptake decreased linearly with decreasing dissolved oxygen tension. This binding of CN^- appeared to be specific for the active intermediate form of cytochrome d and the observed Ki value was much lower than has previously been reported.

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ABBREVIATIONS

AMP, ADP, ATP	- Adenosine 5'-mono-, di-, and triphosphate
BHB	- β-hydroxybutyrate
CoASH	- Coenzyme A
D	- dilution rate
DCPIP	- 2,6-dichlorophenolindophenol
DNP	- Dinitrophenol
d.o.t.	- dissolved oxygen tension
EPR	- Electron paramagnetic resonance
GDP	- Guanosine 5'-diphosphate
FMN	- Flavin mononucleotide
HQNO	- Heptylquinoline N-oxide
NADH	- Nicotinamide adenine dinucleotide (reduced form)
NADPH	- Nicotinamide adenine dinucleotide phosphate (reduced form)
PHB	- Poly-β-hydroxybutyrate
Q	- Ubiquinone
Q_{O_2}	- Respiratory quotient
RI	- Respiratory index
RNA	- Ribonucleic acid
SOD	- Superoxide dismutase
TMPD	- N, N, N', N'-tetramethyl-p-phenylenediamine
UV	- ultraviolet

INTRODUCTION

INTRODUCTION

The process of nitrogen fixation is one of the most important processes occurring in nature. All life forms depend upon the reduction of dinitrogen to ammonia. Yet this process in a biological sense is restricted to a rather small group of organisms. Most of these are anaerobic and thus have no difficulty providing the necessary reductive environment required by the nitrogenase enzyme. Aerobic nitrogen fixers exist and of these Azotobacter species are the most interesting for the simple reason that they are capable of maintaining the required environment for nitrogen fixation yet they are, in fact, strict aerobes. To remove oxygen from the nitrogenase site, this species has an active and versatile respiratory system capable of adjustment to a wide variety of oxygen supply rates. Such adjustments require the cell to be capable of increasing its enzymatic content and/or rates for carbon assimilation, catabolism and oxidation.

My initial work indicated chemostat grown nitrogen-fixing Azotobacter vinelandii could be maintained in a steady-state despite the presence of high dissolved oxygen concentrations. This appeared to be contradictory to previous short-term studies which showed oxygen to have a detrimental effect on viability and nitrogenase activity of A. chroococcum.

My purpose, in this study, was to ellucidate the differences in growth between A. chroococcum and A. vinelandii, and to identify possible toxic effects of oxygen in A. vinelandii. The physiological changes which enable A. vinelandii to overcome such effects were also to be examined.

In order to study the response of Azotobacter to excess dissolved oxygen, I made use of two important "tools". The first was a vortex-

stirred chemostat culture. The stirring rate insured a rapid transfer of atmospheric gases and minimized gradient formation at the cell surface. Yet in this apparatus under an atmosphere of air, Azotobacter are capable of maintaining the dissolved oxygen tension at undetectable concentrations. To permit accumulation of oxygen within the culture fluid, the second "tool" used was extreme carbon limitation. Contrary to previous belief, Azotobacter vinelandii will be shown to be capable of nitrogen fixation in the presence of high dissolved oxygen tensions. Carbon limited cells grown in the presence of excess oxygen demonstrate effects which have not previously been noted. These include a loss of affinity by the terminal oxidases for oxygen and the formation of cytoplasmic organelle-like structures. These structures appear to be derived from other electron dense bodies within the cytoplasm.

Highly stirred carbon-limited chemostat cultures will also be shown to be useful for studies involving carbon assimilation whereby the rapid rates of respiration and oxygen transfer are used to form a system which can show subtle changes in carbon oxidation rates over a period of several hours.

While examining the respiration rates of Azotobacter, it was noted that Krebs cycle intermediates, glycolic acid and lactate were each capable of producing a complete cessation of respiration in Azotobacter vinelandii cells taken from a chemostat and to a lesser degree from batch cultures. This effect was examined and it was shown that these substrates induce an overloading of the reductive potential of the cell which, in turn, produces a reduction of NADH:ferricyanide oxidoreductase activity.

The effect of cyanide on respiration was examined and as had been previouly suggested the binding of cyanide is dependent upon the turnover of the cytochrome oxidase. However the dissociation constant of the inhibitor-enzyme complex appears to be much lower than previous estimates.

HISTORY

HISTORY

1. EFFECTS OF OXYGEN

Moore and Williams (1911) showed that high oxygen tensions will delay or inhibit the growth of many species of aerobic and facultative anaerobic bacteria. Although Azotobacter have the highest known respiration rate of all microorganisms, having a Q_{O_2} value of 4,000 to 5,000 µl O_2 mg dry weight^{-1} hour^{-1} (Williams and Wilson, 1954), members of this genera are still susceptible to the inhibitory effects of oxygen (Tschapek and Giambiagi, 1955). Inhibition of Azotobacter nitrogen fixation by oxygen was first reported by Meyerhof and Burk (1928); however this was later ascribed to a general inhibition of growth (Burk, 1930). That oxygen should be inhibitory to nitrogen fixation was again demonstrated by Parker (1954), when he showed the ideal environment for nitrogen fixation should include low oxygen tensions. Such an inhibition by oxygen was later shown by Parker and Scutt (1960) to be a competitive inhibition, indicating oxygen competes with nitrogen at the active site of nitrogenase. Other possible mechanisms of oxygen toxicity were reported at about this time. Barron (1955) proposed that oxygen inhibits by non-specific oxidation of enzyme sulfhydral groups. Dilworth and Parker (1961) described the maximum respiration rate to occur at 0.25 atms. O_2 and a reduction in the respiration rate of Azotobacter at higher oxygen tensions. This reduction in respiration rate was shown not to occur in Bacillus, Saccharomyces and Rhizobium species or where Azotobacter cells were provided with glycerol as the carbon source. Sulfhydryl stabilizing agents were not effective in preventing this reduction of respiration rates. These workers proposed two opposing

systems; a) a terminal oxidation system which reaches a maximum rate at 0.25 atm oxygen and b) an oxygen sensitive system which is inactivated to a greater extent at high oxygen tensions. Dilworth (1961) demonstrated the production of pyruvate and α-ketoglutarate by cultures of A. vinelandii which had been exposed to oxygen tensions greater than 0.6 atm and suggested that pyruvic oxidase is sensitive to oxygen.

The sensitivity of nitrogenase to oxygen was not neglected. Dalton and Postgate (1969a) suggested the basis for oxygen toxicity in Azotobacter was inhibition of the nitrogenase function. Low concentrations of ammonia were shown to allow better growth of A. chroococcum under high aeration. These workers also demonstrated that the viability of Azotobacter was reduced when cells are exposed to excess oxygen. The percentage of viable cells was determined at 2 hour intervals after exposing these cells to high concentrations of oxygen. They found the nutritional status of the culture to affect their results; for example, phosphate limited cultures were much more sensitive to oxygen poisoning than carbon limited which in turn, were more sensitive than nitrogen limited cultures. This was supported by work done by Lees and Postgate (1973). On the basis of their results Dalton and Postgate (1969a) suggested two mechanisms for the protection of Azotobacter from the toxic effects of oxygen; respiratory protection and conformational protection. Respiratory protection was first observed by Phillips and Johnson (1961). In the presence of excess oxygen A. vinelandii will consume sugars at a greater rate than required for energy supply. Dalton and Postgate (1969) suggested that respiration will balance the oxygen supply, therefore carbon limited cells which cannot provide this balance effect become hypersensitive to the presence of oxygen.

The reason why phosphate limited cells should be the most sensitive to oxygen toxicity was also explained as being due to inadequate respiratory protection. In the presence of high oxygen conditions the ATP:ADP ratio is assumed to be high, thus inhibiting respiration in a manner analogous to that observed in mitochondria (Klingenberg and Schollmeyer, 1960). The inability of respiration to deal with the supply of oxygen would allow oxygen to enter the cytosol of the cell resulting in oxygen damage to nitrogenase, which ultimately may be lethal to the cell.

The second mechanism of oxygen protection was suggested because of the high oxygen tolerance of nitrogenase in crude extracts. Nitrogenase which has been purified to its component parts becomes extremely sensitive to oxygen (Bulen and LeComte, 1966, Kelly et al., 1967). Dalton and Postgate (1969) suggested a steric change may occur in the enzyme in the particulate fraction. Such a conformational change would result in less accessible oxygen sensitive sites or increased stability of such sites.

Contradictory evidence to the explanation of the hypersensitivity of phosphate limited cultures have recently been presented. Leung (1979) was able to maintain a phosphate limited chemostat culture of A. chroococcum under vigorous agitation. Under very extreme phosphate limitation the dissolved oxygen tension in the culture rose above zero, yet the culture growth was still maintained in a steady state. These cells did not demonstrate any extreme sensitivity to oxygen, under conditions which would be much more severe than those described by Dalton and Postgate (1969). Tsai et al. (1979) measured the adenylate nucleotides and found the energy charge to be lower in phosphate limited cultures than in a control. They proposed that oxygen consumption by

phosphate deficient cells was inhibited because the lack of inorganic phosphate prevents the conversion of ADP to ATP; as a result the coupled electron flow to oxygen becomes inhibited.

In light of these results and those of Hine and Lees (1976) who showed Azotobacter growth to be limited by oxygen in a vigorously aerated chemostat culture, it seems necessary to re-examine oxygen toxicity with regard to the genus Azotobacter. Such a study should be able to explain the pyruvate production and inhibition of respiration observed by Dilworth (1961). It is also necessary to determine why the growth of Azotobacter should not continue to increase when a culture is provided with oxygen tensions above 30% oxygen in the atmosphere over the culture (Hine and Lees, 1976). The answer may provide a possible biological source of reduced nitrogen requiring low expenditures of energy. Also, the very high respiration rate of maintaining the oxygen tension of an Azotobacter culture at zero dissolved oxygen, the presumption of toxicity due to high concentrations of oxygen above the culture seems doubtful. It would appear that studies involving oxygen accumulation in the culture are necessary to define the detrimental effects of oxygen on Azotobacter.

2. Free radical formation and oxygen toxicity

Dioxygen may exist in three states, the ground and two excited states. In the ground state molecular oxygen would appear to be a very reactive molecule since it possesses two unpaired electrons in separate outer orbitals. However, the stability of this molecule is the result of the spin of these outer electrons being parallel. In accordance with the Pauli Exclusion principle, two electrons with parallel spin may not enter the same orbital, therefore, before a pair of electrons can be

added to the outer orbitals one of these outer electrons must undergo a change in spin. The change of spin is a slow process compared to the collision time of the two reactants. Therefore, molecular oxygen will normally be a rather unreactive molecule, the reactions being favoured are those which involve one electron transfer (Taube, 1965). This characteristic of molecular oxygen is termed the "spin restriction".

The first excited state is termed singlet oxygen and is designated $^1\Delta$. The outer electrons of singlet oxygen are both in the same orbit and have opposite spins. The second excited state, designated $^3\Sigma$, has two electrons separately occupying the two outer orbitals. The electrons of the second excited state are antiparallel, which allows the outer electron to drop into the inner electron shell resulting in the rapid formation of singlet oxygen. The very short lifetime (1×10^{-11} secs) of the second excited state precludes its role as a possible reactive intermediate of biological consequence. Singlet oxygen has a much longer lifetime (several μ secs) and has been implicated in a number of reactions which may mediate oxygen toxicity.

Singlet oxygen may be formed by a number of reactions outlined in Fig. 1. It has been shown to act as a possible toxic factor in polymorphonuclear leucocytes undergoing phagocytosis (Allen et al., 1972). Under these conditions the cell exhibits chemiluminesence which may be due to the liberation of a photon in the following reaction:

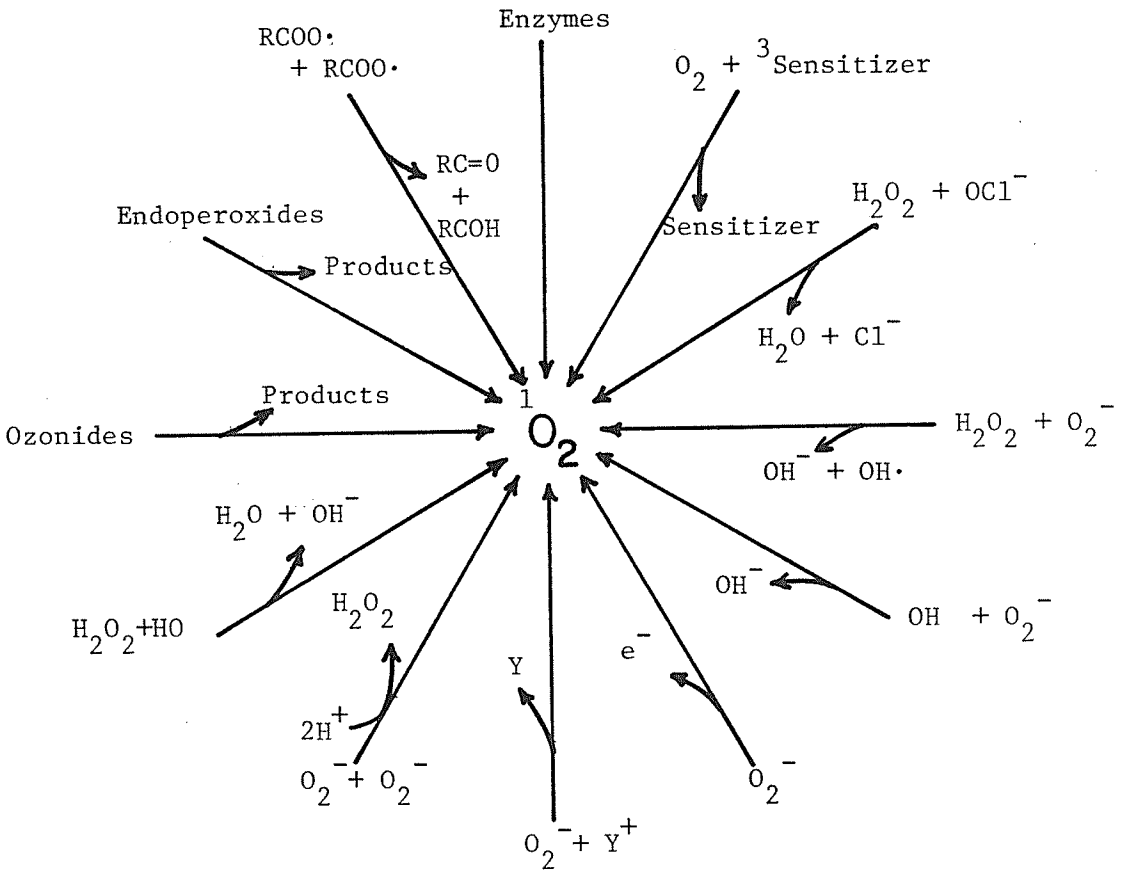
$$^1O_2(^1\Delta) \longrightarrow O_2(^3\Sigma) + h\gamma \ (1269 \text{ nm})$$

or $$2\,^1O_2(^1\Delta) \longrightarrow 2O_2(^3\Sigma) + h\gamma \ (634 \text{ nm})$$

Carotenoids have been shown to provide a protective factor against

Figure 1. Production of $^1O_2$ by photochemical, chemical and biological systems.

(Krinsky 1977)


RCOO·
+ RCOO·
Enzymes
O_2 + ^{3}Sensitizer
RC=O
+
RCOH
Endoperoxides
Products
Sensitizer
H_2O_2 + OCl^-
H_2O + Cl^-
Products
Ozonides
$^1O_2$
H_2O_2 + O_2^-
OH^- + OH·
H_2O + OH^-
H_2O_2+HO
H_2O_2
OH^-
OH + O_2^-
Y
e^-
$2H^+$
O_2^- + O_2^-
O_2^-
O_2^- + Y^+

damage due to $^1O_2$ (Foote and Denny, 1968, Krinsky, 1976). Sarcina lutea mutants which lacked carotenoids were shown to be more susceptible to destruction by light and phagocytosis than wild type cells (Mathews-Roth and Krinsky, 1970, Krinsky, 1970). Membrane damage was also demonstrated by the lysis of liposomes when singlet oxygen was produced by the photosensitized reaction. Catotenoid incorporation in the liposomes prevents such damage (Anderson and Krinsky, 1973).

2.1 Superoxide Anion

The spin restriction of molecular oxygen and the preferential single electron reduction of oxygen provides a problem to aerobic biological systems. Univalent electron transfer of oxygen to water catalyzed by transition metals, results in the formation of species which contain an odd number of electrons, called free radical species. One such species is superoxide anion (O_2^-), which is the first intermediate formed by the univalent reduction of oxygen. It is a weak conjugate base of the perhydroxy radical ($HO_2\cdot$) whose pKa is 4.8. This radical may be produced by a number of reactions including the autooxidation of hydroquinones, leukoflavins (Misra and Fridovich, 1971), catecholamines (Misra and Fridovich, 1972), thiols (Misra, 1974) and tetrahydropterins (Fisher and Kaufman, 1973). Several oxidative enzymes have been shown capable of superoxide production; these include xanthine oxidase (McCord and Fridovich, 1969), adehyde oxidase (Rajagopalan et al., 1962) and dihydroorotic dehydrogenase (Aleman and Handler, 1967). Superoxide may also act as an intermediate in some enzymatic reactions; for example, intestinal tryptophane dioxygenase is inhibited by superoxide dismutase (Hirata and Hayaiski, 1971) and it has been implicated as an intermediate in two hydroxylases from Aspergillus niger (Premakumar et al., 1972).

Superoxide has been shown to be capable of inducing detrimental effects to biological material. For example, it has been shown to inactivate viruses (Lavelle et al., 1973), damage membranes (Goldberg and Stern, 1976), induce lipid peroxidation (Kellog and Fridovich, 1977) and kill cells (Michelson and Buchingham, 1974). Superoxide has also been implicated as the toxic factor produced by the antibiotic streptonigrin on aerobic Escherichia coli (White and Dearman, 1965). The toxic effects of paraquat have also been proposed to be mediated by the superoxide anion (Fridovich and Hassan, 1979). The mode of action of this herbicide is believed to involve it being reduced to a stable free radical intermediate. This intermediate will then react with oxygen to generate the superoxide radical with a rate constant of $7.7 \times 10^{8}\ M^{-1}sec^{-1}$ at 25°C (Farrington et al., 1973).

Paraquat has been shown to induce physiological changes which may be attributed to increased superoxide production. It had also been shown to increase cyanide insensitive respiration of E. coli fourteen fold without any change in total respiration (Fridovich and Hassan, 1979). Other changes induced by paraquat include increased catalase and superoxide dismutase production which reduce the lethality of this compound (Hassan and Fridovich, 1977). Recent work presented by Buchanan (1978) shows the superoxide anion to have a direct affect on nitrogenase activity in whole cells. This effect was prevented by the addition of extra-cellular superoxide dismutase.

2.2 The hydroxyl radical

The superoxide anion and H_2O_2 undergo a reaction which produces a free radical which is the most potent oxidant known. This reaction, called the Haber-Weiss reaction, produces the hydroxyl radical ($OH^{\bullet}$)

(Haber and Weiss, 1934), which is capable of attacking any organic compound in the cell (Neta and Dorfman, 1968). The reaction for formation of the hydroxyl radical being

$$O_2^- + H_2O_2 \longrightarrow OH^- + OH^\bullet + O_2$$

It reacts with other compounds having a rate constant of approximately $10^9\ M^{-1}\ sec^{-1}$. This rate is close to the rate of diffusion and may be diffusion-limited since glycerol addition will decrease the reactivity of the hydroxyl radical.

3. Protective Mechanisms in the Cell

The extreme reactivity of the hydroxyl ion requires that the Haber-Weiss reaction be prevented or minimized. This is accomplished by maintaining a very low concentration of the reactants, superoxide anion and hydrogen peroxide. The superoxide anion will undergo a spontaneous dismutation reaction, catalysed by the enzyme superoxide dismutase, with the release of hydrogen peroxide and oxygen. Hydrogen peroxide is rapidly removed by the enzymes catalase and peroxidase.

3.1 Superoxide dismutase

Superoxide may spontaneously dismutate via one of the reactions:

$$HO_2^\bullet + HO_2^\bullet \longrightarrow H_2O_2 + O_2 \qquad k \approx 8\times10^5\ M^{-1}\ sec^{-1}$$

$$HO_2^\bullet + O_2^- \longrightarrow H_2O_2 + O_2 \qquad k \approx 8\times10^7\ M^{-1}\ sec^{-1}$$

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad k < 0.2\ M^{-1}\ sec^{-1}$$

The spontaneous reaction is therefore most rapid at the pKa of the perhydroxy radical (pH 4.8), with a sharp decrease in rate at higher pH values (Fridovich, 1978). It is the third and slowest reaction which is believed to be the one occuring upon the superoxide dismutase enzyme. The reaction is believed to be speeded up by a metal catalyst

which would accept an electron from O_2^- and transfer it to a second superoxide anion (Klug et al., 1972). The reaction mechanism may therefore be written as:

$$E - Me^n + O_2^- \longrightarrow E - Me^{n-1} + O_2$$

$$E - Me^{n-1} + O_2^- + 2H^+ \longrightarrow E - Me^n + H_2O_2$$

where E denotes the enzyme and Me the metal.

Three types of superoxide dismutase have been found in nature, these are classified on the basis of the metal group believed to be involved in the active site. They are the iron superoxide dismutases (Fe SOD), manganese superoxide dismutase (Mn SOD) and the copper zinc superoxide dismutase (Cu Zn SOD). Procaryotes may contain an Fe SOD and/or Mn SOD, both of these enzymes are closely related in structure, demonstrating amino acid homology (Steinman and Hill, 1973). The Mn SOD and Fe SOD of procaryotes are composed of two subunits, each with a molecular weight of 20,000. There appears to be a preponderance of Mn SOD in Gram positive organisms, whereas Gram negative organisms may contain both Mn and Fe SOD (Britton et al., 1978). When both enzymes are present there appears to be a separation of the enzymes between the cytosol and the periplasmic space. Cells grown under conditions which allow high levels of Fe SOD to be produced are more resistant to extracellularly produced superoxide anions. Therefore, it is likely that the Fe SOD is produced within the periplasmic space, whereas the Mn SOD may be restricted to the cytosol of this cell.

Eucaryotes contain two types of superoxide dismutase, a Mn SOD which demonstrates amino acid sequence homology to the Mn SOD of procaryotes and a CuZn SOD which does not show any amino acid

similarity with the procaryotic form (Steinman and Hill, 1973). The Mn SOD is known to be the only superoxide dismutase found in the mitochondrial matrix, whereas the cytosol of the cell contains both Mn and CuZn SOD (McCord et al., 1977).

The structure of the CuZn SOD has been described by Richardson et al. (1975). Each subunit is believed to exist as a cylinder formed by eight extended segments of linked peptide chain, the interior of the cylinder consists of hydrophobic side chains. Two non-helical loops are believed to project from the ends of the cylinder. These loops then enclose the active site of the enzyme. In the dimeric form the active sites are believed to operate independently.

On the active site, Cu(II) and Zn(II) are located in close proximity being bridged by a histidine residue. The copper atom is exposed to the solvent, whereas, the zinc atom is located behind the copper.

A possible mechanism of catalysis speculated by Fridovich (1978) involves the imidazole group acting in proton conduction. The copper atom may be reduced by superoxide anion to form Cu(I). This reduction is accompanied by the severence of the bond between copper and the imidazole enabling the latter to then take up a proton from the solvent. The copper atom is then reoxidized by O_2^- to Cu(II) with the formation of the imidazole bridge and the transfer of the proton to yield H_2O_2.

3.2 Hydrogen peroxide

Hydrogen peroxide is the most stable intermediate of oxygen reduction. It has been shown to be produced by microsomes (Thurman et al., 1972), illuminated chloroplasts (Halliwell, 1974), mitochondria

(Portwich and Aebi, 1960) and aerobic microorganisms (Zobell and Hittle, 1967). Hydrogen peroxide is not believed to be a toxic agent since aerobes which lack catalase have been reported to secrete it (Gledhill, 1969). However, pneumococci which respire O_2 to H_2O_2 will gradually develop a loss of viability which can be overcome by the addition of catalase (Sevag and Maiwey, 1934).

The removal of hydrogen peroxide is catalized by the hydroperoxidase which include the enzyme catalase and peroxidase. These enzymes are distinguished upon the basis of the reductant; peroxidase may use a wide variety of electron donors whereas catalase will use hydrogen peroxide both as substrate and reductant.

3.3 Peroxidase

Peroxidases are generally hemoproteins capable of reduction by many substrates, the reactions involving one electron transfer from donor molecules (Chance 1950, Yamazaki *et al*. 1960). Such one electron transfer result in the production of free radical species, i.e.

$$\text{Peroxidase} + H_2O_2 \rightarrow \text{Compound I}$$

$$\text{Compound I} + AH_2 \rightarrow \text{Compound II} + AH\cdot$$

$$\text{Compound II} + AH_2 \rightarrow \text{Peroxidase} + AH\cdot$$

The free radicals formed are released freely into solution, (Yamazaki *et al*., 1960), thus requiring the dismutation reaction.

$$2\ AH\cdot \rightarrow A + AH_2$$

Horse radish peroxidase may exist in five stable forms, which are interconvertible upon binding of specific substrates, illustrated by Fig. 2. The normal catalytic cycle involves binding of H_2O_2 to ferriperoxidase. The hydrogen peroxide draws two electrons from the

heme ring resulting in an increase of the effective oxidation number of the heme group to five (compound I). By single electron transfers, compound I is reduced to II and then back to the ferric form (Yamazaki, 1974).

A third compound is believed to be formed in the presence of excess hydrogen peroxide, while oxidizing NADH and dihydroxyfumaric acid (Chance, 1952, Yokota and Yamazaki, 1965). This compound has an oxyferroperoxidase structure. It is capable of undergoing reduction, however it is less reactive than compound I. It is also capable of a spontaneous decay to the ferric enzyme without passing through the intermediate I and II (Wittenberg et al. 1967) i.e.,

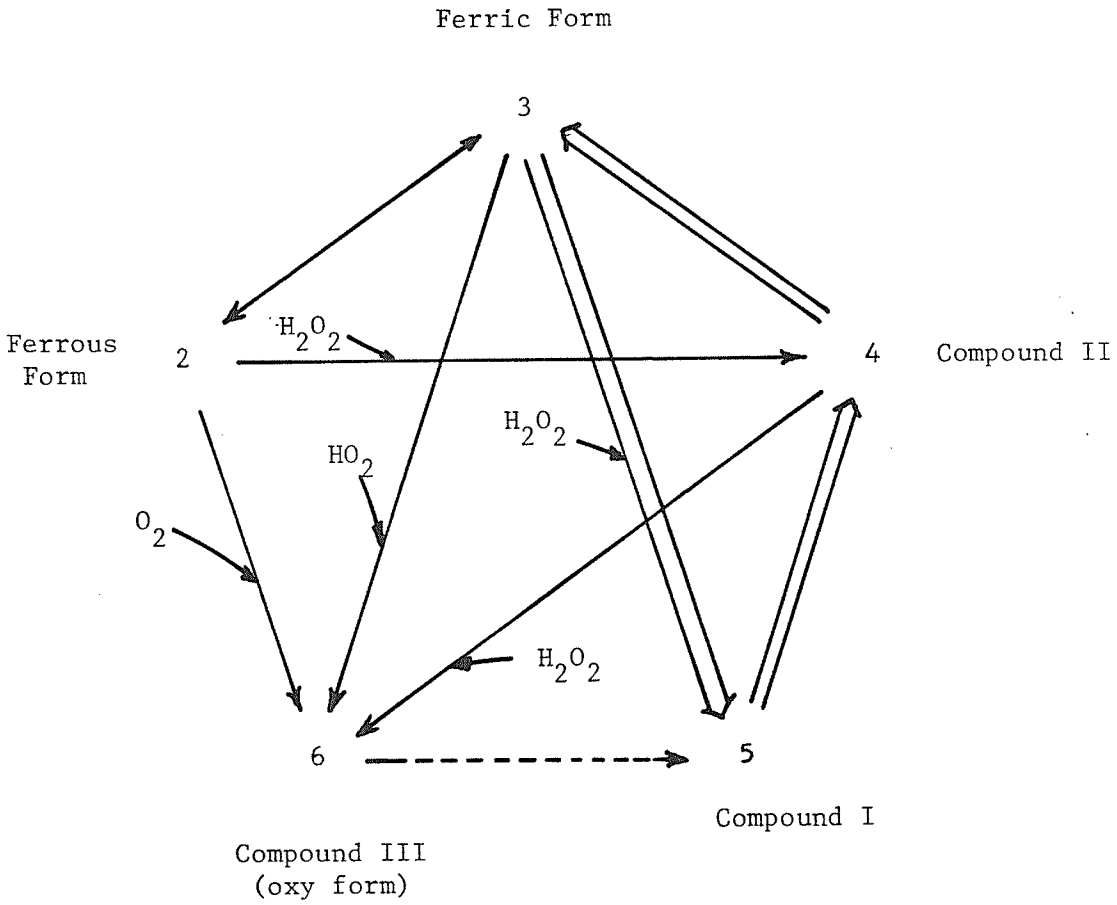
$$\text{Compound III} + 1e^{-} \longrightarrow \text{Peroxidase} + O_2$$

3.4 Catalase

Catalases are generally hemoproteins with a protophyrin IX type heme in a high spin state. Organisms unable to produce heme may have a flavoprotein type of catalase. The catalase enzyme has a molecular weight of approximately 248,000 and it may be denatured by lyophilization to yield two types of products. The first product, called compound I, is believed to be a subunit of catalase with a molecular weight of 82,000-88,000, indicating the native enzyme to be composed of three subunits. These subunits individually have no catalase activity and unlike the native enzyme are susceptible to reduction by dithionite. The second product formed is called compound II. It has a molecular weight approximately equal to that of the native enzyme and demonstrates very low catalase activity. This compound is also susceptible to dithionite reduction indicating that lyophilization has induced a structural change although it has not

Figure 2. Five redox states of horse radish peroxidase. Numbers indicate the oxidation number of the peroxidase heme. $3 \rightarrow 5 \rightarrow 4 \rightarrow 3$ indicate a catalytic cycle.

(Yamazaki 1974)


Ferric Form
3
Ferrous Form
2
H_2O_2
4
Compound II
H_2O_2
HO_2
O_2
H_2O_2
6
5
Compound I
Compound III
(oxy form)

undergone dissociation to subunits.

The hematin prosthetic group of catalase has the fifth and sixth coordinate position available for binding. On the basis of absorption spectra Brill and Williams (1961) suggested a carboxylic anion occupies the fifth position, which may be aspartate or glutamate, the C-terminal of a polypeptide chain, cystein or tyrosine. The sixth position may bind water, but the hydroxyl anion may also be in this position.

The overall reaction mechanism of catalase involves two separate reactions. The first involves the formation of a primary complex between H_2O_2 and the ferric iron of the hematic group, i.e.

$$Fe^{3+} - OH + H_2O_2 \rightleftharpoons Fe^{3+} - OOH + H_2O$$

The involvement of free radicals in the production of the primary complex is unlikely because of its long half-life. Since no free radicals have been detected in solutions undergoing breakdown of H_2O_2, this suggests that the reduction of hydrogen peroxide occurs via a two electron transfer step.

The second reaction generally involves the reduction using hydrogen peroxide as a source of electron, i.e.

$$Fe^{3+} - OOH + H_2O_2 \longrightarrow Fe^{3+} - OH + H_2O + O_2$$

This decomposition does not require hydrogen peroxide as an electron donor; three other alternate reduction mechanisms are possible. The first is the spontaneous decomposition due to the presence of internal hydrogen donors in the enzyme. The second mechanism involves the use of an external hydrogen donor which gives catalase a peroxidase function. This reaction occurs in the presence

of a suitable reductant and low concentration of H_2O_2. The third mechanism is the formation of a compound II which is reduced one equivalent below the compound I and is catalytically inactive.

A hydrogen ion transfer from the donor to the substrate molecule bound at the active site is believed to be the mechanism of reduction in catalase, (Deisseroth et al., 1970) illustrated by Fig. 3. This is supported by the lack of free radical formation and studies involving ^{18}O enriched peroxide. Such ^{18}O enriched peroxides evolved $^{18}O_2$ in a mole fraction similar to the substrate, indicating that the two atoms of oxygen evolved are derived from the same substrate molecule.

The function of catalase in the cell is obviously to remove excess hydrogen peroxide, however it has been postulated to fulfill other roles in the cell. For example, the catalase function in red blood cells is also carried out by glutathione peroxidase. Under normal physiological conditions glutathione peroxidase functions to protect hemoglobin from oxidation (Aebi, 1974). Therefore, the role of catalase in these cells does not appear to be the removal of H_2O_2 and is as yet unknown.

However the peroxidase function of catalase has implicated catalase in oxidation pathways. Deisseroth et al. (1970) suggested that this peroxidase function may be coupled to the oxidation of NADH without conservation of energy. Catalase may also function along with cytochrome c oxidase to remove hydrogen peroxide, i.e.

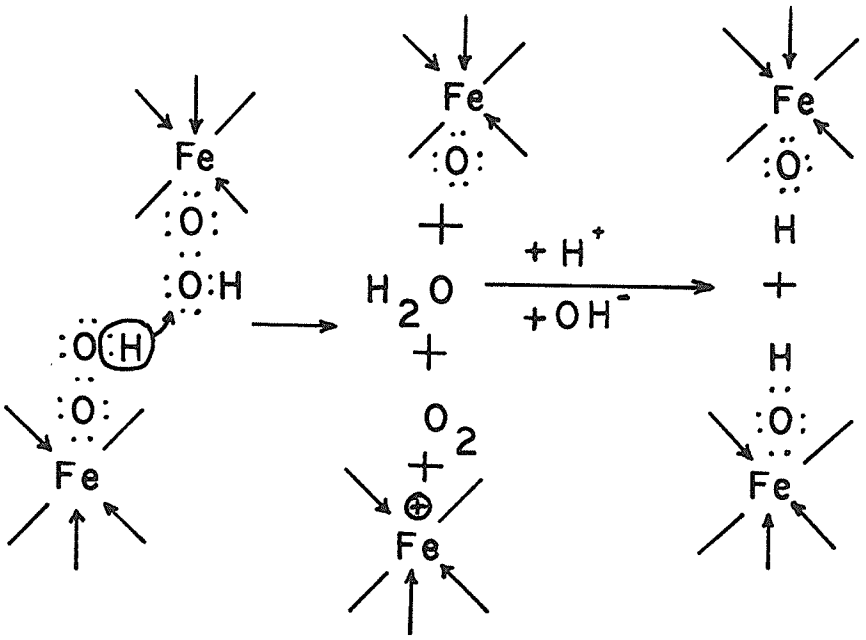
$$4H_2O + 4 \text{ cyt. c} - Fe^{++} + 2\ O_2 \longrightarrow 4 \text{ cyt. c} - Fe^{+++} - OH + 2H_2O_2$$

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

The amount of catalase synthesized has been shown to be regulated

Figure 3. Mechanism of catalase action.

(Deisseroth and Dounce 1970)


Fe
:O:
:O:H
:O:H
:O:
Fe
Fe
:O:
+
H2O
+
O2
+
Fe
+ H+
+ OH-
Fe
:O:
H
+
H
:O:
Fe

by catabolite repression. Catalase positive E. coli which are grown on intermediates of the Krebs cycle or on undefined medium have high levels of catalase, whereas glucose, glycerol, maltose, lactose, xylose, mannitol, sorbitol and trehalose will induce repression of catalase synthesis, (Yoshpe-Purer et al., 1977).

4.0 The Respiratory Pathway of Azotobacter

4.1 Cytochrome content

At least six cytochromes are known to be present in Azotobacter. These are cytochromes c_4 and c_5, an autooxidizable cytochrome b_1 and three cytochrome oxidases a_1, o and d. Cytochromes b, c and d were found by Negelein et al., (1934) and subsequently by Smith (1954), Bruemmer (1957) and Temperli (1960). Cytochrome o and a_1 were shown to be present by Caster and Chance (1959) who also showed that carbon monoxide combined with cytochrome a , o and d indicating their role as cytochrome oxidase. Quantitative studies by Jones and Redfearn (1967) indicated cytochrome d to be the main terminal oxidase, being present in quantities eight fold greater than cytochrome o.

4.2 Quinone component

The quinone component of the respiratory chain in Azotobacter is ubiquinone Q-8 detected by Temperli and Page (1960) and Page et al. (1960). Ubiquinone is present in excess, the levels being six fold greater than levels of the individual cytochrome components. Ubiquinone is also the sole quinone component, no menadione was detected by Jones and Redfearn (1966). Swank and Burris (1969) showed ubiquinone to be essential for the respiration of Azotobacter. These workers extracted ubiquinone with pentane which resulted in a

proportional loss of respiratory activity which could be restored by the addition of native ubiquinone.

4.3 Dehydrogenases

Azotobacter contain a number of active membrane bound dehydrogenases which include NADH dehydrogenase (Bruemmer 1957, Tissiers 1957 and Repaska, 1958), NADPH dehydrogenase (Pandit, 1967, Achrell, 1972), succinate dehydrogenase (Repaske 1954, Alexander, 1955) and malate and lactate dehydrogenase (Hovenkamp, 1959).

The NADH dehydrogenase is the rate limiting compound of NADH oxidase in Azotobacter (Jones et al. 1971). The Km value for NADH of this enzyme is 40 μM. The enzyme contains 1 gram atom of iron and labile sulfur per mole of flavin mononucleotide (DerVottanian, 1972). This ratio may change in iron limited cultures where one gram atom of molybdenum and two of iron and labile sulfur are found per mole of FMN.

Structural changes in the membrane have been shown to have an effect upon the activity of NADH dehydrogenase. For example, brief sonication will result in a momentary increase in activity followed by a decrease. This enzyme is also sensitive to conditions of excess oxygen when the enzyme loses its proton translocation capabilities resulting in loss of energy conservation. Substrate inhibition has been demonstrated with mitochondrial NADH dehydrogenase in that concentration of NADH greater than 0.1 mM resulted in partial inhibition of activity (Hatefi and Stempel, 1969).

NADPH dehydrogenase is not believed to be an important electron source to the electron transport system of Azotobacter. This enzyme is an allosteric enzyme and has a poor affinity for its substrate, the $S_{0.5}$ being 440 μM (Ackrell et al., 1972). Unlike NADH dehydrogenase,

its activity is not coupled to proton translocation and ATP synthesis. However, to ensure efficiency an active transhydrogenase will transfer equivalents to NAD^+ from NADPH. The activity of NADPH dehydrogenase is competitively inhibited by adenine nucleotides, with the maximum activity at low energy charge (Ackrell, 1972).

4.4 Effects of inhibitors on respiration

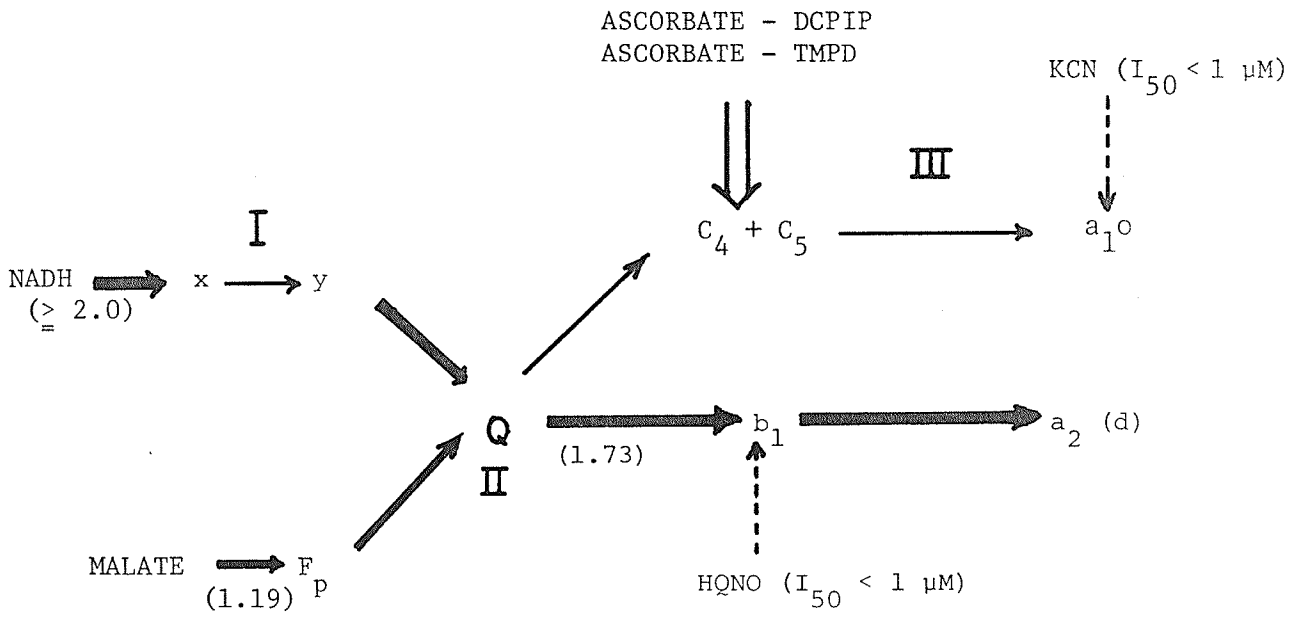
Jones and Redfearn (1967) found the oxidation of physiological substrates to be tolerant of high concentrations of KCN (Ki of 115 μM). However the oxidation of artificial electron donors was found to be inhibited by very low levels of KCN (Ki of 0.5 μM). These results suggest two separate cytochrome oxidases functioning in Azotobacter, one for physiological substrates, the second for artificial electron donors.

Oxidation reduction kinetics (Jones and Redfearn, 1967) indicated that DCPIP - ascorbate donated electrons at the level of cytochrome c. These studies also indicated cytochrome c_4 and c_5 to be on a different path than cytochrome b_1, since the c-type cytochromes show a greater reduction in the aerobic steady state than the b type cytochrome.

These workers also described CO action spectra indicating DCPIP - ascorbate inhibition was much more sensitive to relief by light than NADH oxidase. Low concentrations of KCN, which abolish the relief by white light of CO inhibition utilizing artificial electron donors, had no effect on the light relief of NADH oxidase. The inability of KCN to abolish light relief may reflect the insensitivity of cytochrome d to the inhibitor.

Jones and Redfearn (1967) proposed a branched chain electron transport system to explain their results (Fig. 4). The cytochrome d

Figure 4. The respiratory system of *A. vinelandii*. I, II and III represent the three energy coupling sites. Numbers in parentheses refer to electron transfer rates (μmoles substrate oxidized/min mg protein) in absence of ADP.
Broken arrows indicate inhibitor action.
Solid arrows indicate physiological electron transfer.
Open arrows indicate artificial electron transfer.
(Jones *et al.*, 1971)


ASCORBATE - DCPIP
ASCORBATE - TMPD
KCN (I_{50} < 1 μM)
III
I
$C_4 + C_5$
a_1 o
NADH
(≥ 2.0)
x
y
Q
II
(1.73)
b_1
a_2 (d)
MALATE
(1.19)
F_p
HQNO (I_{50} < 1 μM)

($\underline{a}_2$) is the main terminal oxidase of the branched chain system. The second branch of the system is believed to carry a much lower flux of electrons and to terminate with the $\underline{o}$ and $\underline{a}_1$ terminal oxidase. This path is very sensitive to inhibition by KCN, but unlike the major path is insensitive to 2-n-alkyl-4-hydroxyquinoline (HQNO), an inhibitor believed to prevent the reoxidation of $\underline{b}$ type cytochromes. Parallelism of the $\underline{c}$ type cytochromes with cytochrome $\underline{b}$ is shown by the inability of HQNO to affect the steady state reduction of cytochrome $\underline{c}$. Therefore, electrons flowing through the minor branch are transferred to oxygen by:

$$\text{hydroquinone} \rightarrow \text{cytochrome } \underline{c}_4 + \underline{c}_5 \rightarrow \underline{a}_1\underline{o} \rightarrow O_2$$

This minor path cannot support more than 30% of the total flux of the respiratory chain (Sagi-Eisenberg and Gutman, 1979).

The minor branch may be further subdivided to cytochrome $\underline{c}_4 \rightarrow O_2$ and cytochrome $\underline{c}_5 \rightarrow O_2$ on the basis that purified $\underline{c}_4$ and $\underline{c}_5$ yielded different P/O ratios when used as electron mediators between ascorbate and the respiratory chain (Ackrell and Jones, 1971a), i.e. $\underline{c}_5 \rightarrow \underline{a}_1\underline{o}$ may be non-phosphorylating.

Other sites of phosphorylation are believed to occur at the level of NADH dehydrogenase (Eilermann, 1970) and in the ubiquinone region.

In the absence of ADP or inorganic phosphate the flow of electrons through a coupled site may be inhibited, this inhibition is indicative of the degree of coupling occurring at this site and is termed respiratory control. Such control occurs at the level of NADH dehydrogenase and in the ubiquinone region; however, this control is dependent upon the electron pressure at the phosphorylation site. For example, NADH as substrate elicits respiratory control at site I. Control at site II

was only observed when the ubiquinone region was made rate limiting by use of the inhibitor HQNO, which slows down the flow of electrons, or by using a pair of substrates such as lactate and malate (Eilermann et al., 1971, Jones, 1971a,b).

The third site of phosphorylation occuring on the minor pathway does not elicit any respiratory control, indicating there may be a very low degree of energy coupling on this branch. However, Ackrell and Jones (1971) demonstrated a stimulation of 0.20 in the P/O ratio in the presence of HQNO; the stimulation was inhibited by low concentrations of KCN. However, these authors concluded that the minor pathway does not contribute significantly to the overall phosphorylation of the cell since no significant change in overall P/O ratio was observed with low KCN compared to cells without KCN. Studies on the stoichiometry of respiratory-driven proton translocation (the H^+/O ratio) exhibited by whole cells of A. vinelandii were more consistent with two sites of phosphorylation rather than three as was proposed.

The phosphorylating capability of the three sites is affected by the oxygen tension in the medium. For example, the efficiency at site I increases as the oxygen tension decreases and is maximal when oxygen is rate limiting (Ackrell and Jones, 1971b). When cells enter oxygen limitation the respiratory carrier concentration increases, as does the activity of the primary dehydrogenase and the activity of the minor path increases nine to thirteen fold (Ackrell and Jones, 1971b). The increased activity of the minor path under oxygen limited conditions suggest the minor path may act to increase the efficiency of energy conservation during conditions when the cell is energy limited.

The possibility that the coupling observed at site III could be

due to artificial coupling was proposed by Sagi-Eisenberg and Gutman (1979). They noted that the oxidation of TMPD is coupled when carried out by intact A. vinelandii cells, but not by membrane vesicles. The oxidation of ascorbate on the outside of the intact cell would liberate protons while protons are removed from the interior by the terminal oxidase which is provided with electrons from reduced TMPD. The pH difference generated may be sufficient to produce an artificial loop of energy conservation. Membrane fragments, which do not demonstrate energy coupling to TMPD oxidation, would form inside-out vesicles which would prevent the formation of a pH gradiant.

Studies on mutants devoid of the ability to oxidize TMPD-ascorbate were carried out by Hoffman et al. (1979). This mutant was unable to oxidize reduced $c_4 + c_5$ by a_1o. This did not affect the respiration rate of the mutant compared to the wild type cell. No effect was observed on either the phosphorylating efficiency or on the H^+/O ratio which was about 4.0 for both the wild type and mutant. These workers also examined the oxygen uptake kinetics of the wild type cell. They found these kinetics to be solely due to the cytochrome d pathway when at very low oxygen tensions. Addition of TMPD-ascorbate to wild type cell illustrated the oxidase of the minor path had a greater affinity for oxygen although it had a much lower Vmax value than the major path. Because of the inability to detect the kinetic characteristics of the minor path even at low oxygen tensions, Hoffman et al. (1979) proposed this path as unlikely to oxidize even small quantities of NADH.

5. The Major Cytochrome Oxidases: Cytochrome o and d

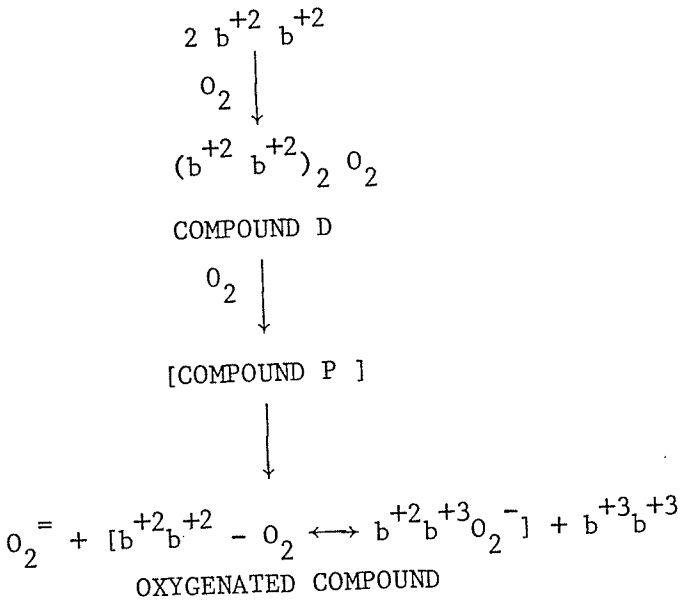
5.1 Cytochrome o

Studies of the cytochrome o in Azotobacter are complicated by the presence of two other cytochrome oxidases. Since most work done on this cytochrome oxidase has dealt with the cytochrome o of Vitreoscilla, which contains cytochrome o as the sole terminal oxidase, I shall discuss some observations which may possibly apply to Azotobacter cytochrome o.

The Vitreoscilla cytochrome o has a molecular weight of 27,000 and consists of two identical polypeptide chains. Each molecule of cytochrome o has two molecules of protoheme IX, each capable of binding CN^- independently (Tyree and Webster, 1978). The midpoint potential of the two hemes are +0.118 volts and -0.122 volts (Tyree and Webster, 1978b). Binding studies of CO to reduced cytochrome o resulted in a sigmoidal curve with a Hill plot slope of 2.0 indicating cooperativity between the binding sites with at least two binding sites functioning (Tyree and Webster 1978a). However, $K^{14}CN$ binding studies to oxidized cytochrome o indicated that no such interaction occured between the two subunits. Therefore it appears that in the reduced form there is cooperativity between the subunits, and upon oxidation this cooperativity is lost. Oxidation of the purified reduced cytochrome o requires approximately 0.5 mole oxygen per cytochrome molecule, this oxidized form called compound D, has absorbance maximum of 516,548 and 418 nm. Addition of more oxygen results in the formation of an oxygenated species with absorbance maximum at 576,543 and 414 nm. This oxygenated

Figure 5. Proposed model for the reaction of reduced cytochrome o with molecular oxygen.

(Tyree and Webster 1978)


2 b+2 b+2
O2
(b+2 b+2)2 O2
COMPOUND D
O2
[COMPOUND P]
O2= + [b+2b+2 - O2 ⟷ b+2b+3O2-] + b+3b+3
OXYGENATED COMPOUND

form was easily converted to the compound D by NADH catalysed by the enzyme NADH-cytochrome o reductase or by dithionite. The oxygenated form is the predominant form of cytochrome o during catalytic turnover in whole cell suspensions (Webster and Oris, 1977).

Tyree and Webster (1978) proposed a model for the reaction of reduced cytochrome o with oxygen (Fig. 5). The first intermediate, Compound D, is believed to have one bound oxygen molecule between two molecules of cytochrome o without any significant transfer of electrons from the iron, thus remaining in the ferrous form. Further addition of oxygen results in the formation of a postulated intermediate (Compound P), which is converted to the oxygenated form. This oxygenated form contains half ferrous and half ferric iron. The oxidized cytochrome may then be reduced and react with the oxygenated form to form Compound D, thus completing the catalytic cycle.

The A. vinelandii cytochrome o also has been shown to have an oxygenated form (Yang and Jurtshuk, 1978). This form occurs rapidly when purified cytochrome o is reduced by small amounts of dithionite and subsequently aerated for about 30 secs. Untreated cytochrome o had a soret maxima at 412 nm whereas the reduced form had this peak at 425 nm. When the reduced form was oxygenated, the peak shifted back to 414 nm with a decrease in absorbance. The oxygenated form was very stable suggesting a possible functional role similar to that of mammalian oxidase (Yang and Jurtshuk, 1978).

5.2 Cytochrome d

Cytochrome d is the major functional oxidase in Azotobacter. It is rapidly oxidized and does not reach the steady state reduced form till anaerobosis is maintained (Jones and Redfearn, 1967).

The absorption spectrum of this cytochrome shows a peak in the red end of the spectrum, the reduced peak at 631 nm and the oxidized peak at 648 nm. The oxidized peak can be further resolved into three gaussian curves with peaks at 635, 648 and 670 nm. The reduced cytochrome absorption peak at 631 nm is composed of a single peak. The ratio of the 648 and 670 nm peak varies depending upon the preparation and may be composed of two different compounds. However, the peak at 648 is believed to be the same compound as that producing the peak at 631 nm.

An intermediate is believed to be formed when the oxidized cytochrome d, exhibiting the peak at 648, is produced from the reduced form. This was shown by a gradual oxidation of the reduced cytochrome d when oxygen was added slowly to A. vinelandii cells. The peak at 631 nm slowly disappeared, however no increase in the peak at 648 was observed till there was excess oxygen in solution. The intermediate formed did not absorb light in the red end of the spectrum and may be the normal oxidized form of cytochrome d (Kauffman and Van Gelder, 1973a). The formation of the compound absorbing at 648 nm was proposed to be due to a charge transfer of an electron from the oxygen to the iron of the heme, thus giving a reduced type of spectrum.

Kauffman and Van Gelder (1973b) proposed the binding of CN^- to the cytochrome d occurs at the level of this intermediate compound. Upon addition of CN^- to reduced cytochrome d no binding was observed. However, addition of CN^- to the oxidized cytochrome d results in the loss of the peak at 648 nm with no formation of a 631 nm peak. The two other oxidized peaks reacted differently to CN^-, the 670 nm peak

decreased more slowly whereas the 635 nm peak was not affected. The binding of CN^- to the intermediate may prevent the charge transfer which produces the 648 nm band, i.e.

$$\underline{d}_{648} \rightleftharpoons \underline{dx} \rightleftharpoons \underline{dx}\text{-}CN^-$$

6. Carbon Metabolism in Azotobacter

6.1 Major catabolic pathways

In order to maintain the high rates of respiration necessary for the added energy demands of nitrogen fixation and respiratory protection, Azotobacter must possess very efficient means of carbon assimilation and catabolism.

The major pathways of Azotobacter for carbon substrate oxidation are the Entner Doudoroff pathway, the pentose phosphate pathway (Mortenson and Wilson, 1954, Mortenson et al., 1955, Still and Wang, 1964, Haaker and Veeger, 1976) and the TCA pathway. Glycolysis does not function as a complete path because of the absence of a phosphofructokinase (Stephenson et al., 1978).

The control of carbon oxidation depends to an extent upon the environment of the cell. For example, Haaker and Veeger (1976) described differences between cells grown in the presence of excess oxygen and those grown under low oxygen as to their ability to oxidise sucrose. Cells taken from an oxygen limited culture are capable of oxidizing pyruvate much more rapidly than sucrose, i.e. 300-600 versus 20-140 nmoles O_2 min^{-1} mg^{-1}. However, cells taken from a culture with excess oxygen had about the same rate of substrate oxidation for both pyruvate and sucrose.

These authors also found higher levels of NADH dehydrogenase at

high O_2 concentrations, but because they detected only very low levels of reduced pyridine nucleotides in the cell under these conditions, they dismissed the possibility that this would have any controlling effect over the enzymes of the Entner Doudoroff or pentose phosphate pathway. They also excluded the possibility of control by energy charge since there was no significant change between the different types of cell examined. Therefore Haaker and Veeger (1976) suggested the rate of sucrose oxidation is determined by the activity of the sucrose translocator enzyme. The uptake mechanism may be coupled to the activity of the glucose-6-phosphate dehydrogenase since this enzyme increases three to four times in the presence of excess oxygen (Haaker and Veeger, 1976).

Glucose transport has also been proposed to be coupled primarily to malate oxidation at a low potential site in membrane vesicles from Azotobacter (Barnes, 1973). He also found the transport of glucose to be coupled to TMPD oxidation, which occurs at a higher redox potential. This led Barnes to propose two sites of coupling for the transport of glucose in these cells, one the low potential site and a second on the minor branch of the electron transport chain.

The control by reduced pyridine nucleotides and by energy charge, although unlikely for cells in the presence of excess oxygen, may occur in oxygen limited cells. Senior and Dawes (1971) found control is exerted by NAD(P)H on glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, citrate synthetase and isocitrate dehydrogenase in A. beijerinkii. Stephenson and Dawes (1978) found acetyl-CoA and NADH to inhibit the pyruvate dehydrogenase enzyme. Therefore high levels of NADH and acetyl CoA may inhibit the flow of carbon

with a possible detrimental effect to the cell. The cell may remove the extra reducing equivalents and acetyl CoA by sequestering them in a carbon and energy sink which may be the function of poly-β-hydroxybutyrate.

6.2 Poly-β-hydroxybutyrate

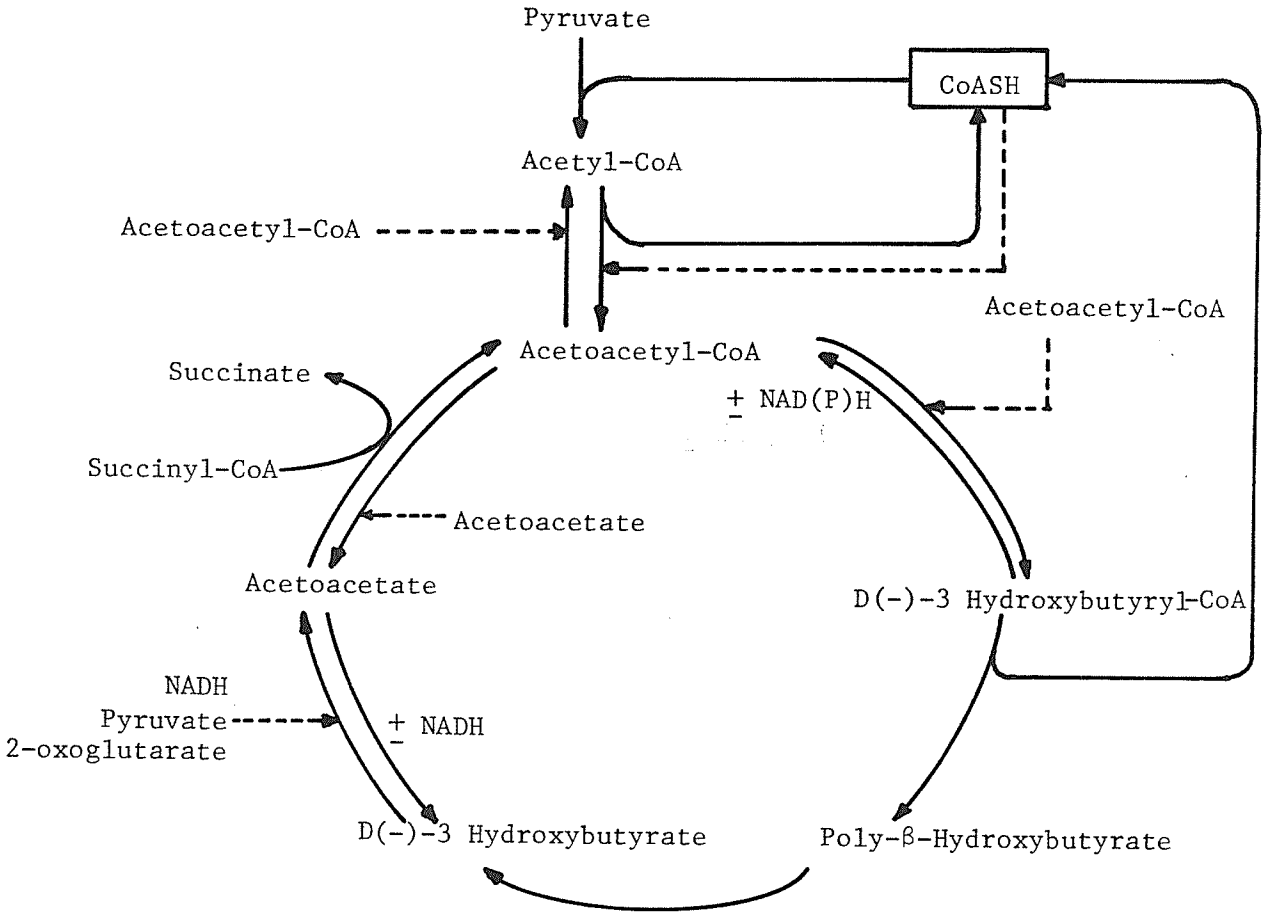
Poly-β-hydroxybutyrate is the sole storage form of carbon and reducing power in Azotobacter. A. beijerinkii in batch and oxygen limited continuous cultures, has been shown capable of accumulating up to 70% of its dry weight in the form of this polymer. The initiating factor for polymer synthesis has been shown to be oxygen limitation rather than nitrogen limitation. The growth rate has also been shown to affect synthesis since the PHB content of oxygen limited cultures is inversely related to the growth rate (Senior, 1972).

Senior and Dawes (1973) suggested a cyclic mechanism for the production and degradation of PHB in Azotobacter (Fig. 6). Acetyl CoA is the substrate for both citrate synthase of the TCA cycle and β-ketothiolase, the initiating enzyme for polymer synthesis. During conditions of abundant oxygen supply acetyl CoA is rapidly consumed by citrate synthesis with a resulting high intracellular CoASH level. High CoASH levels have an inhibitory effect upon β-ketothiolase resulting in negligible PHB synthesis. When the cell is placed in oxygen limited conditions, the levels of reduced pyridine nucleotides increase which has a negative effect on the citrate synthase activity. Resulting high levels of acetyl-CoA and low CoASH which allow greater activity of β-ketothiolase, resulted in increased carbon flow toward polymer synthesis.

Acetoacetyl CoA, formed by the action of β-ketothiolase, is

Figure 6. Regulation of poly-β-hydroxybutyrate metabolism in *A. beijerinckii*. Broken lines indicate inhibition.

(Senior and Dawes 1972)


Pyruvate
CoASH
Acetyl-CoA
Acetoacetyl-CoA
Acetoacetyl-CoA
Acetoacetyl-CoA
Succinate
± NAD(P)H
Succinyl-CoA
Acetoacetate
Acetoacetate
D(-)-3 Hydroxybutyryl-CoA
NADH
Pyruvate
2-oxoglutarate
± NADH
D(-)-3 Hydroxybutyrate
Poly-β-Hydroxybutyrate

reduced to D(-)-3-hydroxybutyryl CoA by acetoacetyl-CoA (Ritchie, 1968, Ritchie et al., 1971, Senior, 1972). Such inhibition was suggested to occur to prevent wasteful cycling in the system (Senior and Dawes, 1973). Polymerization of D(-)-3-hydroxybutyryl CoA would result in release of CoASH and formation of PHB.

Degradation of PHB was proposed to be controlled by NADH, pyruvate and α-ketoglutarate, all being inhibitory to the D(-)-3-hydroxybutyrate dehydrogenase. Therefore, under conditions of high oxygen tension resulting in a high NAD^+:NADH ratio, this enzyme may still be inhibited by pyruvate and α-ketoglutarate. Such inhibition would insure that the cell utilizes all extracellular readily utilizable carbon sources. Only when these are exhausted, resulting in decreased intracellular intermediate levels, would PHB degradation occur. Senior and Dawes (1973) had also discovered an acetoacetate succinate CoA transferase enzyme which complete the cycle. This enzyme is inhibited by acetoacetate for the succinate stimulated degradation of acetoacetyl CoA. The equilibrium constant for this reaction does not favour PHB degradation, however under conditions of low NAD(P)H, high CoASH and low acetoacetyl-CoA, this reaction would proceed toward acetoacetyl-CoA formation.

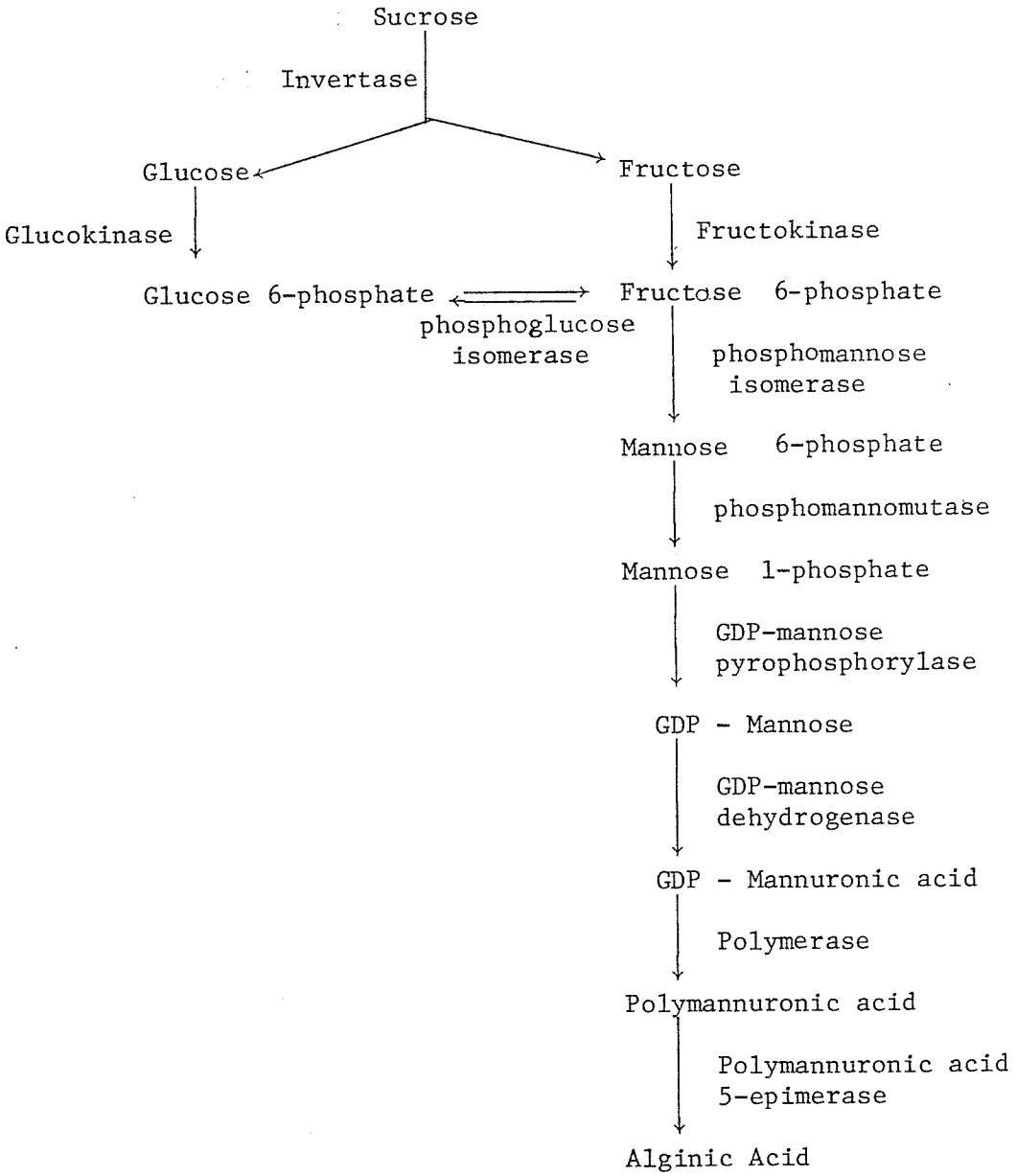
6.3 Polysaccharide production in Azotobacter

A. vinelandii normally produce a polysaccharide which has been identified as an alginic acid, a partially acetylated linear copolymer composed mainly of D-mannuronic acid and smaller amounts of L-guluronic acid (Gorin and Spencer, 1966, Larsen and Haug, 1971). Larsen and Haug (1970) found that the formation of this polysaccharide appears to depend upon a Ca^{++} dependent epimerase to convert D-manuronic acid residues to L-guluronic acid residues. Cells grown under

conditions of low Ca^{++} concentrations, have higher D-mannuronic acid to L-guluronic acid ratios and increased Ca^{++} results in a decrease of this ratio. Energy for this conversion was proposed to be provided by coupling the strong binding of Ca^{++} to the L-guluronic acid. Pindar and Bucke (1975) proposed a sequence of reactions leading to the production of alginic acid in A. vinlandii from sucrose (Fig. 7). The overall pathway however does not appear to differ very much from alginic acid production by the brown algae Fucus gardneri (Lin and Hassid, 19766 a,b), except the A. vinlandii guluronic acid units do not appear to act as precursors to the polymer synthesis. Therefore it appears that the calcium dependent epimerase of Azotobacter acts on the mannuronic acid polymer. The role of this polymer is not clear; however several possibilities exist as to its function. These include its possible role in protection against dessication, phagocytosis, phage attack or high oxygen tensions (Williamson 1958, Sutherland, 1972, Postgate 1974). It may be involved also in metal ion uptake (Williamson, 1958, Sutherland, 1972), act as an adhesive agent (Johnson et al., 1977), or an ATP sink (Neijssel and Tempest, 1976). It is clear however that this polysaccharide is required for the encystment of Azotobacter, since lack of Ca^{++} which will prevent the formation of L-guluronic acid residues results in abortive encystment.

Figure 7. Pathway for the biosynthesis of alginic acid in *A. vinelandii*.

(Pindar and Bucke 1975)


Sucrose
Invertase
Glucose
Fructose
Glucokinase
Fructokinase
Glucose 6-phosphate
phosphoglucose isomerase
Fructose 6-phosphate
phosphomannose isomerase
Mannose 6-phosphate
phosphomannomutase
Mannose 1-phosphate
GDP-mannose pyrophosphorylase
GDP - Mannose
GDP-mannose dehydrogenase
GDP - Mannuronic acid
Polymerase
Polymannuronic acid
Polymannuronic acid 5-epimerase
Alginic Acid

7. Encystment of Azotobacter

7.1 Characteristics of encysting cells

Cyst production by Azotobacter was first described by Batchinskya (1935), where she noted the formation of spherical structures much different from the oblong vegetative cell. Winogradsky (1938) showed that cyst formation is induced by growth on butanol or ethanol, while readily oxidizable sugars such as glucose or sucrose suppressed its formation. He noted that the central body is encased by an inner coat called the intine and an outer coat called the exine. Lipid in the form of poly-β-hydroxybutyrate accumulates within the central body (Forsyth et al., 1958, Stevenson and Socolofsky, 1966). Socolofsky and Wyss (1962) demonstrated that Azotobacter cysts are resistant to the lethal effects of UV light, sonication and dessication yet showed no significant resistance to heat.

Another method of inducing cyst formation was described by Layne and Johnson (1964), who showed increased cyst production when A. vinelandii was exposed to severe mineral deficiency. However these cysts differed from the cysts produced by growth on butanol. Unlike the previously reported cysts, these lacked both intine and exine yet were reported resistant to sonic oscillation and dissication, casting doubts upon the role of the exine and intine coats as protective structures. However Parker et al. (1966) demonstrated the importance of the exine in providing resistance to the cyst. Cyst exines can be ruptured by chelating agents releasing the central body. The resistance of this central body to dessication was determined to be much less than the intact cyst. Therefore, the exine appears to be required by the central body to confer resistance. The mechanism

of resistance of the mineral deficient cells is not known.

Lin and Sadoff (1968) demonstrated that much faster rates of encystation were possible when β-hydroxybutyrate or crotonate were added to washed glucose grown cells. Butyraldehyde, hydroxybutyraldehyde, butyrate and ethyl acetoacetate were not effective inducers. Also prior growth in acetate prevented induction of encystation. Washing of the cells was essential to prevent the release of copious amounts of polysaccaride which has been termed abortive encystment. This release of polysaccaride occurs without any cyst formation. Lack of Ca^{++} has also been shown to induce abortive encystment (Page and Sadoff, 1975, Stevenson and Socolfsky, 1972).

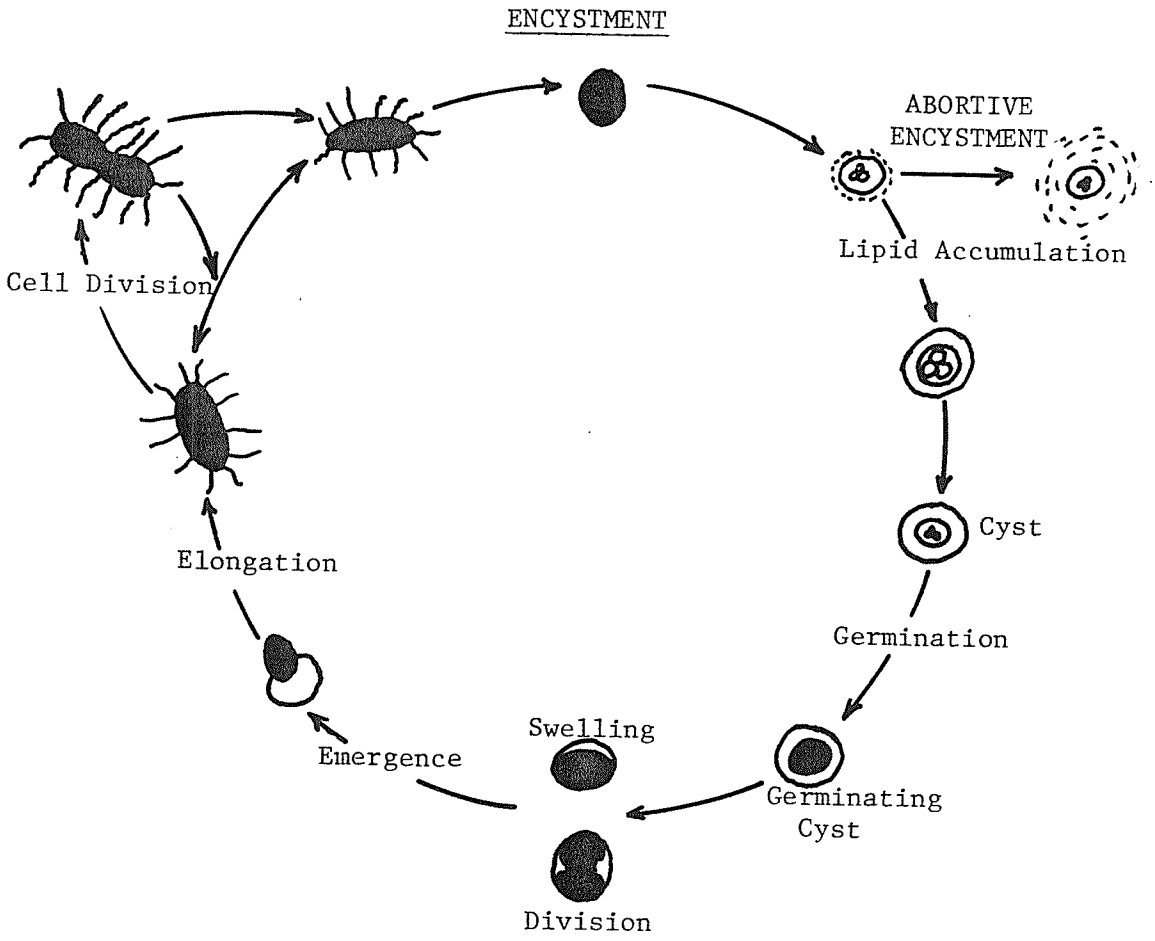
Once a cell has been induced to encyst it undergoes a series of physiological changes which have been described in terms of a life cycle by Sadoff *et al*. (1971), (Fig. 8). After induction of encystment the cells lose their mobility, become spherical and the walls become thickened. They undergo a final division yielding two daughter cells. At six hours membrane blebs are secreted from the surface of the encysting cell which break free and form sheets embedding in the capsular material. The sheets give the exine a layered or shingled appearance (Cagle, 1972, Koo, 1969).

The cyst once formed is metabolically dormant being capable of surviving ten years in dry soil (Vela, 1974).

Germination occurs at 30°C in B_6 medium with glucose, sucrose or acetate as the carbon source (Loperfido, 1973). The central body swells and occupies the entire volume. Respiration proceeds immediately upon addition of the carbon source followed by RNA and protein synthesis. By eight hours the exine develops a fracture with the release of the

Figure 8. Life cycle of *A. vinelandii*.

(Sadoff, 1975)


ENCYSTMENT
ABORTIVE
ENCYSTMENT
Lipid Accumulation
Cyst
Germination
Germinating
Cyst
Swelling
Division
Emergence
Elongation
Cell Division

typical vegetative cell. Motility returns before the first cell division.

When the cell undergoes encystment with BHB, the cell must shift metabolism so as to utilize this new carbon source. Gluconeogenic enzymes and glyoxylate pathway enzymes must be produced, whereas glucose-6-phosphate dehydrogenase activity immediately starts to decrease (Hitchins and Sadoff, 1973). A bimodal pattern is seen in the activity of the glyoxylate pathway enzymes and gluconeogenic enzymes with a low peak at approximately three hours and a large peak at twenty-five hours.

7.2 Polysaccharide components of the cyst

D-mannuronic and L-guluronic acids are the only uronic acids present in isolated exine and intine fractions. The exine is rich in guluronic acid residue whereas the intine contains a high percentage of mannuronic acid. L-guluronic acid residues are scarce in polysaccaride from cells which have undergone abortive encystement. The reason for the preponderance of L-guluronic acid residues in the exine may be due to the tertiary structure of the molecules. X-ray fiber diffraction studies and polarised infrared spectroscopy studies indicate distinct differences between the poly-mannurinate and poly-gulurionate (Atkins, 1971). Poly-mannurinate has a flat ribbon-like structure with H-bonding between the O^3-H and the O^5 ring oxygen of the next mannuronic acid residue. This structure does not induce strong binding of Ca^{++} (Grant et al., 1973). Polygulurionate has a stiff rod-like structure. It is stabilized by one H_2O per acid residue with H-bonding between $-O^2$-H and either oxygen of this next carboxyl unit. This structure allows four oxygens to participate in coordination complexes with

divalent cations.

It is easily seen why chelators will have a destructive effect on the exine. Removal of the Ca^{++} will result in loss of the coordinate complex resulting in destabilization of the tertiary structure. Abortive encystment caused by lack of Ca^{++}, brought about by chelation, may be the direct affect of either lack of energy due to the requirement of Ca^{++} binding to the guluronic acid residue to drive the epimerization reaction or the inability to form a strong exine because of the absence of Ca^{++} to maintain structure.

8. Nitrogen Fixation

8.1 Relationship of Nitrogen fixation to oxygen

One common and important characteristic of all N_2 fixers is the anaerobic environment required by their nitrogenase, in order to function. This requirement places severe restrictions on cells which normally grow in the presence of oxygen. For example nitrogen fixing cyanobacteria such as Anabaena cylindrica must compartmentalize the nitrogenase enzymes into specialized cells called hetrocysts which lack the photosynthetic capability to generate oxygen, thus allowing N_2 fixation in the cells when those around them are actively photosynthesizing. Rhizobium must enter the environment of a root nodule or grow under low oxygen tension in vitro, before nitrogen fixation can proceed; the root nodule provides protection from oxygen since leghemoglobin appears to bind excess oxygen from the environment of the bacteroids. Finally, Azotobacter must develop extraordinary respiration capabilities and induce conformational protection in order to protect its nitrogenase from the deleterious effects of oxygen (Dalton and Postgate, 1969). Since nitrogenase has been reported as the site of

oxygen toxicity to Azotobacter, a close examination of the function of this enzyme is required in a study examining the toxicity of oxygen to Azotobacter.

8.2 Biological N_2 fixation

The reduction of dinitrogen to ammonia, catalysed by the enzyme nitrogenase, is carried out by a wide variety of organisms including strict anaerobes such as Clostridium pasteurianium or Desulfovibrio desulfuricans, facultative anerobes: Bacillus and members of the genus Klebsiella, Citrobacter, Enterobacter and Erwinia, and strict aerobes such as Azotobacter, Azomonas, Azotococcus, Beijerinkii and Derxia (Postgate, 1978).

The contribution of biological nitrogen fixation to the overall world input of fixed nitrogen is believed to be enormous. It has been estimated that 15% of the fixed nitrogen is produced by the Haber Bosch process and approximately 10% is contributed by nitrous oxide formation in the air, this leaves over 70% or approximately 14 x 10^7 tonnes of nitrogen to be contributed by biological nitrogen fixation. The most important species in terms of N_2 fixed, is believed to be the Rhizobium species; in symbiosis with legumes it is capable of producing about 100 to 300 kg N per hectare per year (Dalton and Mortenson, 1972). Of the free living nitrogen fixers, the photosynthetic capabilities combined with nitrogen fixation makes the Cyanobacteria the most effective producers of fixed nitrogen as well as the most ubiquitous. Heterotrophic nitrogen fixation is not believed to contribute much to the overall global fixed nitrogen because of the requirement for an oxidizable carbon source. However the ease of growth and the high cell yields make the study of

heterotrophic nitrogen fixation much more convenient, so it is from these organisms that most of the knowledge of nitrogen fixation is derived.

8.3 The nitrogenase enzyme

The nitrogenase complex consists of two types of proteins both essential for catalysis. These are a large molybdenium iron protein with molecular weight of 270,000 and a smaller protein which has a molecular weight of 56,000 to 67,000. These proteins are respectively termed the MoFe protein and the FeS protein. The larger protein may consist of four subunits of tetramer of $\alpha_2\beta_2$ structure and has a large number of iron sulfur centers with approximately 24 Fe + 24 S per 2 Mo atoms. Mossbauer spectroscopy suggests the Fe atoms are grouped in at least three and possibly four distinct units, however during turnover only one center changes properties. The overall redox potential of the MoFe protein is less than -260 mV and may be as low as -460 mV during nitrogen fixation.

The FeS protein from Clostridium and Azotobacter is cold labile and irreversibly damaged by oxygen. It will bind ATP and ADP producing a well studied conformational change in the protein. This can be detected by EPR spectrum studies which have shown that the effect is specific for adenosine di- and tri-phosphates. EPR spectrum studies also indicate two molecules of Mg-ATP must bind to the Fe protein to give a complete conformational change. This change also appears to open the conformation of the protein making its iron moiety more accessible to chelating agents. It is also accompanied by a shift of the redox potential from -294 to -402 mV.

8.4 Requirements for catalysis

For full activity the nitrogenase enzyme requires a source of electrons, an electron carrier, ATP and Mg^{++}. The electron source in strict anaerobes has been identified by Mortenson et al. (1963) as pyruvate. Electrons are accepted by ferredoxin from pyruvate dehydrogenase via the phosphoroclastic reaction. The source of electrons for nitrogenase in aerobic bacteria is not as clear, some possibilities include NAD(P)H via flavodoxin or ferredoxin (Benneman et al. 1971), a similar type of phosphoroclastic reaction as in anaerobes (Bresters et al., 1972) and a membrane bound flavodoxin reductase (Haaker and Veeger, 1976).

The involvement of a membrane bound flavodoxin reductase is likely to be the mechanism involved in reducing flavodoxin which then transfers reducing equivalents to nitrogenase. Haaker and Veeger (1974) demonstrated the involvement of the highly energized cytoplasmic membrane by use of an uncoupler and acetate which does not require energy to be assimilated. In these cells the ATP and ADP levels are about the same as the control, but nitrogenase activity is almost completely suppressed. Nitrogenase also requires an electron carrier, but attempts at using physiological substrates as electron sources have not been very successful (Yates and Jones, 1974). Yoch and Arnon (1970) were the first to use a physiological electron source when they donated electrons to nitrogenase from heated spinach chloroplasts. Nitrogenase activity occurred only in the presence of ATP and an electron carrier such as ferrodoxin or flavodoxin. The flavodoxin of Azotobacter exists in three oxidation states i.e. oxidized, semiquinone and hydroquinone. The hydroquinone is the form believed to be

capable of supporting nitrogenase activity (Yates, 1972). Oxidation of the hydroquinone may be detrimental to the activity of nitrogenase since flavodoxin from Azotobacter is easily oxidized by molecular oxygen. Therefore the occurrence of oxygen in the cell may allow the diversion of the flow of reducing equivalents from the nitrogenase and thus cause the switch-off in activity seen in the presence of excess oxygen (Yates and Jones, 1974).

8.5 ATP requirements

Although the overall reduction of nitrogen

$$N_2 + 3H_2 \longrightarrow 2NH_3 \qquad \Delta G^\circ = 7.98$$

is exothermic (Steifel, 1977), the enzyme requires large amounts of ATP to function. Dalton and Postgate (1969b) suggested the maximum amount of energy required is four ATP per mole N_2 fixed in Azotobacter. Generally, it is believed that not less than four ATP molecules per two electron transferred are required (Watt et al., 1976). Higher values for ATP utilization were determined by Daesch and Mortenson (1968) with C. pasteurianium which appears to use twenty ATP per N_2 fixed.

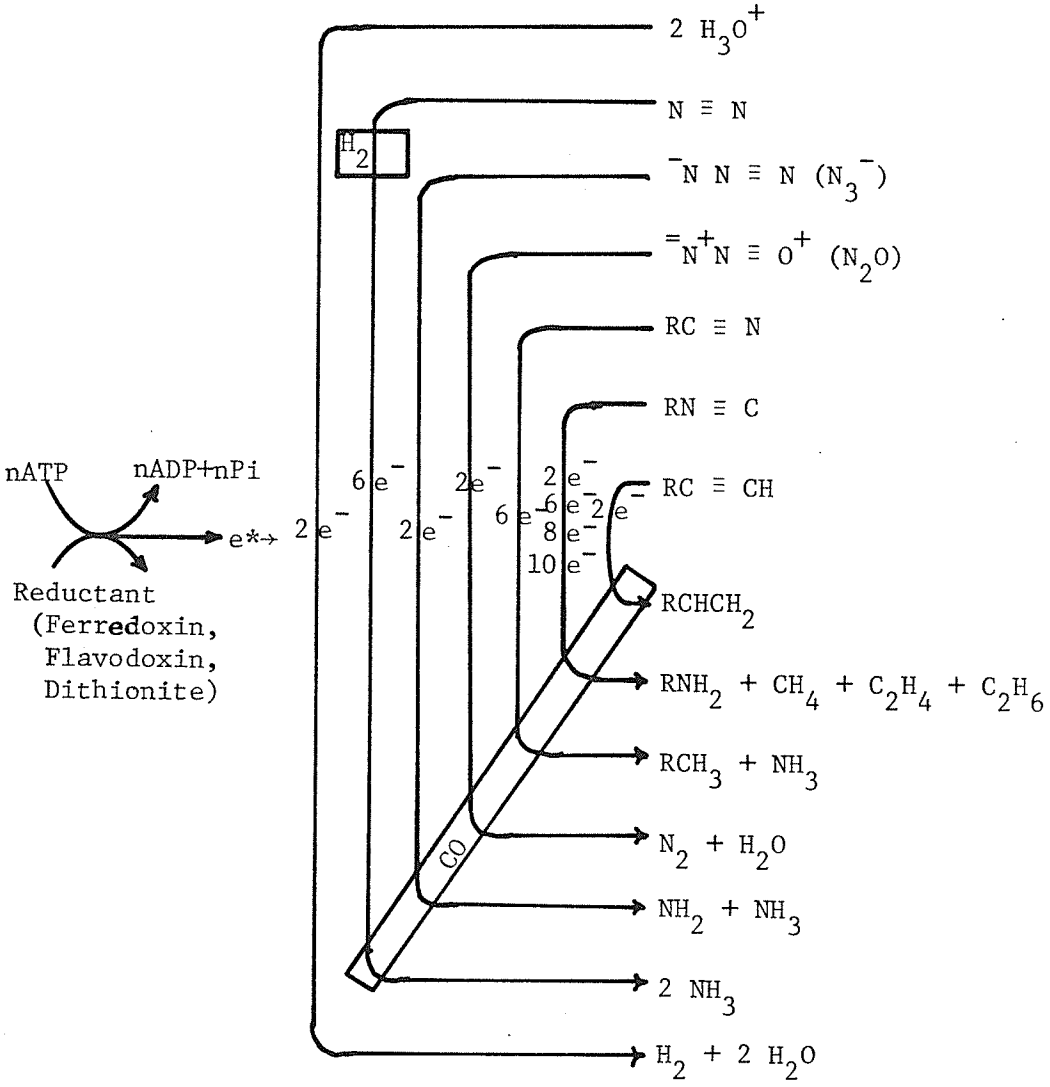
8.6 Reactions occurring on Nitrogenase

The reduction of dinitrogen is not the sole reduction which nitrogenase is capable of catalyzing. Fig. 9 shows the other substrates which may be reduced by this enzyme (Burns and Hardy, 1975). These substrates are generally cylindrical with a terminal triple bond (with the exception of H_3O^+). The reduction of all substrates, except H_3O^+, is non-competitively inhibited by CO (Rivera-Ortiz and

Figure 9. Reactions of nitrogenase

$\boxed{H_2}$ and $\boxed{CO}$ indicate reactions inhibited by these gases.

(Burns and Hardy, 1975)


nATP
nADP+nPi
e*→
Reductant
(Ferredoxin,
Flavodoxin,
Dithionite)
2 e⁻
6 e⁻
2 e⁻
2 e⁻
6 e⁻
2 e⁻
6 e⁻
8 e⁻
10 e⁻
2 H3O+
N ≡ N
H2
⁻N N ≡ N (N3⁻)
⁼N⁺N ≡ O⁺ (N2O)
RC ≡ N
RN ≡ C
RC ≡ CH
RCHCH2
RNH2 + CH4 + C2H4 + C2H6
RCH3 + NH3
N2 + H2O
CO
NH2 + NH3
2 NH3
H2 + 2 H2O

Burris, 1975). Competitive inhibition of N_2 reduction by H_2 occurs (Wilson, 1969) and appears to be specific for the substrate. Acetylene is also non-competitive with respect to N_2 and azide and is the only substrate capable of completely blocking H_2 formation.

8.7 Hydrogenase function of Nitrogenase

Nitrogenase undergoes three reactions which involve hydrogen gas; these are the hydrogenase reaction which results in the release of hydrogen gas, an exchange reaction between D_2O and H_2 with the release of HD and inhibition of N_2 reduction

The hydrogenase reaction reduces protons at the expense of ATP and occurs best in purified nitrogenase preparations given a reductant and ATP in the absence of N_2. This reaction still occurs but to a lesser extent in the presence of N_2 (Bulen et al., 1965). At saturating levels of N_2 the amount of H_2 gas formed is equal to the amount of N_2 gas reduced (Hadfield, 1970, Rivera-Ortiz and Burris, 1975).

Nitrogenase is also capable of exchanging protons from the solvent with hydrogen gas. This exchange was first demonstrated by Hock et al. (1957) in soybean nodules. This exchange reaction requires N_2 (Hock, 1960) and is inhibited by CO. Bulen (1976) suggested that N_2 may act as a catalyst for HD formation and the HD formed may account for a portion of the electrons consumed.

H_2 gas is also a well known inhibitor of N_2 fixation (Wilson and Umbreit, 1937). Inhibition of N_2 reduction by D_2 does not result in a greater production of hydrogen gas as would occur in the absence of N_2, rather, the flow of the reducing power is believed to go toward the production of HD (Bulen, 1976).

Steifel (1977) suggested a scheme to explain the formation of

Figure 10. Scheme for N_2 reduction, H_2 evolution and C_2H_2 reduction by nitrogenase.

(Stiefel 1977)

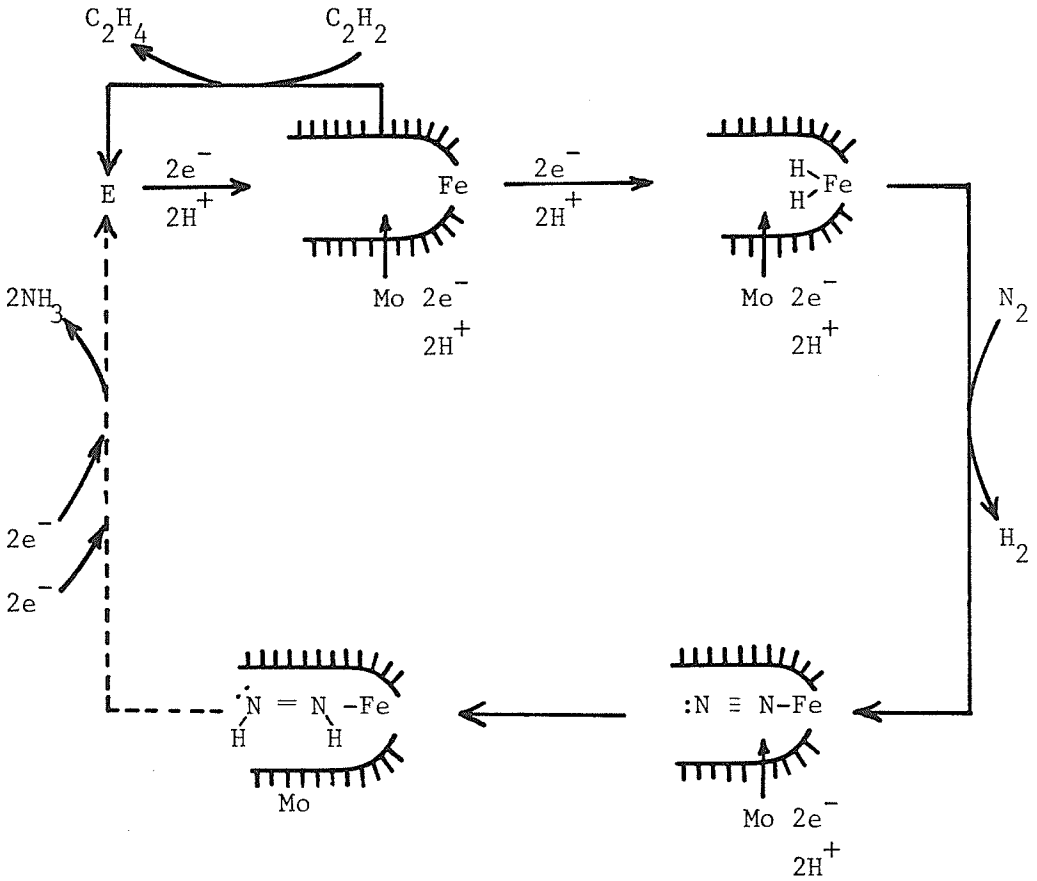

C_2H_4
C_2H_2
E
$2e^-$
$2H^+$
Fe
Mo
$2NH_3$
$2e^-$
$2e^-$
H
Fe
N_2
H_2
N = N -Fe
:N ≡ N-Fe

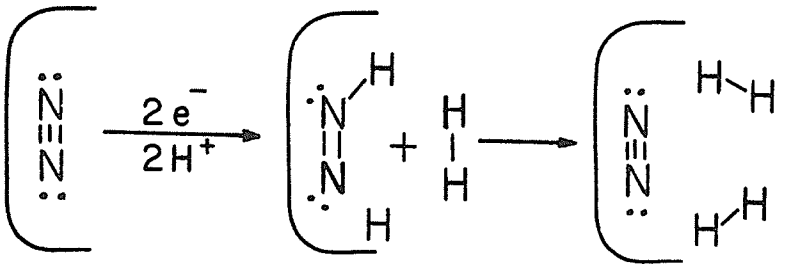
Figure 11. Proposed mechanism for

A. H_2 inhibition of N_2 reduction

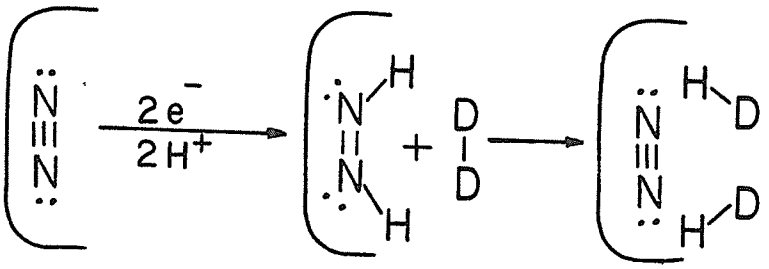
B. HD formation by nitrogenase

(Stiefel 1977)

A.



B.



hydrogen gas during catalysis as well as the inhibition of N_2 fixation by H_2 and the HD exchange reaction, (Fig. 10). The reduction of acetylene requires only a two electron transfer and is assumed to occur at the Mo site. A second metal, possibly iron which is essential for nitrogen fixation, but not for acetylene reduction, is also in the active site. Reduction of the Mo site occurs before reduction of the Fe site, and reduction of the Fe site produces a dihydride at the Fe site. Upon binding of N_2 to the Fe site the dihydride is released as H_2 gas accounting for the one to one ratio of H_2 produced to N_2 fixed. The intermediates N_2H_2 and N_2H_4 are held at the active site while the site is reactivated. The inhibition of H_2 and HD exchange are proposed to occur on the diamide intermediate (Fig. 11).

9. Bacterial Growth in a Chemostat

Analysis of bacterial metabolism in batch cultures is hindered by the many variable physical and chemical parameters introduced by a growing bacterial population to its environment. For example, increased growth can be expected to be accompanied by a reduction in oxygen supply as well as an accumulation of metabolic by-products and intracellular storage compounds as well as increased cell lysis. Such systems are always approaching zero growth and therefore are always in transient states. (Pirt 1975.)

Monod (1949) had described the specific growth rate of bacteria to be dependent upon the substrate concentration by the equation:

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

μ = specific growth

s = substrate concentration

K_s = substrate concentration at half saturation.

Most bacteria have a very high affinity for the substrate, that is K_s is very small, resulting in the growth rate being equal to the maximum growth rate even at very low concentrations of substrate. When the substrate concentration becomes limiting, the cells quickly consume it and growth ceases (Pirt 1975). It becomes practically impossible to examine cells which are in some degree of nutrient limitation and are still actively growing.

The chemostat was developed to overcome many of the problems encountered in batch cultures. This method of culturing bacteria allows the maintenance of a substrate limited culture, at a defined growth rate under conditions which allow the experimenter to have control over parameters such as cell density, substrate concentration and dissolved oxygen tension. The basic apparatus of a chemostat is shown in Fig. 12. It consists of a culture vessel with a constant volume (V) maintained by removal of culture fluid via an effluent port. Liquid medium is pumped into the culture vessel at a defined flow rate (f), the number of culture volumes pumped in per hour is the dilution rate. This medium is composed of ample concentrations of all nutrients required for cell growth, except for one which is deliberately provided in low concentrations. When bacteria grow in the chemostat the growth rate is dependent upon the rate of addition of the limiting nutrient. The rate of change of the biomass is expressed by the difference between the rate of cell production (μx) and the rate of cell loss through the effluent port (Dx).

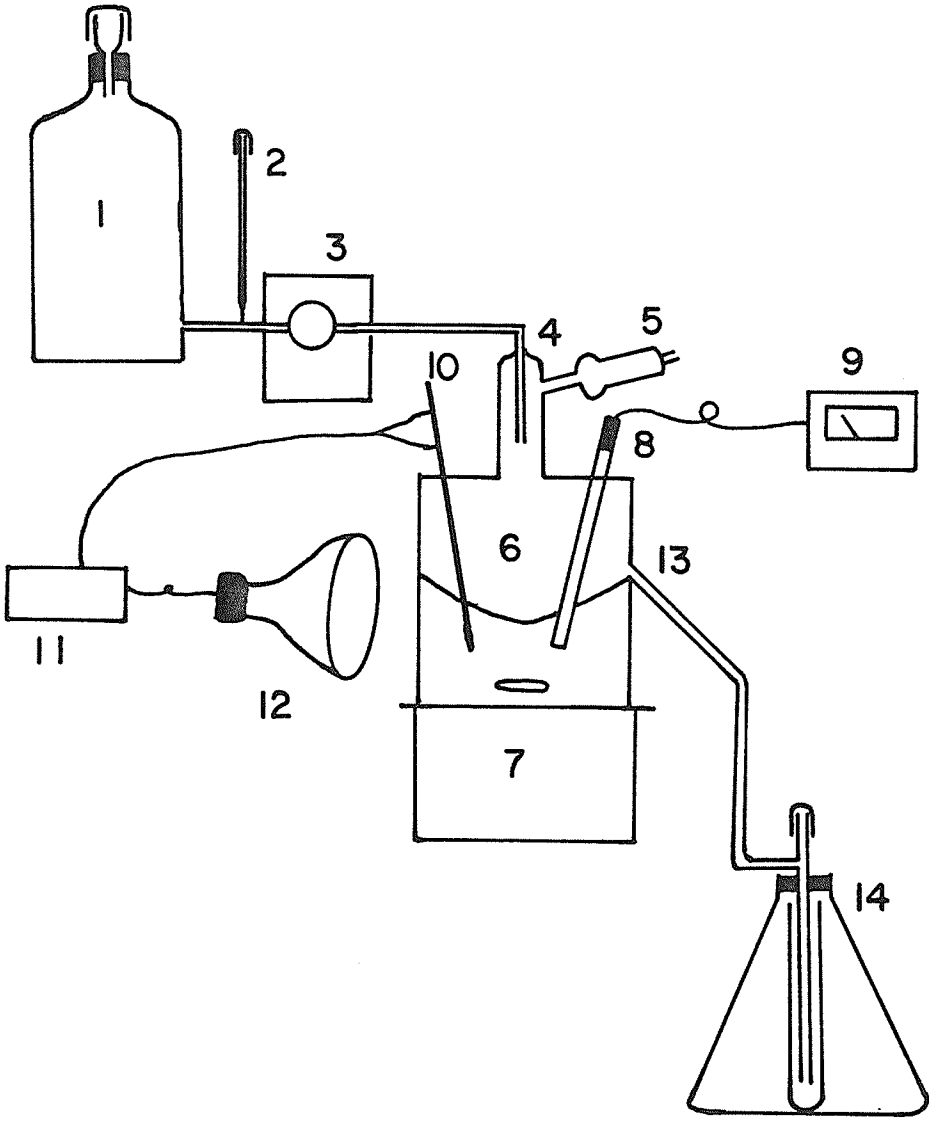
$$\frac{dx}{dt} = \mu x - Dx$$

$$= x(\mu - D).$$

The culture will eventually reach a steady state, whereby $\frac{dx}{dt} = 0$ and

Fig. 12. Basic chemostat culture apparatus

1) 8 liter liquid medium reservoir
2) 5 ml pipette
3) Peristaltic pump
4) Nutrient and gas input
5) Gas scrubber
6) Reaction vessel
7) Magnetic stirrer
8) Oxygen probe
9) Oxygen meter
10) Thermostat
11) Relay switch
12) Infrared heat lamp
13) Liquid and gas outlet
14) Collection flask


1
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most importantly $\mu = D$. An important characteristic of the chemostat is the self balancing, i.e.

$$\frac{dx}{dt} = \mu \text{ max } \frac{s}{K_s + s} \; x - Dx$$

At high substrate concentrations the rate of cell growth will approach the maximum growth rate, and the culture will rapidly increase in biomass. The substrate concentration will not be lowered below that which allows $\mu = D$, since a negative change in production may occur, resulting in the wash-out of the biomass till μ can again equal D.

9.1 Oxygen limited continuous cultures

The solution rate of gas into a liquid, can be described by the stationary liquid film theory (Pirt 1975). At the surface of a liquid, a thin layer is presumed to occur, which has a concentration gradient of dissolved gas. At the surface of the gas, the liquid is presumed to be saturated with the gas, at a concentration c_s, and in the bulk of the liquid, the gas concentration is expressed as c. A unit volume of liquid with an interfacial area "a" has a rate of gas absorption (Rs).

$$Rs = \frac{dc}{dt} = KLa \; (c_s - c).$$

$(c_s - c)$ therefore represents the driving force for the diffusion of the gas.

The KLa constant can be measured in a chemostat as the oxygen balance:

$$\frac{dc}{dt} = KLa(c_s - c) - xq_{O_2}$$

where xq_{O_2} is the rate of oxygen uptake by biomass. The above equation, for simplicity, ignores the amount of oxygen entering and

leaving in the liquid medium; this is presumed to have little effect on the overall oxygen balance in a highly aerated culture.

At steady state $$\frac{dc}{dt} = 0$$

and $$\bar{c} = c_s - \bar{x}q_{O_2}/KLa \ .$$

If $\bar{x}q_{O_2}$ is a constant over a variety of culture oxygen tensions, a plot of c against c_s will have a slope -1/KLa.

In an oxygen limited culture; in steady state

$$\frac{dc}{dt} = 0$$

therefore $$\bar{x} = \frac{KLa}{q_{O_2}} (c_s - c)$$

substituting the growth yield from oxygen i.e. $q_{O_2} = \frac{D}{Yo}$

$$\bar{x} = \frac{KLa \ Yo}{D} (c_s - \bar{c}) \ .$$

This last equation demonstrates the inverse relationship between biomass and dilution rate to be expected in oxygen limited chemostat cultures.

MATERIAL AND METHODS

MATERIALS AND METHODS

1. Growth Conditions

1.1 Organisms

The organisms used in this study were Azotobacter vinelandii (ATCC 9047) and Azotobacter chroococum (ATCC 74º3). Stock cultures underwent bimonthly subculturing and were stored at 4°C in sealed tubes containing B_6 medium with 1% mannitol (see section 1.2). Initially the stock cultures were streaked onto nitrogen-free agar containing B_6 medium with 1% mannitol and incubated at 30°C. From the agar plates colonial material was selected to inoculate approximately 10 ml B_6, 1% mannitol liquid medium. Before use as an inoculum for a chemostat this culture was grown at 30°C and checked for purity by microscopic examination and by streaking onto nutrient agar.

1.2 The Chemostat

The continuous culture apparatus was similar to the one described by Baker (1968). B_6 medium containing 1.0% mannitol was used, however when required the medium strength was increased by one half or one and shall be referred to as 1.5 x B_6 or 2.0 x B_6. B_6, 1% mannitol medium has the following composition (grams/liter) Mannitol, 10; K_2HPO_4, 0.64; KH_2PO_4, 0.16; NaCl, 0.20; $MgSO_4 \cdot 7H_2O$, 0.20; nitrolotriacetic acid (tri-Na^+ salt), 0.144; $CaCl_2$, 0.10. The trace elements solution was composed of (g/liter): $FeSO_4 \cdot 7H_2O$, 2.5; H_3BO_3, 2.9; $CoSO_4 \cdot 7H_2O$, 1.2; $CuSO_4 \cdot 5H_2O$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.9; $Na_2MoO_4 \cdot 2H_2O$, 2.5; $ZnSO_4 \cdot 7H_2O$, 2.1. 1.0 ml of this trace elements solution was added per liter of B_6 medium.

Sulfur-limited continuous cultures were provided with the chloride

salts replacing the sulfate salts in the above constituents in molar equivalent amounts, i.e. (grams/l): K_2HPO_4, 0.64; KH_2PO_4, 0.16; NaCl, 0.20; $MgCl_2 \cdot 6H_2O$, 0.165; nitrolotriacetic acid (tri-Na^+ salt), 0.144; $CaCl_2$, 0.10. The trace elements consisted of $FeCl_2 \cdot 4H_2O$, 1.79; H_3BO_3, 2.9; $CoCl_2 \cdot 6H_2O$, 1.0; $CuCl_2 \cdot 2H_2O$, 0.068; $MnCl_2 \cdot 4H_2O$, 0.09; $Na_2MoO_4 \cdot 2H_2O$, 2.5; $ZnCl_2$, 1.0. Sulfur was provided from a stock solution of 0.10 M Na_2SO_4 using 1 ml per liter for 0.10 mM sulfur.

Carbon-limited cultures were grown on B_6 medium with reduced concentrations of mannitol, the exact amount depending upon the cultural requirements.

The physical conditions in the chemostat were maintained constant by a variety of mechanisms. Temperature was maintained at 30°C by an infrared heat lamp coupled through a relay. During conditions which induced high temperature, cold water was passed through rubber tubing looped around the chemostat vessel. This was effective in cooling the medium. The gas flow rate to the culture, be it air or a gas mixture, was maintained at 200 ml/mim and monitored by means of a gas flow meter. The culture was stirred at vortex rate (i.e. the stirring bar rotated at a speed of 1750 rpm propelled by a Coleman 9x9 magnetic stirrer). To maintain a constant vortex, no probes in the culture fluid were removed, replaced or repositioned in any way during the course of an experiment. If required, the pH of the culture was maintained constant by the addition of acid or base controlled by an automatic titrator.

To begin an experiment approximately 10 ml of batch culture was aseptically added to approximately 200 ml of medium in the chemostat culture vessel. The culture was allowed to grow up overnight

as a closed system under a low stirring rate. The next day, the atmosphere was diluted by N_2 to approximately 5% oxygen. The dilution rate was adjusted to 0.10 hr^{-1} and the stirring rate was increased to a vortex rate. This latter treatment raised the dissolved oxygen tension in the culture medium. As the culture grew and respired the d.o.t. in the medium gradually decreased to zero. At this time the oxygen tension was increased in 5% increments to 20% oxygen over eight hours. Approximately twenty-four hours after increasing the oxygen tension to 20%, the dilution rate was increased to 0.20 hr^{-1}.

1.3 Analysis of growth and metabolism

1.3.1 Sample Collection: Three methods of sample collection were used, depending on the volume of sample required.
For small volumes a port was incorporated into the side of the culture vessel. This was capped by a suba seal to allow the use of a syringe to extract samples or to inject chemicals into the culture. This method provided easy accessibility to the culture with very little disturbance to the vortex and the oxygenation of the culture. The second method was as described by Baker (1968) for collecting volumes as large as 20 ml. The glass tube, connected to a universal collection bottle with a gas outlet, was pushed into the culture liquid. To transfer liquid to the collection bottle the pressure inside the vessel was increased by clamping off the effluent port. After collection the clamp was removed and the glass tube was raised from the culture fluid. This method is very convenient, however introduction of a glass tube into the medium and removal of approximately 10% of the culture fluid does affect the vortex with a resultant increase in the dissolved oxygen concentration in the culture medium.

The last method used to collect cells was for large volumes up to one liter. The effluent from the chemostat was collected overnight in a one liter collection bottle packed in ice. This method was used to collect samples to prepare crude cell-free extracts and for spectral analysis.

1.3.2 Dissolved oxygen measurements: The dissolved oxygen tension was measured by use of a silver-lead galvanic oxygen electrode type 50225 (L.H. Engineering Company Ltd., Bells Hill, Stoke Pages, Bucks, England). The electrical current produced by the electrode was determined by use of an ammeter, connected to a Rustrak recorder.

The sterilized oxygen electrode could be conveniently calibrated in the cultural medium prior to inoculation. The ammeter was zeroed by simply disconnecting the electrode. After reconnection, the electrode was placed in the B_6 medium which had been stirred in a vortex at 30°C under air. The meter was calibrated at 240 µM O_2 under these conditions. This method allows precise calibration since it takes into account temperature differences which affect the rate of reaction occurring within the electrode. The oxygen electrode must be relied upon to be stable since once the culture has been innoculated the electrode cannot be accurately recalibrated in situ.

1.3.3 Measurement of CO_2 production: The amount of CO_2 produced within the chemostat was determined by passing the effluent gas through a percolating apparatus containing a saturated solution of $BaOH_2$ (Lees and Postgate 1973). Titration of the remaining base with 0.1 N HCl after one-half hour was used to calculate the amount of CO_2 evolved from a culture during this time.

1.3.4 Biomass: The biomass was determined by measuring the dry weight of a washed suspension of bacteria. Ten mls of culture was centrifuged at 27,000 g for 10 mins, the pellet was then washed once in distilled water and recentrifuged. The cell pellet was resuspended in distilled water and transferred to preweighed aluminum dish and dried at 95°C to a constant weight.

1.3.5 Residual Mannitol: The amount of residual mannitol in the supernatent fraction was determined by the method of Neish (1950).

1.3.6 Total Nitrogen: Total nitrogen was determined by Kjeldahl digestion. A solution consisting of 1 ml of sample, 1 ml of concentrated H_2SO_4 and a pinch of CuSe catalyst was heated over a hot flame for 2.5 hours in a Kjeldahl digestion apparatus. After digestion the liquid in each flask was made up to a total volume of 10 ml with distilled water. A 2.0 ml sample of the diluted digestion mixture was placed in a Markham still along with 4.0 ml 40% NaOH. To prevent loss of ammonia it was imperative that the apparatus was immediately sealed after addition of base. The NH_4OH so formed was distilled into a solution of Conway's borate buffer (Dawson et al. 1974) and back titrated with N/140 HCl.

1.3.7 Carbohydrate: Carbohydrate was determined by the anthrone method.

1.3.8 Uronic Acids: The amount of uronic acids produced were determined by the method of Bitter and Muir (1962). One ml of sample was added to 5 ml Na^+ tetraborate (0.025 M in concentrated H_2SO_4). This mixture was heated to 100°C for ten minutes and allowed to cool to room temperature. A 0.20 ml volume of carbazole (0.125%) in ethanol)

was added to the mixture. It was then kept at 100°C for fifteen minutes after which the absorbance at 530 nm was determined.

1.3.9 RNA: The concentration of RNA was determined by the method of Herbert et al. (1971). The orcinol reagent could not be used with Azotobacter because the presence of uronic acids interfere with the reaction. To determine the pentose concentration a solution of 110 ml glacial acetic acid, 2 ml concentrated HCl, and 5 ml phloroglucinol (0.8%) was prepared. To 5 ml of this solution 0.4 ml of sample was added. This was kept at 100°C for fifteen minutes, cooled and the optical density was determined at 552 nm.

1.3.10 Cytochrome levels: Cytochrome levels in whole cells were determined by the visible light absorption spectrum. Cells were collected overnight at 4°C and approximately 250 mls of culture was centrifuged and washed in 50 mM phosphate buffer (pH 7.2) then resuspended to a concentration of approximately 100 mg/ml. The spectra were obtained from a Shimadzu multipurpose recording spectrophotometer MPS-5/OL. The dithionite reduced spectrum was used to estimate the levels of cytochrome $\underline{c}_4 + \underline{c}_5$ and cytochrome $\underline{b}$. Reduced cytochrome $\underline{c}_4 + \underline{c}_5$ shows a peak in absorbance at 551 and 554 nm respectively. The difference between this peak at 551 and the trough at 540 nm, εmM $\underline{c}_4 = 23.8$ (Tissieres 1956), $\underline{c}_5 = 9.6$ (Swank and Burris, 1969) was used to estimate the levels of these cytochromes. Reduced cytochrome $\underline{b}_1$ has a peak at 558 nm, which appears as a shoulder on the cytochrome $\underline{c}_4 + \underline{c}_5$ peak, to measure this cytochrome the difference between the absorbance at 558 and 565 nm was determined. Cytochrome $\underline{d}$ levels were estimated by the difference spectrum obtained from dithionite reduced and oxidized samples. Samples were oxidized by lightly sparging the washed cell

preparation with pure oxygen for two minutes. Unlike the other oxidized cytochromes in A. vinelandii which do not absorb a significant amount of light in the visible region, the oxidized cytochrome d molecules absorb light at 648 nm. Reduction of the cytochrome by dithionite shifts the absorption peak to 631 nm. These peaks show as a peak and a trough in the oxidized minus reduced difference spectrum. The area within the peak and enclosed by a line from 610 to 650 nm plus the area within the trough and enclosed by the line from 630 to 670 nm was used to describe the relative amount of this cytochrome present.

The other cytochrome oxidases, cytochrome o and cytochrome a_1 could be assayed from the CO-reduced minus reduced spectrum. Cytochrome o absorbs at 417 nm, with a trough at 432 nm, and the difference between the trough and peak is used to quantitate this cytochrome; εmM = 170 (Daniel 1970). Cytochrome a_1 was also assayed in this difference spectrum, using the absorbance at 427 nm (Chance 1953) (εmM 120).

The concentration of cells present was estimated by the absorption at 700 nm.

1.3.11 Poly β-hydroxybutyrate: The measurement of PHB quantities was carried out by the disk assay method of Ward and Dawes (1973). A 0.20 ml aliquot of bacterial culture was added to washed glass fiber disks (GF/A, 2.5 mm). After drying, the cells were hydrolysed until colourless by treatment with sodium hypochlorite (10-14%). Several applications of hot chloroform were added to the disks to anneal the polymer to the glass fibers. The disks were immersed in sodium hypochlorite (37°C) overnight and then were washed separately with H_2O, ethanol, acetone and diethyl ether. The dry disks were placed

in clean test tubes along with 5 ml H_2SO_4 and placed in a boiling water bath for ten minutes. The amount of crotonic acid, formed by the dehydration reaction, was estimated by its absorption at 235 nm.

1.3.12 Oxygen uptake and Catalase activity by whole cells: The rate of oxygen uptake was determined at 30°C by the decrease in dissolved oxygen measured in a Gilson oxygraph Model KM. To the well of the oxygraph was added 1.8 ml B_6 medium containing 1% mannitol and 0.20 ml culture. After a short lag period, generally the rate of oxygen uptake was constant and could be expressed in units of µmoles O_2 consumed min^{-1} mg dry $weight^{-1}$.

The catalase activity was determined on these same cells by the release of O_2 after addition of 0.20 ml hydrogen peroxide (0.10% v/v). The release of oxygen occurred with no lag period and catalase activity was expressed in units of µmoles O_2 release min^{-1} mg dry $weight^{-1}$.

1.3.13 Mannitol uptake rate: The rate of mannitol translocation was estimated by the rate of ^{14}C-mannitol incorporation into washed cells. A 10 ml sample of culture was washed twice by centrifugation, in B_6 medium lacking a carbon and energy source. To exhaust the endogenous carbon supplies these cells were resuspended in this medium and left at 30°C for two hours, with occasional stirring. A 0.25 ml amount of these starved cells was transferred to 10 ml B_6 medium containing 0.10 µCi ^{14}C-mannitol at 30°C. After addition of cells, 0.20 ml samples were taken at exactly one minute and then two minute intervals thereafter. The cells were collected on a millipore filter (Metricel, 0.45 µM pore size, 25 mm diameter) and were rapidly washed twice with distilled water. The filters were dried at 60°C in scintillation vials, after which 10 ml of Scintiverse scintillation fluid were added

to the vial, and the amount of isotope present was determined in a Beckman scintillation counter.

1.3.14 Total carbon analysis: Total organic carbon was estimated by use of the Beckman model 915A total organic carbon analyser. This apparatus oxidizes all carbon to CO_2 in pure oxygen at 975°C. The quantity of CO_2 was determined by its absorbance of infrared light using a Beckman infrared analyser Model 865. Standard solutions of potassium biphthalate and sodium bicarbonate were used to calibrate the apparatus. Total inorganic carbon was determined by the release of CO_2 in a low temperature oven at 150°C, and the value for the inorganic carbon was substracted from the value for the amount of total organic carbon.

1.4 Electron microscopy

1.4.1 Fixation: Samples for examination by electron microscopy were either prepared fresh or stored at -70°C before fixation. To prepare for fixation cells were centrifuged at 4,000 g for five minutes to a pellet, suspended in 0.10 M cacodylate buffer (pH 7.3) and again centrifuged. Fixation was carried out by suspending the pellet for four hours in a mixture of acrolein (0.15 ml); 25% glutaradehyde (0.60 ml); H_2O (1.75 ml) and 0.2 M cacodylate buffer (pH 7.3) (2.50 ml). After fixation the cells were washed four times in 0.10 M cacodylate buffer (pH 7.3). The cells were then embedded in 3% melted and cooled agar which was subsequently cut into tiny blocks of approximately 0.5 mm^3. These blocks were placed in a solution of 1% osmium tetraoxide in 0.1 M cacodylate buffer (pH 7.3) at 4°C for two hours followed by washing four times in distilled water for fifteen minutes per wash. The samples were then placed in a 0.5% uranyl

acetate solution overnight at 4°C.

1.4.2 Dehydration: Dehydration of the samples was carried out at room temperature, by placing the agar blocks in increasing concentrations of ethyl alcohol. The blocks were placed for fifteen minutes in each of a 30, 50, 70, and 90% alcohol solutions, followed by two standings of ½ hour duration in absolute alcohol.

1.4.3 Embedding: The embedding plastic was a vinyl cyclohexene dioxide epoxy resin as described by Spurr (1969), and will be referred to as "spurr". 40 mls of "spurr" was prepared from the following,

10 grams vinyl cyclohexene dioxide (VCD)

6 grams diglycidyl ether of polypropylene glycol (DER)

26 grams non-enyl succinic anhydride (NSA).

These agents were stirred for ten minutes before the addition of 0.4 grams dimethyl amino ethanol (DMAE).

To embed the sample most of the absolute alcohol was decanted from the agar blocks. A volume of "spurr" equal to that of the remaining alcohol was added. After one half hour this volume was again doubled by the addition of "spurr" and left for one hour. The "spurr" alcohol mixture was decanted and replaced by pure "spurr". This was left for one hour and again replaced with pure "spurr". The sample in "spurr" was then placed on a multi-purpose rotor (Scientific Industries Inc.) overnight to ensure good penetration.

1.4.4 Polymerization: The plastic moulding forms were partially filled with "spurr" and to each well an agar cube was added. The cubes were left in the liquid spurr for three hours before polymerization. Polymerization was accomplished by placing the plastic forms containing spurr and samples in an oven at 60° C under vacuum

for twelve hours.

1.4.5 Sectioning: Sections were cut from the sample using glass knives on a Riechert OM U2 ultramicrotome (Stockholm, Sweden). They were collected on 300-mesh copper grids, air dried and examined and photographed on an EM 6B (AEI) electron microscope (Harlow, Essex England).

1.5 Analysis of extracellular products

1.5.1 Free Ammonia: The possibility that ammonia could be excreted by Azotobacter was tested for by the use of Nesseler's reagent. One ml of culture supernatant was added to 5 ml of Nessler's reagent, (Gilcreas, 1965) and after one hour the absorbance was determined at 490 nm.

1.5.2 Fructose: The concentration of fructose in the culture medium supernatant was determined by its reaction with cysteine - HCl in sulfuric acid (Dische and Devi, 1960). To 0.5 ml of culture supernate were added 0.10 ml cysteine HCl (25% aq) and 6.0 ml H_2SO_4 (75%). The mixture was shaken and allowed to stand at room temperature. The difference between the optical density at 412 and 380 nm was determined, this difference being proportional to the concentration of ketohexose present.

1.5.3 Pyruvate determination: The amount of pyruvate in the cultural fluid was determined by the formation of the 2,4-dinitrophenyl-hydrazone derivative. A 2 ml quantity of the culture supernatant was mixed with 1 ml of 2,4-dinitrophenylhydrazine (0.5% in 2N HCl). The deriviative formed was extracted with 5 ml ethyl acetate and the aqueous layer was discarded. The ethyl acetate was extracted with three, five ml aliquots of 10% sodium carbonate. The sodium carbonate fractions

were combined and made to a total volume of 25 ml and the OD at 235 nm was determined.

1.6 Preparation of 2,4-dinitrophenylhydrazine derivatives

A. vinelandii cultures could be induced to produce acid after a long period of mannitol limitation simply by rapidly increasing the mannitol concentration in the medium to 0.5%. The acid formed was concentrated by passing the culture supernate through Dowex-I anion exchange resin. The resin was washed with deionized water, and the acid was collected off the resin by passing through the column an aqueous solution at 1.0 N HCl. The eluate was then treated with an excess of 2,4-dinitrophenylhydrazine (1% in 2 N HCl). The resulting precipitate was collected by suction filtration on paper and washed several times in cold distilled water. This derivative was used for chromatography and melting point determination.

1.6.1 Melting Point: The melting point of the 2,4-dinitrophenylhydrzaone derivative was determined as described by Vogel (1966).

1.6.2 Chromatographic analysis of the 2,4-dinitrophenylhydrazine derivative: The derivative was chromatographed by descending chromatography, on Whatman no. 1 paper. The four solvent systems used were butanol, ethanol, water (70/10/20); isopropanol, water NH_4OH (200/20/10); 1% sodium carbonate; and 0.1 M glycine - NaOH buffer pH 4.85 with trace phenol (Dilworth, 1962). Prior to running the chromatogram, the atmosphere was allowed to become saturated with solvent vapour.

1.7 Nitrogenase assay

The method used for the determination of nitrogenase activity was similar to that described by Hardy et al. (1968) and Postgate (1971). The exact volumes of 25 ml erlenmeyer flasks with a rubber stopper in place, were determined. The air in the sealed flasks was replaced with a gas mixture of 78% Argon and 22% oxygen. One ml of culture and 1 ml of pure acetylene gas were injected through the stopper and the flask was shaken at 110 strokes per min at 30°C. At exactly one hour after the start of incubation the cells were killed by the injection of 5 drops of concentrated H_2SO_4. The gas mixture above the culture was then analysed by flame ionization gas chromatography.

The gas chromatograph was a Pye Series 104 chromatograph fitted with a 6' by 1/8 inch glass tube containing Porasil-C (phenyl isocyanate). The column was maintained at 45°C and was flushed with a carrier gas of N_2 at 50 ml/min. To the ionization chamber the flow of air was 630 ml/min and H_2 50 ml/min. The activity of the nitrogenase was determined by the amount of ethylene produced, the quantity being determined by comparison to a standard ethylene gas injection.

1.8 Preparation of crude cell-free extracts for enzyme assay

Approximately one liter of culture was collected overnight on ice. These cells were washed twice in 50 mM phosphate buffer containing 1.0 mM EDTA and resuspended in this buffer to a final concentration of approximately 100 mg/ml (wet weight). Disruption of the cells was done immediately using a Raytheon Sonicator equipped with a water cooled jacket which maintained the temperature at 4°C. The cells were sonicated for 5 minutes and were then immediately frozen. When

required the thawed sonicate was centrifuged at 37,000 x g for fifteen minutes and the supernatent fraction was analyzed for enzyme activity.

1.9 Protein

Protein was determined by the method of Lowry et al. (1951) or, when protein concentration was fairly high, by the Biuret method (Gornall et al., 1949).

1.10 Superoxide dismutase

The levels of activity of superoxidase dismutase were determined by the method of Buchanan and Lees (1976) using acetylated cytochrome c. To a 1 ml quartz cuvette were added 0.25 ml acetylated cytochrome c (25 µM), 50 µl xanthine (0.01 mM), 0.55 ml Na-borate buffer (pH 9.0) and 0.10 ml crude extract. The reaction was started by the addition of 0.10 ml xanthine oxidase and monitored by the change in optical density at 550 nm. One unit of enzyme activity is that which reduces the reduction rate of acetylated cytochrome c to 25% of that occurring in the absence of S.O.D.

1.11 NADH and NADPH oxidase

The activity of NADH and NADPH oxidases were determined by the decrease in concentration of the reduced form of these nucleotides. To a 1 ml quartz cuvette was added 0.10 ml NADH (2.5 mM), 0.80 ml PO_4^{-3} buffer (10 mM pH 7.5) and 0.10 ml crude cell-free extract. The reaction was monitored by the reduction in absorbance at 340 nm.

1.12 Isocitrate lyase

The activity of this enzyme was determined by the formation of a phenylhydrazine derivative of the glyoxylate formed (Dixon and Kornberg 1959). To a 1.5 ml cuvette the following solutions were added, 0.10 ml tris-HCl, (0.50 M, pH 7.5); 0.10 ml phenylhydrazine-HCl, (0.10 M); 0.05 ml cysteine-HCl, (1.0 mM); 0.05 ml $MgCl_2$, (0.10 M); 1.05 ml H_2O and 0.10 ml crude cell-free extract. The reaction was initiated by the addition of 0.05 ml isocitrate-K^+ (20 M, allo-free) and was monitored by the change in OD at 325 nm. One unit of enzyme activity is equal to a change of OD of 11.54 per minute (Reeves et al., 1969).

1.13 Isocitrate dehydrogenase

The activity of this enzyme was determined as described by Jackson and Dawes (1976). To a 1 ml cuvette was added 0.60 ml tris-HCl, (0.15 M, pH 7.5); 0.10 ml $NADP^+$, (3.75 mM); 0.10 ml $MnCL_2$, (20 mM); 0.10 ml crude cell-free extract and to initiate the reaction 0.10 ml isocitrate (5 mM). The reaction is monitored by the change in OD at 340 nm.

1.14 NADH dehydrogenase

The activity of NADH dehydrogenase was assayed for by reduction of ferricyanide in the presence of NADH. To a 1.0 ml cuvette was added 0.1 ml of cell free extract (10 x diluted), 0.1 ml of 1.0 mM ferricyanide and 0.8 ml of 50 mM phosphate buffer (pH 7.2). The change in OD was followed at 420 nm (molar extinction coefficient is 1.0×10^{-3} cm^{-1}).

RESULTS

RESULTS

1. Growth of Azotobacter vinelandii in a chemostat

A large amount of research previously done in this laboratory has concentrated on the species A. chroococcum. This organism was extensively studied in highly stirred (Vortex) chemostat cultures and its growth under such conditions is well defined (Hine 1975, Tsim 1976, Dawson 1977, Buchanan 1978, Leung 1979). However initial comparisons of A. chroococcum and A. vinelandii under these conditions reveal large differences with respect to carbon dioxide and biomass production rates between two otherwise similar cultures. It is therefore appropriate, before initiating studies of oxygen toxicity in A. vinelandii, to define its growth and respiration in a highly stirred chemostat and how these results compare to A. chroococcum.

1.1 Effect of dilution rate

a) On biomass production rates

Both species were cultured in similar chemostats and their response with respect to cellular production and respiration was compared.

The conditions for growth were as outlined in the Materials and Methods section; both cultures were provided with nitrogen-free B_6 medium containing 1.5% mannitol. The atmosphere above each culture was sterile air introduced at a rate of 150 ml per min. The dissolved oxygen tension (d.o.t.) remained at zero throughout the course of the experiment for both cultures. Slight differences in volume existed between the two cultures, the A. vinelandii culture occupying a 250 ml volume under vortex conditions whereas the A. chroococcum culture

was contained in 220 ml. No attempt was made to control the pH beyond the buffering capacity of the medium. To insure each culture had reached a steady state growth at the applied dilution rate, it was allowed to stabilize for 48 hours before sampling. Each dilution rate was then examined in duplicate, the average of these measurements is reported.

Fig. 13 illustrates the relationship between biomass and dilution rate for both A. chroococcum and A. vinelandii. The curve representing the response of A. chroococcum is a rectangular hyperbola, the arithmetic product of biomass and dilution rate being equal to a constant production rate of 0.12 mg ml^{-1} hr^{-1} indicating gas limitation (see History section 9.1). However such a constant relationship between biomass and dilution rate is not observed in cultures of A. vinelandii, which show a response similar to what is to be expected if the culture growth is limited by a lack of some nutrient in the medium.

To attempt to overcome possible nutrient limitation of A. vinelandii, the concentration of mannitol was doubled. This increased the cellular production rate to 0.21 mg ml^{-1} hr^{-1}, from 0.12 mg ml^{-1} hr^{-1} at 0.14 hr^{-1}, however this production rate was still far below that observed at higher dilution rates, i.e. 0.34 mg ml^{-1} hr^{-1} at 0.26 hr^{-1}. Increasing the concentration of the inorganic ion components as well as increasing the mannitol concentration to 3.0% was effective in overcoming the nutrient limitation. The response of a second continuous culture of A. vinelandii to various dilution rates resulted in constant production rates of 245 mg l^{-1} hr^{-1} (Fig. 14).

b) On respiration

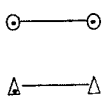
Because Azotobacter species are strict aerobes and capable of maintaining

the d.o.t. at zero, the rate of oxygen solution into a culture would equal the rate of carbon dioxide evolution plus a small amount of assimilated CO_2 (Hine and Lees, 1976). Therefore we can approximate the rate of oxygen uptake of such a culture by measuring its rate of CO_2 evolution (Senior et al. 1972). Table 1 shows the rate of CO_2 evolution for A. chroococcum and A. vinelandii at each dilution rate tested. Contrary to the results of Hine and Lees (1976) the rate of CO_2 evolution in both cultures decreases at higher dilution rates. The highest rates of CO_2 production occurs at 0.20 hr^{-1}.

A comparison of the two species reveals a two-fold greater rate of CO_2 evolution by A. vinelandii, indicating that A. chroococcum cultures are unable to absorb as much oxygen from the air as the A. vinelandii. This difference is probably due to the very large amount of polysaccaride generally produced by A. chroococcum. Comparing the cell pellets of centrifuged culture reveals a thick layer of polysaccharide accompanying the A. chroococcum cells, which occurs, but to a much less extent in A. vinelandii. Such high levels of polysaccharide may increase the viscosity of the culture, resulting in a reduced vortex and lower oxygen solution rates.

The efficiency of respiration in terms of cellular production is described by the respiratory index value (RI), which represents the carbon dioxide produced in a unit of time per biomass unit produced in the same time interval (Hine and Lees, 1976). Under conditions of nutrient limitation where the respiration would be uncoupled from the growth, the RI value would increase. Table 1 also shows the RI values for both species of Azotobacter. Under these conditions the RI is low and shows little variation with dilution rate. A. vinelandii

Fig. 13. Variation in the biomass with different dilution rates of continuous cultures of A. chroococcum and A. vinelandii, at 20% O_2 with 1 x B_6, 1.5% mannitol medium. D = 0.20 hr^{-1}.



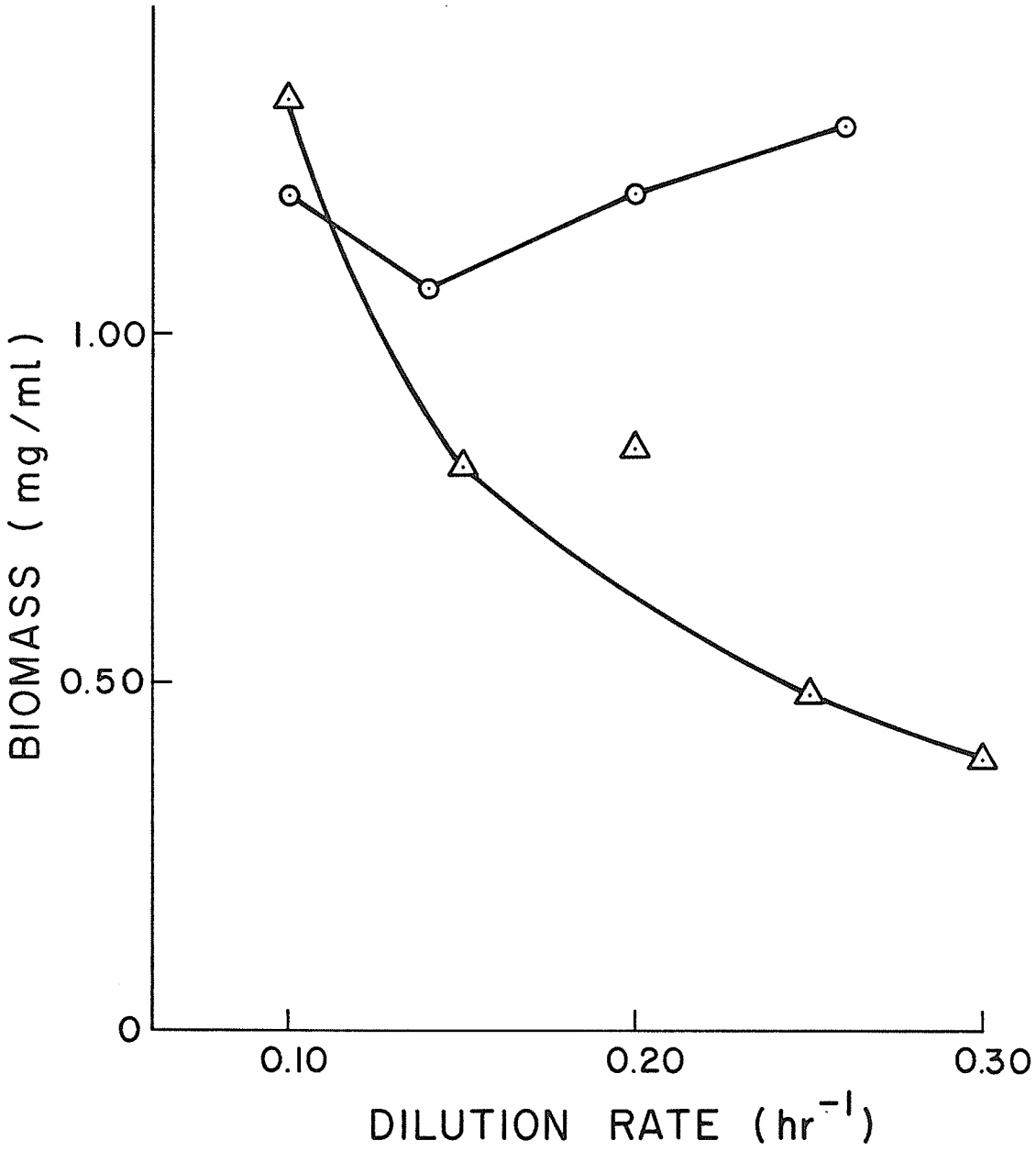

BIOMASS (mg/ml)
1.00
0.50
0
0.10
0.20
0.30
DILUTION RATE (hr^{-1})

Fig. 14. Variation in the biomass with different dilution rates of a continuous culture of A. vinelandii, at 20% O_2 with 1.5 x B_6, 3.0% mannitol medium.

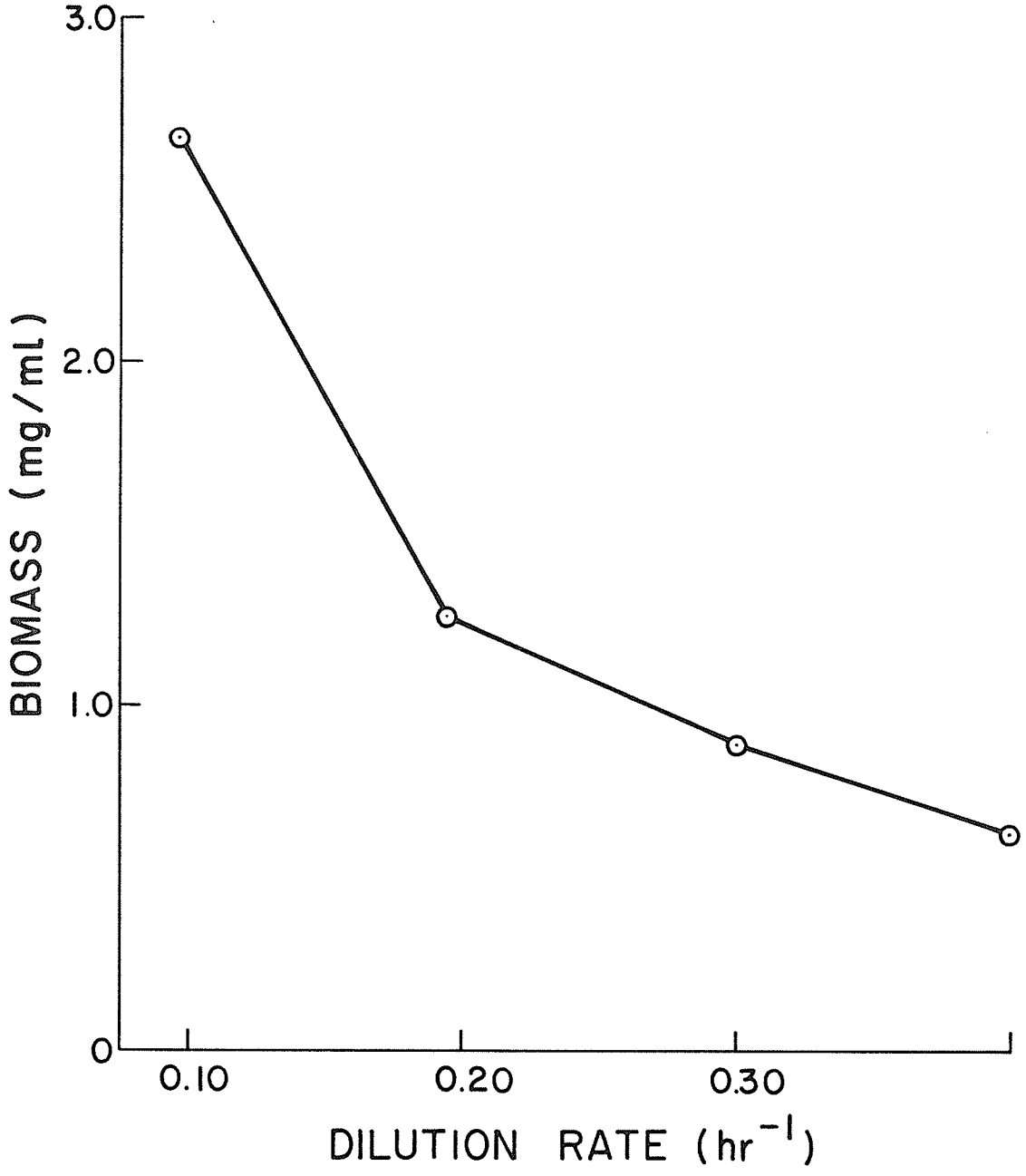

3.0
2.0
1.0
0
BIOMASS (mg/mL)
0.10
0.20
0.30
DILUTION RATE (hr^{-1})

TABLE 1

Parameters of growth and respiration at various dilution rates for *A. chroococcum* and *A. vinelandii* chemostat cultures

Dilution rate (hr^{-1})	Cellular production ($mg \cdot l^{-1} \cdot hr^{-1}$)	CO_2 production ($mmoles \cdot l^{-1} \cdot hr^{-1}$)	R.I.[b] ($mmoles \cdot mg^{-1}$)	pHB[a]
A. chroococcum				
0.10	135	16.6	0.123	
0.15	122	16.4	0.134	
0.20	180	16.9	0.094	
0.25	139	13.0	0.100	
0.30	120	13.1	0.109	
A. vinelandii				
0.10	218	28.6	0.131	7.3
0.20	288	35.2	0.122	0.7
0.30	297	32.1	0.108	0.4
0.40	247	27.1	0.110	0.4

[a] Poly-β-hydroxybutyrate (expressed as a percentage of dry weight).

[b] respiratory index.

and A. chroococum have similar RI values indicating comparable efficiency of coupling growth to respiration.

1.2 Effect of NH_4^+ on growth and respiration

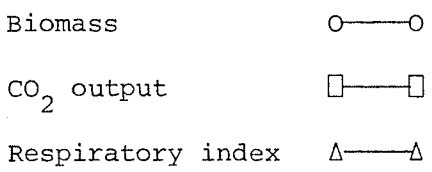
The process of N_2 fixation is an expensive energy consuming process, requiring high levels of both ATP and reducing power. To determine the amount of energy required by A. vinelandii to fix N_2, the culture was provided with various concentrations of $(NH_4)_2SO_4$ and the difference in the degree of respiratory coupling to growth was determined.

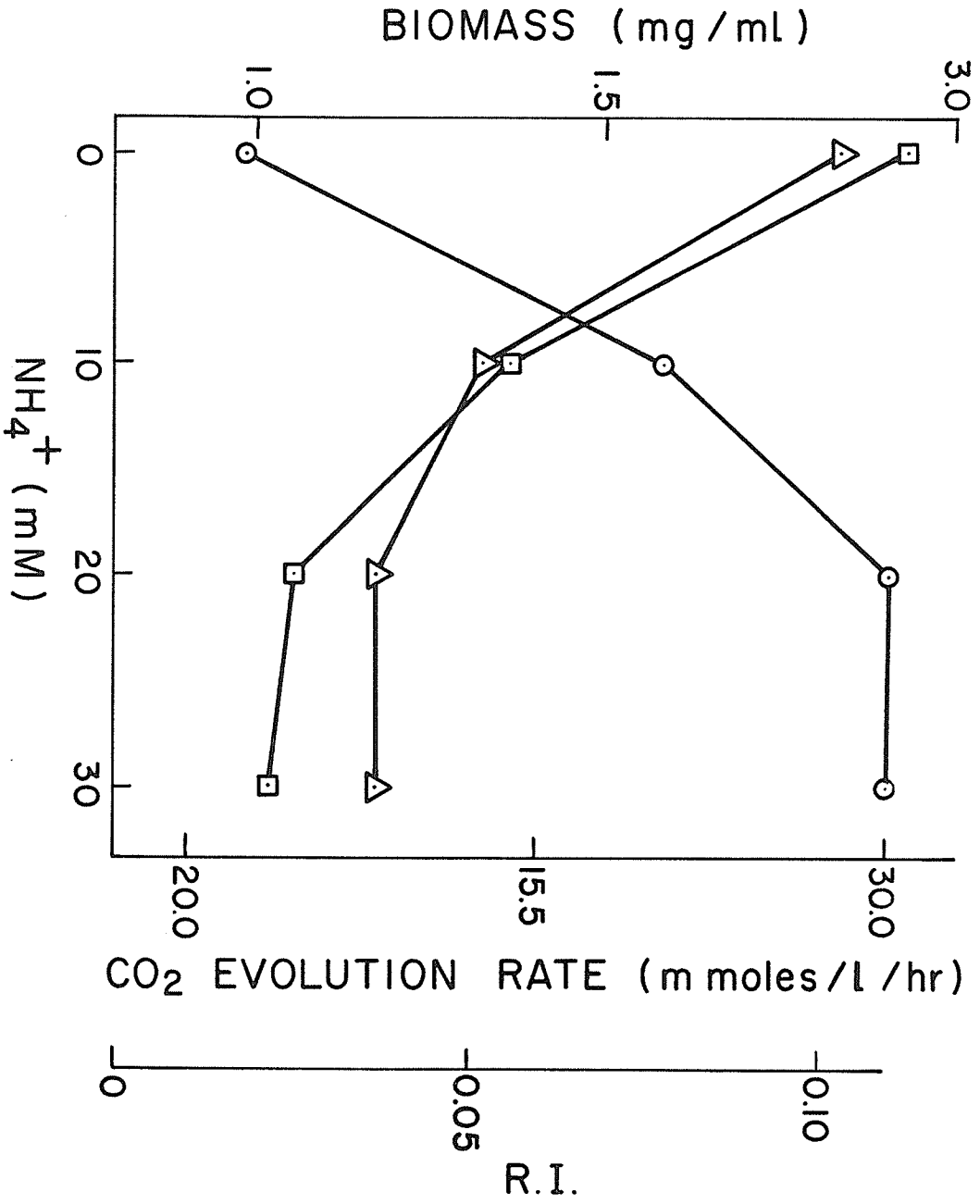
The A. vinelandii culture was grown at a dilution rate of 0.30 hr^{-1}. $(NH_4)_2SO_4$ was added to the medium to final concentrations of 0, 10, 20 and 30 mM NH_4^+.

The change in biomass with increasing NH_4^+ concentration is shown in Fig. 15. Addition of 20 mM NH_4^+ produced almost a two-fold increase of biomass which was accompanied by a decrease in CO_2 evolution, resulting in a large decrease in RI values. The growth above 20 mM NH_4^+ could be limited by the lack of some essential nutrient, however this possibility was not investigated. Excess NH_4^+ was found to be present in the supernate only at 30 mM NH_4^+, by a positive reaction with Nessler's reagent.

The amount of energy required to fix nitrogen can be calculated from the difference in RI between N_2-fixing cells and those provided with excess NH_4^+ where nitrogenase activity is expected to be essentially zero. The difference in RI is assumed to be equal to the amount of oxygen reduced for the production of energy for nitrogen fixation plus the number of electron pairs transferred to N_2. Assuming 15% of the cellular dry weight is nitrogen the difference in

Fig. 15. Variation in growth and respiration of A. vinelandii with different concentrations of NH_4^+ in a continuous culture, at 20% O_2, 1.5 x B_6 medium 3.0% mannitol. D = 0.30 hr^{-1}.




BIOMASS (mg/ml)
1.0
1.5
3.0
0
10
20
30
NH_4^+ (mM)
20.0
15.5
30.0
CO_2 EVOLUTION RATE (m moles/l/hr)
0
0.05
0.10
R.I.

RI (0.066 mmoles CO_2·mg dry wt^{-1}) can be expressed as 6.17 moles CO_2 per mole NH_3 formed. The requirement for reducing power by the nitrogenase is six reducing equivalents per nitrogen fixed which would correspond to 1.39 moles of CO_2 per mole of nitrogen fixed. The remaining $RI_{(N)}$ value (4.76 moles CO_2 per mole N_2 fixed) represents the energy requirements for nitrogenase, be it energy for enzyme production or ATP use in catalysis. If the cell is capable of coupling the reduction of oxygen to two coupling sites (Hoffman et al. 1979) and each CO_2 indicates production of 4.3 ATP when oxidizing mannitol, then the ATP requirement would be 20.6 ATP per mole of N_2 fixed.

1.3 The growth of A. vinelandii under high oxygen tension

The constant cellular production rates of A. vinelandii chemostat cultures at different dilution rates when provided with sufficient nutrient (Fig. 14) indicates gas limitation (Pirt 1975). For A. chroococum this was shown to be due to oxygen limitation by Hine and Lees (1976). We wished to determine if A. vinelandii undergoes the same type of oxygen limitation as opposed to possible nitrogen limitation. Also, the very high cellular production rates of A. vinelandii (Table 1) raises the possibility that this rate is approaching the maximum and it was therefore necessary to determine if the rates of biomass production would increase substantially with increasing oxygen tension above the culture.

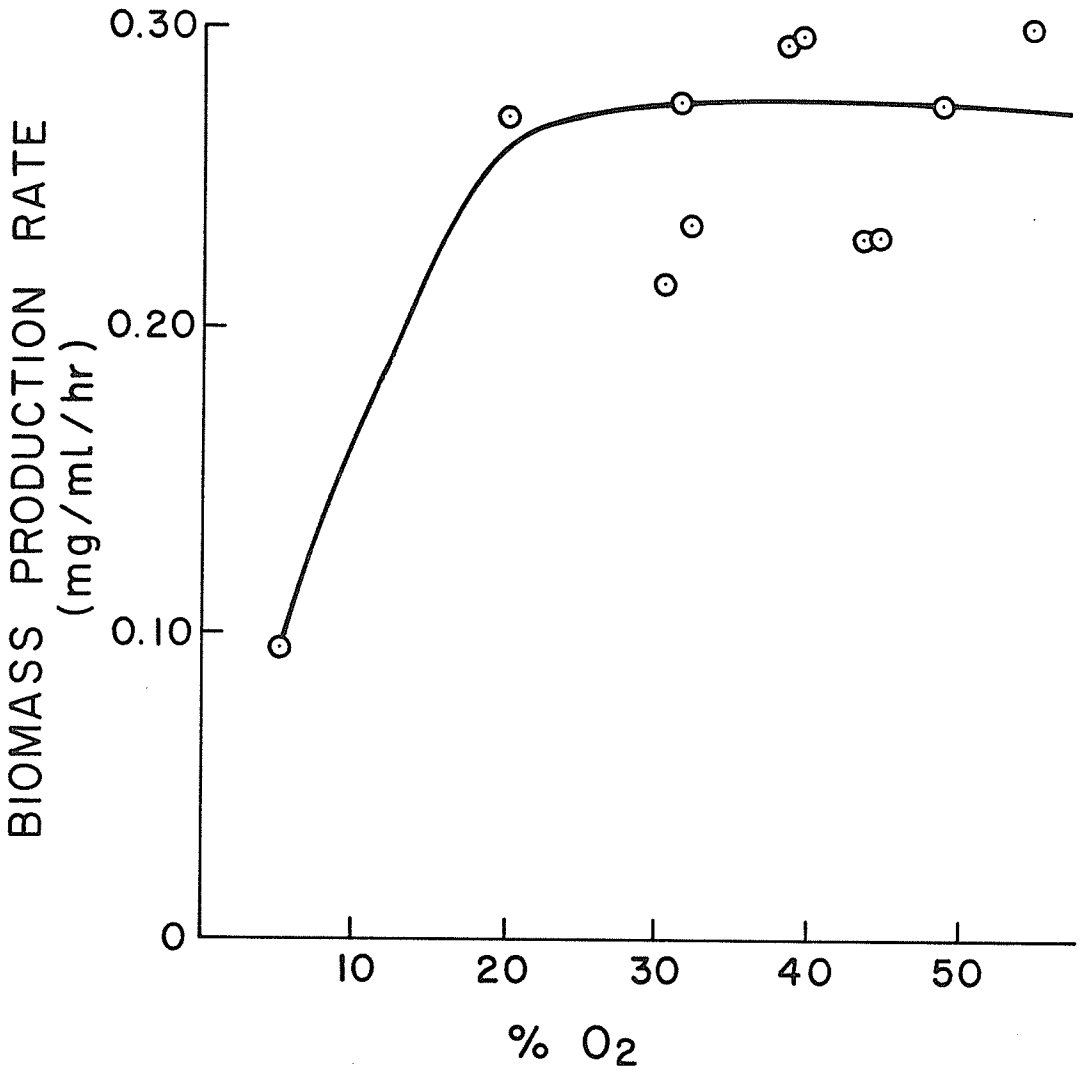
This experiment was carried out twice, once with B_6 medium plus 3% mannitol at a dilution rate of 0.20 hr^{-1} and the second time with 2 x B_6 medium plus 2% mannitol and a dilution rate of 0.30 hr^{-1}. The results of both are reported. This first experiment demonstrated

the nutrient limitations of B_6 medium which may be incorrectly interpreted as being due to oxygen toxicity. Fig. 16 shows the response of biomass production to increasing oxygen tension above the culture. Below 20% oxygen the rate of biomass production increases with increasing O_2, however above 20% oxygen the rate of production is independent of the oxygen tension. Hine and Lees (1976) had also observed a reduction in the increase in the biomass production rate when the atmosphere above the culture rose above 30% O_2. To try to avoid possible nutrient limitation effects, the experiment was repeated with the 2 x B_6, 2% mannitol medium and a higher dilution rate. Because these cultures are limited by the gas solution rate, cellular production rates would be independent of dilution rates. Thus a comparison of cellular production rates from cultures grown at different dilution rates is valid.

The higher dilution rate maintained a lower biomass concentration. This was necessary to prevent interference with the oxygen solution rate and also to prevent plugging of the exit ports, both effects being a consequence of the higher viscosity accompanying greater cell yields.

The results are shown in Fig. 17. Using this concentrated medium and varying the oxygen concentration the biomass production rates increased from 0.46 mg ml^{-1} hr^{-1} at 20% oxygen to 0.66 mg ml^{-1} hr^{-1} at 40% oxygen. Undoubtedly this A. vinelandii culture was oxygen limited at 20% oxygen and cellular production was not affected by the toxic effects of oxygen up to 40% O_2. The biomass production rate did decrease above 40% oxygen; later analysis showed this to be due to carbon limitation at high oxygen tension. Interestingly, these carbon

Fig. 16. Variation in the biomass production rate with different oxygen concentrations in the gas phase above a continuous culture of A. vinelandii. 1 x B_6 medium, 3% mannitol. D = 0.20 hr^{-1}.


BIOMASS PRODUCTION RATE
(mg/ml/hr)
0.30
0.20
0.10
0
10
20
30
40
50
% O2

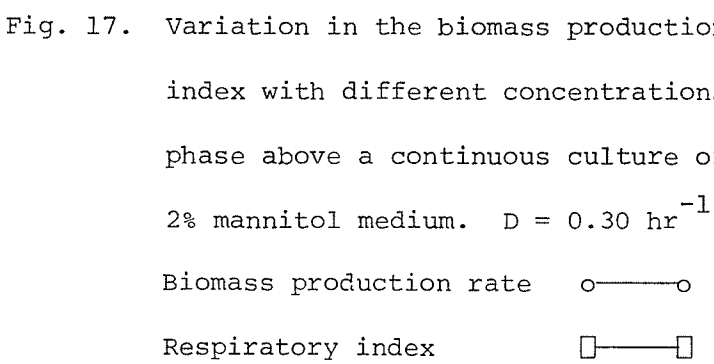
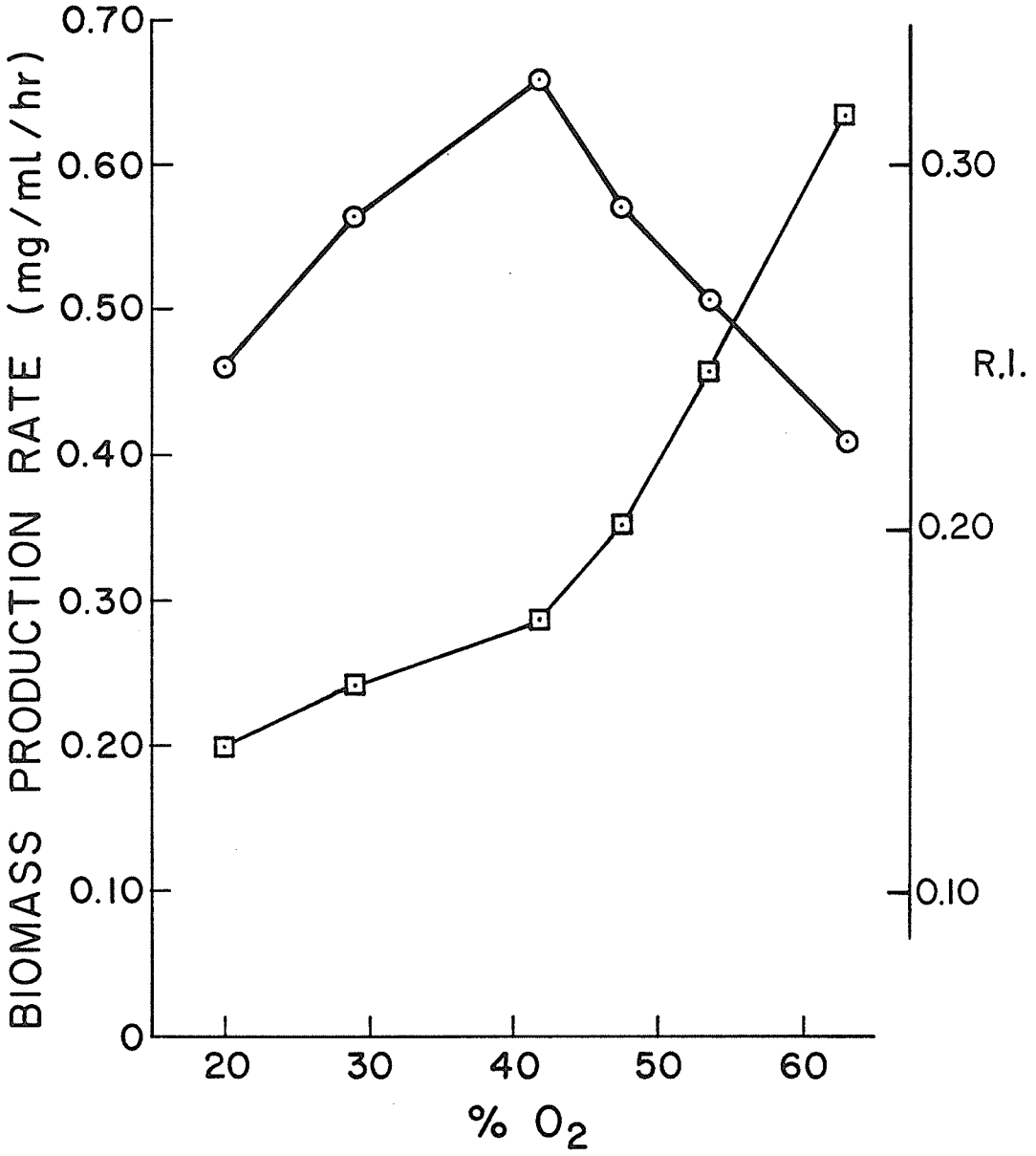


Fig. 17. Variation in the biomass production rate and respiratory index with different concentrations of oxygen in the gas phase above a continuous culture of A. vinelandii. 2 x B_6, 2% mannitol medium. D = 0.30 hr^{-1}.


BIOMASS PRODUCTION RATE (mg/ml/hr)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
R.I.
0.30
0.20
0.10
20
30
40
50
60
% O_2

limited cells have higher RI values indicating they will use a greater proportion of carbon for respiration than for cell production compared to carbon-sufficient cells. This priority given to high respiration, which is quite wasteful in terms of cell growth, indicates the importance to these cells of maintaining the oxygen tension as low as possible at the cell surface. Therefore toxic effects of oxygen can be expected to occur in these cells; however, to observe these effects some method must be used to overcome the high respiration rate of this organism.

1.4 Comparison of different carbon sources as substrates for the growth of A. vinelandii.

A. vinelandii were grown in 1.5 x B_6 medium with mannitol, glycerol, Na-lactate or glucose (2.0%) as the sole carbon and energy sourse. The results with respect to growth and respiration are shown in Table 2. Mannitol and glycerol oxidizing cultures are very similar with regard to CO_2 production and biomass production. The slightly greater biomass production rate of mannitol grown cells may reflect the fact that both types of cells are energy limited and the extra energy required to transport glycerol reduces the amount of energy available for biosynthesis.

Cultures which are utilizing Na-lactate as the sole carbon and energy source tend to accumulate NaOH, and require the use of automatic pH control. On this carbon source, CO_2 and biomass production are reduced and the efficiency of growth to respiration as indicated by the RI value is slightly lower than mannitol oxidizing cells. The poor CO_2 production is not reflected by an increase above zero of the d.o.t., indicating that growth on lactate induces a change in the

Table 2. Variation in growth and respiration of A. vinelandii oxidizing different carbon sources. Substrates were provided in 1.5 x B_6 medium at a concentration of 2.0% D = 0.20 hr^{-1}.

Substrate	pH	CO_2 production ($mmoles \cdot l^{-1} \cdot hr^{-1}$)	Biomass production ($mg \cdot l^{-1} \cdot hr^{-1}$)	R.I. ($mmoles \cdot mg^{-1}$)
Mannitol	6.68	39.2	290	0.135
Glycerol	6.71	38.8	248	0.156
Lactate	8.10	26.6	180	0.148
	6.60*	28.8	156	0.185
Glucose	5.95	27.1	180	0.151
	6.55*	56.7	246	0.230

*Indicates the pH was automatically controlled.

medium (probably and increase in polysaccharide excretion) which reduces the oxygen solution rate.

Glucose grown cultures continuously excreted acid, thus reducing the pH of the medium to 5.95. Initially the CO_2 production rate of this culture was low, possibly due to the presence of residual lactate from the previous experiment. The following day, with the pH automatically controlled, the CO_2 production rate of this culture had doubled and the biomass production rate was similar to glycerol grown cells. Efficiency of growth to respiration was poor and may reflect the loss of energy occurring when the carbon is only partially oxidized.

1.5 Discussion

The above results with respect to the biomass production rates of A. vinelandii at low dilution rates and high oxygen tension indicate the importance of identifying the limiting nutrient and ensuring an adequate supply of all other growth requirements to a chemostat. The maximum biomass which B_6 medium can support is approximately 1.5 mg/ml if the culture is provided with 3% mannitol. Attempts to increase the cellular production by increasing the oxygen supply would result in eventual salt or carbon limitation which may be mistaken for oxygen inhibition of growth.

A comparison of the A. vinelandii and A. chroococcum cultures indicate two important differences. Firstly A. chroococcum cultures have much lower rates of CO_2 production than A. vinelandii cultures. This difference in rate is proposed to be due to a greater production of polysaccharide by A. chroococcum which decreases O_2 solution rates. Secondly, the cellular production rates of

A. vinelandii are twice that of A. chroococcum reflecting the large difference in O_2 solution rates between these oxygen limited cultures.

A similarity between the respiratory index values for both O_2-limited species demonstrates that both produce approximately the same amount of cell material with respect to their respiration. Therefore it is likely that both have the same degree of efficiency of energy production with respect to oxygen reduced.

The response of A. vinelandii to the presence of fixed nitrogen gives some important insights to the energy budget of the cell. The reduction of the amount of CO_2 evolved for biosynthesis was used to determine the amount of ATP required for the reduction of dinitrogen to ammonia. The value determined was 20.6 ATP per N_2 fixed. This value is similar to the value for ATP required by particulate nitrogenase preparations uncorrected for H_2 evolution (Hwang and Burris 1972).

The response of a vortex stirred culture to various oxidizable carbon sources demonstrates that the carbon source for a given culture of A. vinelandii may dictate the respiration and growth characteristics. Glucose which is not immediately oxidized does not induce a reduction in the oxygen solution rate as was observed for lactate oxidizing cells. Since lactate is oxidized by a membrane bound dehydrogenase (Jones and Redfearn 1967) the redox potential at the cell membrane may dictate the amount of polysaccharide excreted by the cell.

Mannitol and glycerol appear to be intermediary. Both substrates are immediately oxidized, but since the CO_2 production rate of these cultures is greater than lactate oxidizing cultures the initial oxidation may not have a significant reductive effect on the cell membrane. The fact that CO_2 production by mannitol and glycerol grown cells is less than glucose oxidizing cells may be a consequence of a faster rate of glucose uptake resulting in high rates of glucose catabolism and low amounts of available carbon for polysaccharide production.

2.0 Growth in the presence of excess dissolved oxygen

The oxygen limited nature of A. vinelandii grown with 20% O_2 above the culture, in addition to its adaptable respiration must be taken into account for the study of oxygen toxicity. To simply increase the oxygen tension in the atmosphere above the culture would result in greater cellular production rates which would be eventually limited by either carbon or mineral supply. Even at high oxygen concentrations in the atmosphere above the culture the respiration rate is sufficiently high so as to maintain the d.o.t. at zero or slightly above zero. Therefore to study the effects of oxygen on Azotobacter the cells must be exposed to high d.o.t. which can only be brought about by either reducing the respiration rates or by the exposure to high oxygen tension after growth at a lower oxygen tension where respiratory protection is low. The first method was used for long term studies whereby a culture was exposed to severe carbon limitation with a resultant constant high d.o.t. in the culture. The exposure to high oxygen tensions was then used for short term analysis.

2.1 Response of A. vinelandii and A. chroococum to a high dissolved oxygen tension.

In order to compare the ability of each species to withstand the presence of excess dissolved oxygen in the culture, two cultures, one A. vinelandii and the other A. chroococum, were placed in a severely carbon limited state by stopping the mannitol flow to the culture. Initially both cultures maintained the d.o.t. at zero but after all residual mannitol was oxidized the d.o.t. in the medium increased. Mannitol was then supplied to the culture at a slow rate allowing respiration to decrease the d.o.t. When the d.o.t. had decreased to a constant level the concentration was recorded and the mannitol supply rate was further increased. The results are shown in Fig. 18.

The A. vinelandii culture is capable of reducing the dissolved oxygen tension with increasing mannitol supply, to a minimum of 7.2 μM, given mannitol at a rate of 0.40 g hr^{-1}. Addition of mannitol at a higher rate did not allow the culture to reduce the d.o.t. below this 7.2 μM value. Therefore, such exposure to excess dissolved oxygen appears to induce a change, which reduces the affinity of the cytochrome oxidase for oxygen.

The response of A. chroococum is also shown in Fig. 18. This culture is initially capable of respiration at high d.o.t., however when given mannitol at a rate of 0.33 g hr^{-1} in the presence of excess dissolved oxygen the respiration of this culture completely ceases. This inhibition of respiration was not relieved by the addition of more mannitol or by the addition of acetate which is believed to be taken into the cell without the expenditure of energy (Haaker and Veeger 1976). The culture of A. chroococum remained in this inhibited

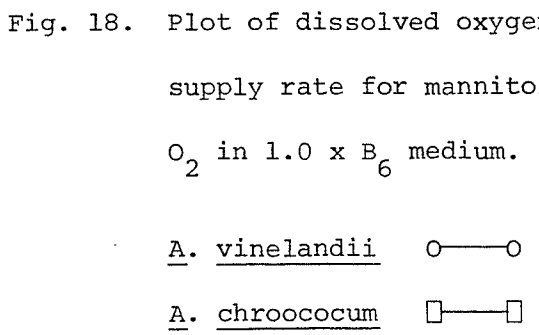
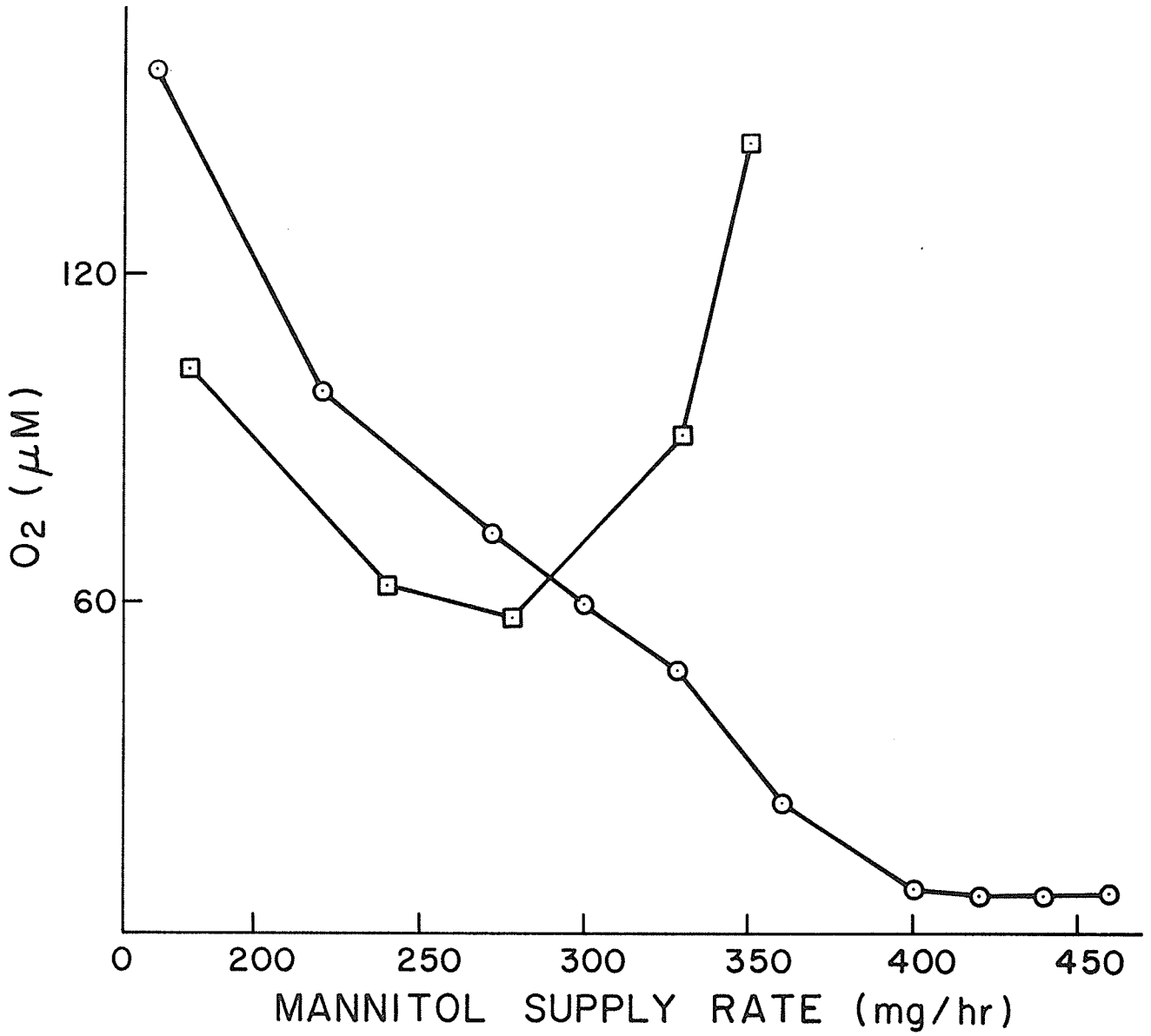


Fig. 18. Plot of dissolved oxygen tension against mannitol supply rate for mannitol limited cultures at 20% O_2 in 1.0 x B_6 medium.

A. vinelandii o——o

A. chroococum □——□


120
60
O2 (μM)
0
200
250
300
350
400
450
MANNITOL SUPPLY RATE (mg/hr)

state for approximately 14 hours, at which time respiration gradually increased.

The shift which occurred in A. chroococcum respiration makes the growth of this organism under conditions of excess oxygen difficult and therefore attempts to study this organism under a long term condition of extreme carbon limitation were not pursued.

2.2 Growth of A. vinelandii in extreme carbon limitation.

The long term maintenance of an excess dissolved oxygen tension in a culture of A. vinelandii growing under vortex-stirred conditions can only be accomplished by maintaining the culture under severe mineral or carbon limitation. The effect of such limitation may be either to reduce the cellular concentration to a low density so the culture no longer has the respiratory capability to balance the solution rate of oxygen into the culture, or by using carbon limitation, to decrease the amount of reducing power produced so that it would be insufficient to meet the demands of respiration. One important difference between carbon and inorganic ion limitation would be the interference in respiration by carbon limitation, which may allow oxygen to accumulate within the cell. Such an effect would not be expected in cells which are under inorganic ion limitation since respiration could be maintained at very high rates independent of growth.

A. vinelandii was cultured in a nitrogen free medium with insufficient mannitol to allow the respiration to equal the oxygen solution rate with consequent accumulation of excess oxygen in solution. In addition to the carbon limitation another factor was operating under these conditions which would enhance the toxicity of the dissolved oxygen. This was the intermittant nature of the dropwise addition

of medium to the culture supplied through a peristaltic pump which utilized three pins rotating at approximately one rpm to move the medium through silicon tubing. This pump moved medium at a pulsed rate; that is, medium would be added quickly followed by a slight pause before more medium was added. During the period of this pause respiration ceased due to lack of carbon substrate and on the addition of more medium, the d.o.t. decreased again, with resultant regular fluctuations in the d.o.t. This repeated start and stop in respiration provides a means by which excess oxygen could accumulate in the cytosol of the cell during carbon starvation.

Cultures which have been maintained in a carbon limited state for several weeks show characteristic differences to those grown fresh from the stock culture. This is not surprising in view of the selection process occurring in a chemostat which would favour cells with highest growth rate. Therefore to initiate the experiment a fresh culture of A. vinelandii was inoculated into the sterile chemostat vessel. The culture was then allowed to grow as a batch culture to reduce the mannitol concentration to zero. When the dissolved oxygen tension rose, indicating carbon limitation, the culture was provided with 1.5 x B_6 media with 0.475% mannitol and maintained at a dilution rate of 0.20 hr^{-1}. Over a period of 5 weeks the mannitol concentration in the medium was increased from 0.475 to 0.700% by 0.025% increments. The range of mannitol concentrations which were fed to the culture correspond to mannitol supply rates of 0.218 to 0.330 grams per hr. The culture was analyzed in duplicate on consecutive days after it had been allowed to reach a steady state.

a) Effect of carbon starvation on dissolved oxygen concentration, respiration and growth.

The concentration of dissolved oxygen which accumulated in the medium at various mannitol concentrations is shown in Fig. 19. This decreased gradually to 18 μM oxygen at 0.293 g mannitol hr^{-1}; further increases in mannitol supply were accompanied by a rapid decrease to zero d.o.t.

Accompanying the decrease in the dissolved oxygen was an increase in the carbon dioxide evolution (Fig. 19). The ratio of the difference in CO_2 evolution at 0.218 to 0.293 g mannitol hr^{-1} is 0.80, similar to the difference in the rate of oxygen uptake which is 0.87.

The biomass production at various degrees of carbon starvation is shown in Fig. 19. This biomass production effectively doubles over the period from extreme carbon limitation (0.218 g mannitol hr^{-1}) to where the mannitol is sufficient to maintain the d.o.t. at zero (0.305 g hr^{-1}). The respiratory index for the various mannitol concentrations is plotted in fig. 20. As had occurred at very high oxygen concentration, respiration is given priority for carbon flow under these extremely carbon limited conditions. The RI value decreases rapidly as the dissolved oxygen tension decreases and reaches its minimum value when the d.o.t. equals zero. The relationship between RI and d.o.t. is shown in Fig. 21. Above 6 μM oxygen there appears to be a linear relationship between the d.o.t. and the efficiency of respiration indicating that these cells respond to the levels of dissolved oxygen rather than simply to its presence or absence.

Fig. 19. Variation in biomass, CO_2 evolution and dissolved oxygen tension with different mannitol supply rates to a continuous culture of A. vinelandii at 20% O_2 with 1.5 x B_6 medium.

Biomass o——o

D.O.T. □——□

CO_2 evolution Δ——Δ

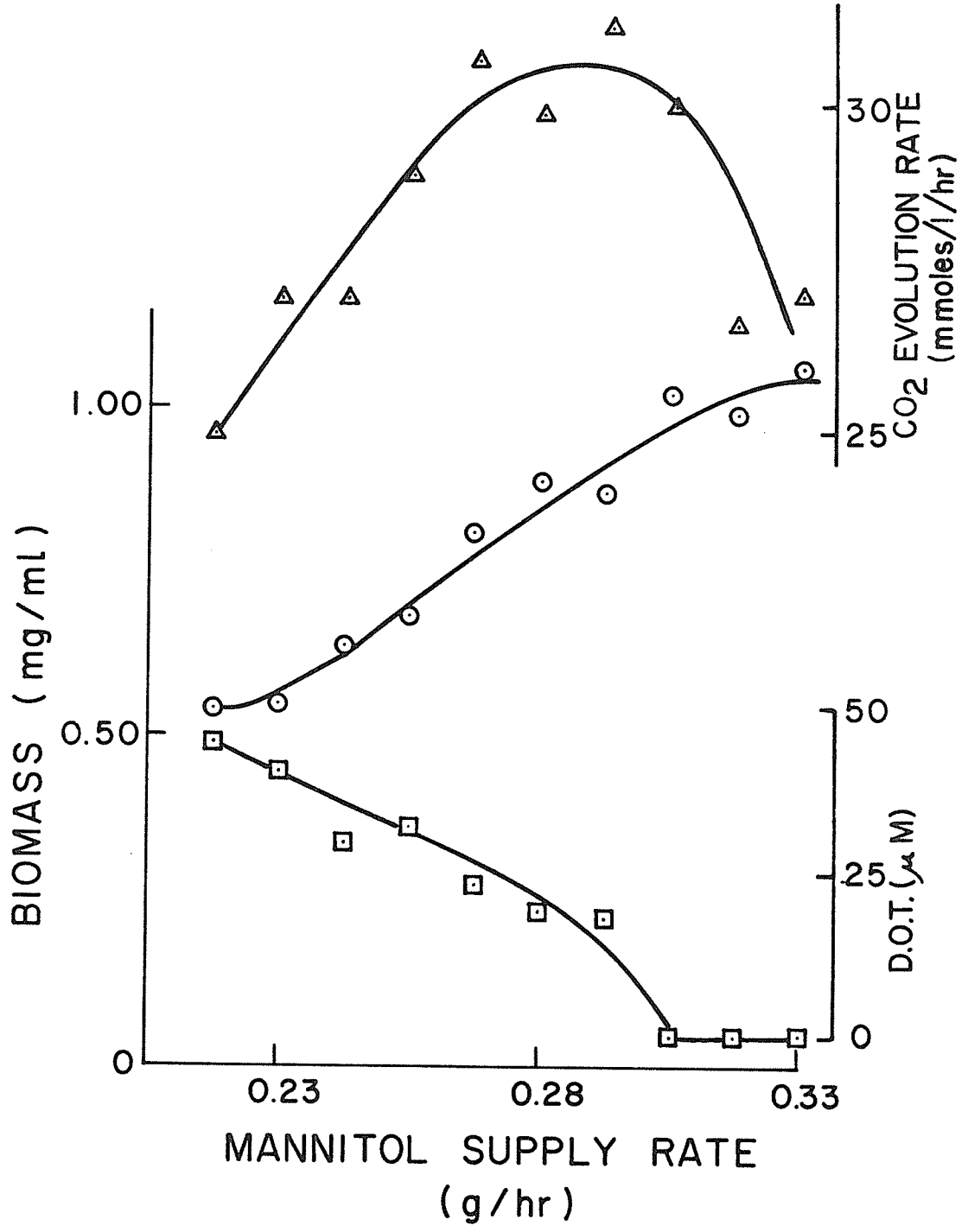

BIOMASS (mg/ml)
1.00
0.50
0
30
25
CO_2 EVOLUTION RATE (mmoles/l/hr)
50
25
0
D.O.T. (μM)
0.23
0.28
0.33
MANNITOL SUPPLY RATE (g/hr)

Fig. 20. Variation in the respiratory index (mole CO_2 per gram dry weight produced) with different mannitol supply rates to a continuous culture of A. vinelandii, at 20% O_2 with 1.5 x B_6 medium.

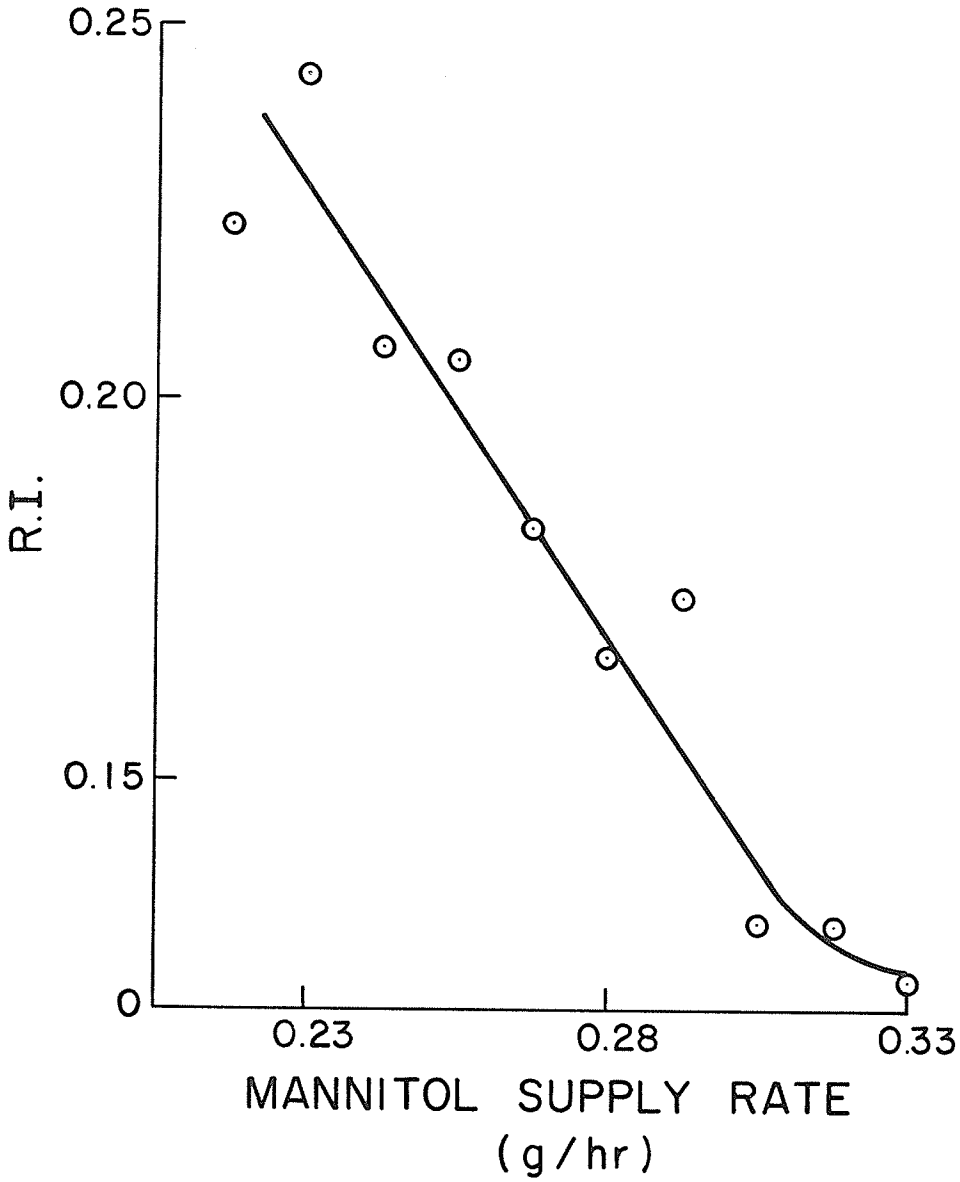
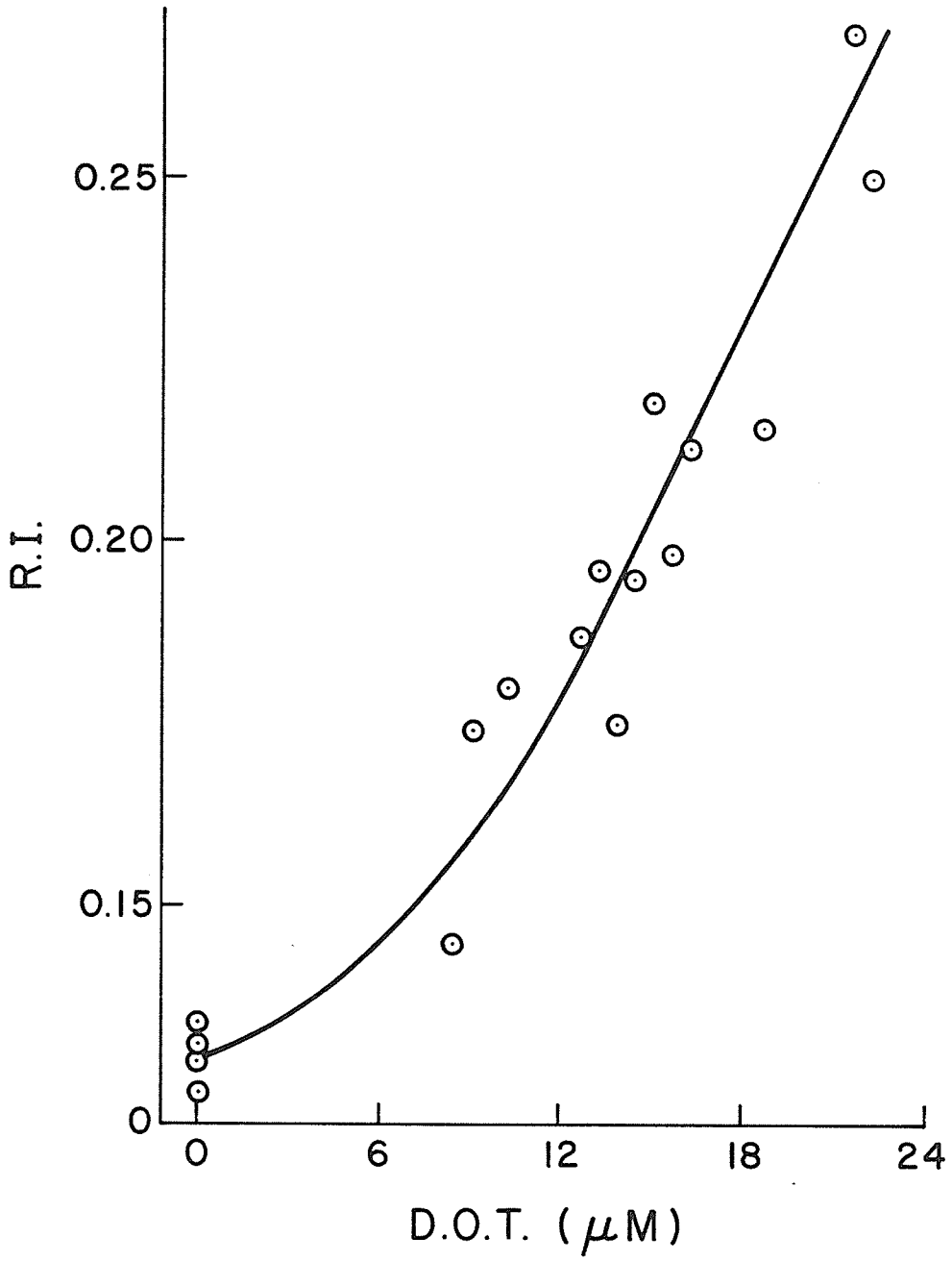

0.25
0.20
0.15
0
R.I.
0.23
0.28
0.33
MANNITOL SUPPLY RATE
(g/hr)

Fig. 21. Relationship between the respiratory index (moles CO_2 per gram dry weight produced) and the dissolved oxygen tension for a continuous culture of A. vinelandii, at 20% O_2, with 1.5 x B_6 medium, D = 0.20 hr^{-1}.


0.25
0.20
0.15
0
R.I.
0
6
12
18
24
D.O.T. (μM)

b) Oxygen and carbon uptake rates of carbon starved cells.

The rate of oxygen uptake was determined for the cells at the different degrees of carbon limitation. These results are shown in Fig. 22. The rate declines almost linearly as the mannitol supply is increased from 0.218 to 0.304 mg hr^{-1}. Veeger and Haaker (1976) claim that the rate limiting step in carbon limited cells as to oxygen uptake is the transport of the carbon into the cell. The rate of carbon uptake was determined; the results are shown in Fig. 23. A comparison of Fig. 22 and 23 makes it appear unlikely that under the conditions used to measure oxygen uptake with a plentiful supply of carbon substrate, the rate of oxygen uptake would be limited by the rate of mannitol transport. The ratio of the translocase activity at 0.218 to 0.304 g hr^{-1} indicates an almost 3-fold decrease in translocase activity. However over the same mannitol concentrations, the rate of oxygen uptake, measured in the presence of 1% mannitol is only reduced by one-third. If the translocase activity determines the rate of O_2 uptake in the oxygraph, a similar ratio in activities would be expected. Undoubtedly the rate of respiration of carbon limited cells in the chemostat is dependent upon the rate of carbon uptake; however, when these cells are given sufficient carbon under the conditions in an oxygraph the rate of O_2 uptake appears to be determined by the rate of some intermediate enzyme.

c) NADH oxidase

The NADH oxidase activity represents the overall rate of electron flow from NADH dehydrogenase to the cytochrome oxidases. The results of the assay for the enzyme activity are shown in Fig. 24. During extreme carbon limitation, the NADH oxidase activity is highest;

Fig. 22. Variation in the oxygen uptake rate with changes in mannitol supply rate to a continuous culture of A. vinelandii, at 20% O_2 with 1.5 x B_6 medium, D - 0.20 hr^{-1}.

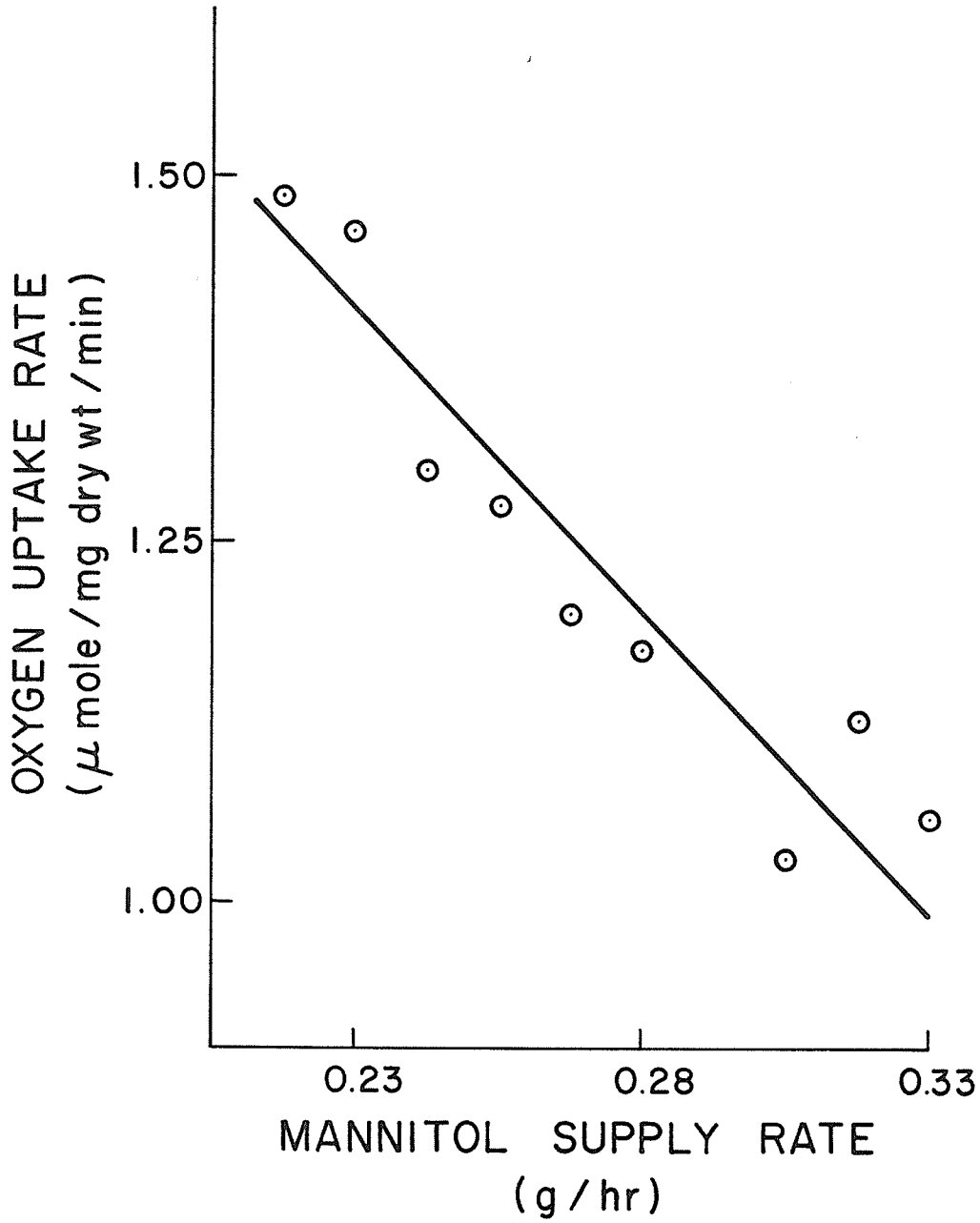

OXYGEN UPTAKE RATE
(μ mole / mg dry wt / min)
1.50
1.25
1.00
0.23
0.28
0.33
MANNITOL SUPPLY RATE
(g / hr)

Fig. 23. Variation in the mannitol uptake rate (Δ cpm·min^{-1}) with different mannitol uptake rate to a continuous culture of A. vinelandii, at 20% O_2 with 1.5 x B_6 medium, D = 0.20 hr^{-1}.

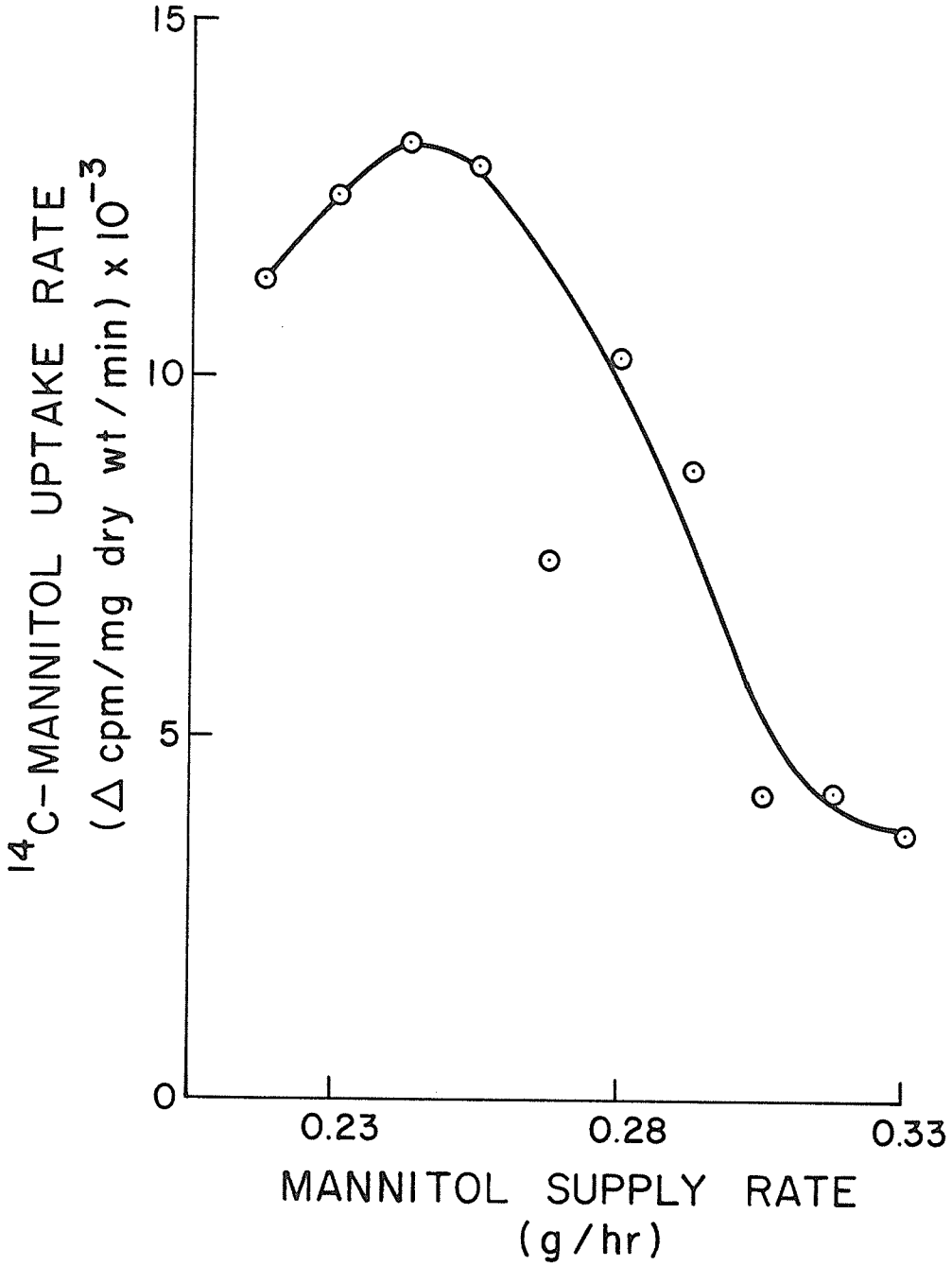
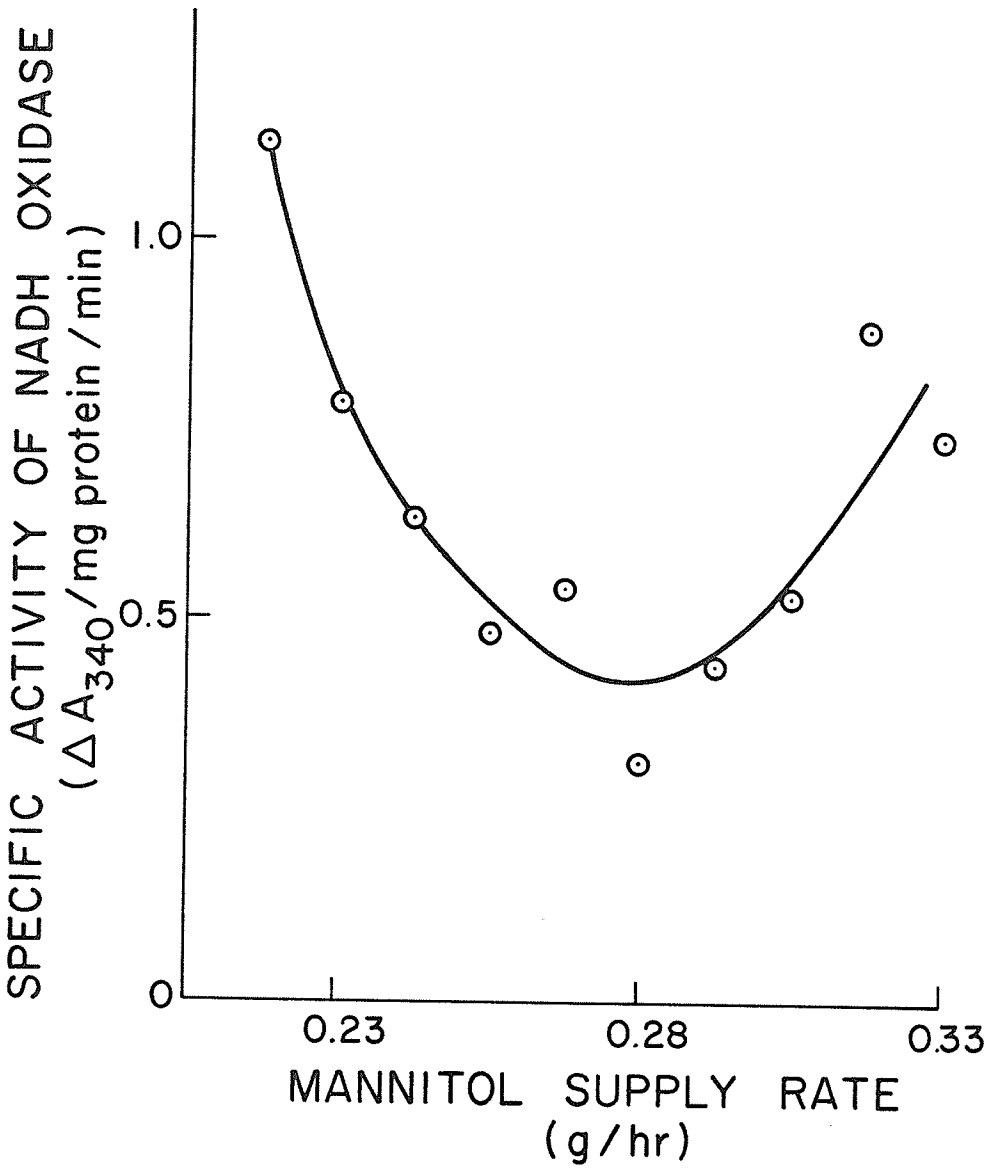

15
10
5
0
0.23
0.28
0.33
^{14}C-MANNITOL UPTAKE RATE
(Δ cpm/mg dry wt/min) x 10^{-3}
MANNITOL SUPPLY RATE
(g/hr)

Fig. 24. Variation in the activity of NADH oxidase with different mannitol supply rates to a continuous culture of A. vinelandii, at 20% O_2 with 1.5 x B_6 medium, D = 0.20 hr^{-1}.


SPECIFIC ACTIVITY OF NADH OXIDASE
(ΔA_{340}/mg protein/min)
1.0
0.5
0
0.23
0.28
0.33
MANNITOL SUPPLY RATE
(g/hr)

it then decreases rapidly to a minimum value of 0.28 g mannitol hr^{-1}. At this rate of mannitol addition the d.o.t. is still relatively high being 24 μM oxygen (Fig. 19). Further increases in mannitol supply resulted in an increasing activity for this enzyme.

If the respiration of Azotobacter is competitive with growth as has been suggested (Phillips and Johnson, 1961), the control of NADH oxidase would allow the cell to divert reducing power away from respiration under conditions where it is desirable to have this reducing power go toward growth. The high activity of NADH oxidase during extreme mannitol limited growth would provide a competitive advantage to respiration in conditions of excess dissolved oxygen. When the amount of dissolved oxygen is reduced, this enzyme activity is lower, thus giving more of a competitive advantage to enzymes involved in biosynthesis. The increased activity at high mannitol concentration may reflect the fact that these cells are becoming oxygen limited and in an effort to produce more energy more reducing equivalents are directed toward the respiratory chain.

d) Superoxide Dismutase

The activity of superoxide dismutase was determined in cell-free extracts of samples taken from the mannitol limited cultures. The results are shown in Fig. 25. The activity of this enzyme is greatest during extreme carbon limitation (i.e. between .218 and .243 g of mannitol per hr) and decreases with increasing mannitol supply. Thus Azotobacter appear to be capable of inducing the synthesis of this enzyme under conditions of excess oxygen. This result is apparently contrary to the results of Buchanan (1978) who found reduced S.O.D. activities per mg of protein when the oxygen tension above the

culture was increased from 5 to 30%.

e) Catalase Activity

The catalase activity measured as the rate of oxygen production from hydrogen peroxide is markedly dependent upon the dissolved oxygen tension (Fig. 26 and Fig. 27). The activity at zero dissolved oxygen is similar to that described by Buchanan (1978). However the remarkably high catalase activity in the presence of high dissolved oxygen tensions is a result of the necessity to experimentally increase this dissolved oxygen tension in order to obtain noticeable affects of oxygen in this organism.

An interesting comparison can be made between the activities of catalase and that of the mannitol translocase. The enzyme activities show a similar pattern in response to increasing mannitol supply. At low mannitol supply rates activities are high and increase slightly with increased mannitol supply. Both activities decrease and reach their lowest levels when the d.o.t. reaches zero. It is tempting to speculate that these enzymes may be affected by a common factor, as yet undetermined.

f) Effect of high dissolved oxygen on the activity of isocitrate dehydrogenase.

The activity of the Krebs cycle enzyme isocitrate dehydrogenase was determined from cells taken from various degrees of carbon limitation (Fig. 28). The activity of this enzyme does not appear to be affected by either carbon limitation or the excess dissolved oxygen tension.

g) Isocitrate lyase

The activity of isocitrate lyase was determined by measuring the formation of the glyoxylate phenylhydrazine derivative (Fig. 29).

Fig. 25. Variations in the superoxide dismutase activity with different mannitol supply rates to a continuous culture of A. vinelandii, at 20% O_2, with 1.5 x B_6 medium, D = 0.20 hr^{-1}.

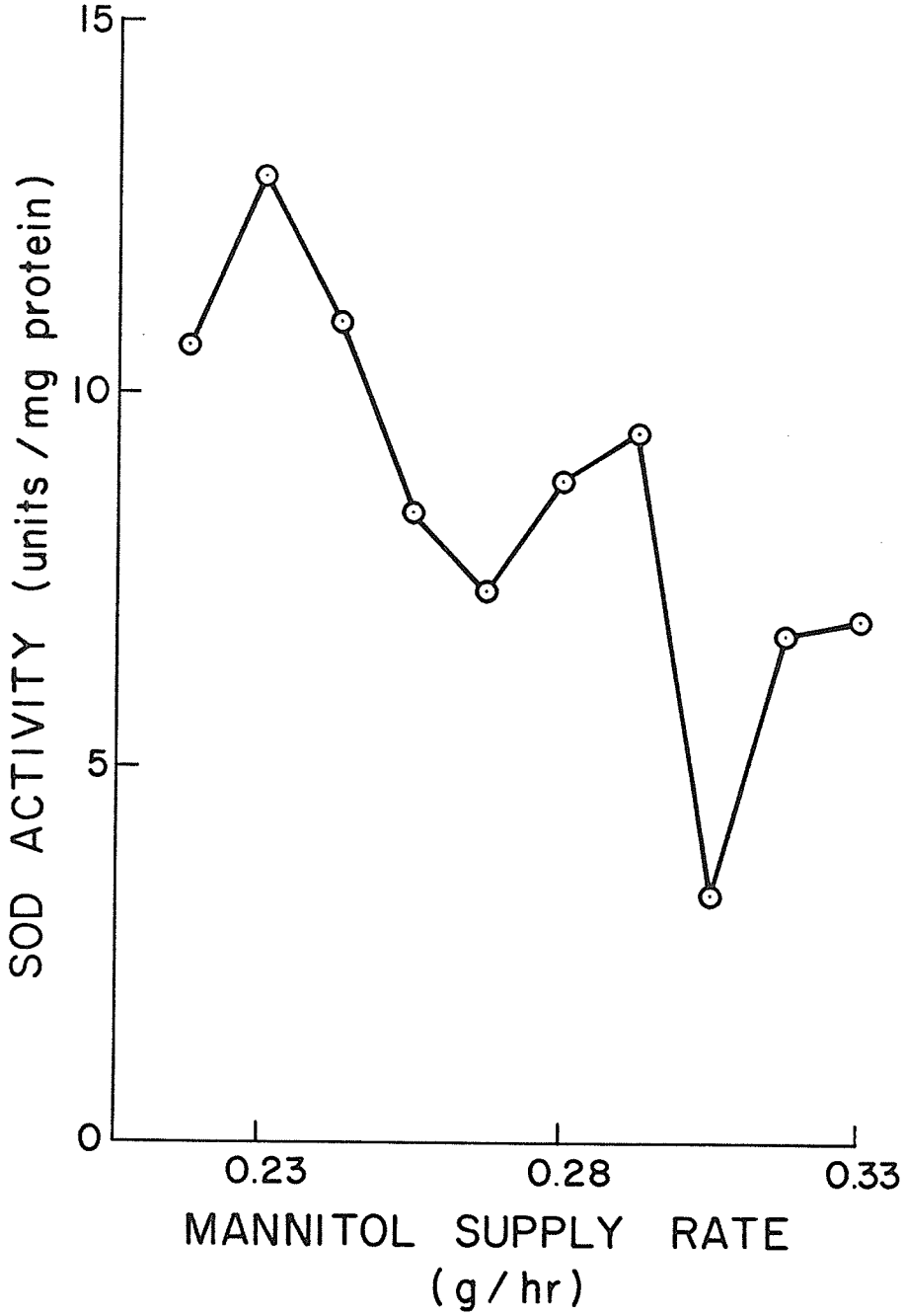

15
10
5
0
SOD ACTIVITY (units / mg protein)
0.23
0.28
0.33
MANNITOL SUPPLY RATE
(g / hr)

Fig. 26. Variation in the catalase activity (μ moles O_2 released x min^{-1} x mg dry $weight^{-1}$) with different mannitol supply rates to a continuous culture of A. vinelandii at 20% O_2 with 1.5 x B_6 medium, D = 0.20 hr^{-1}.

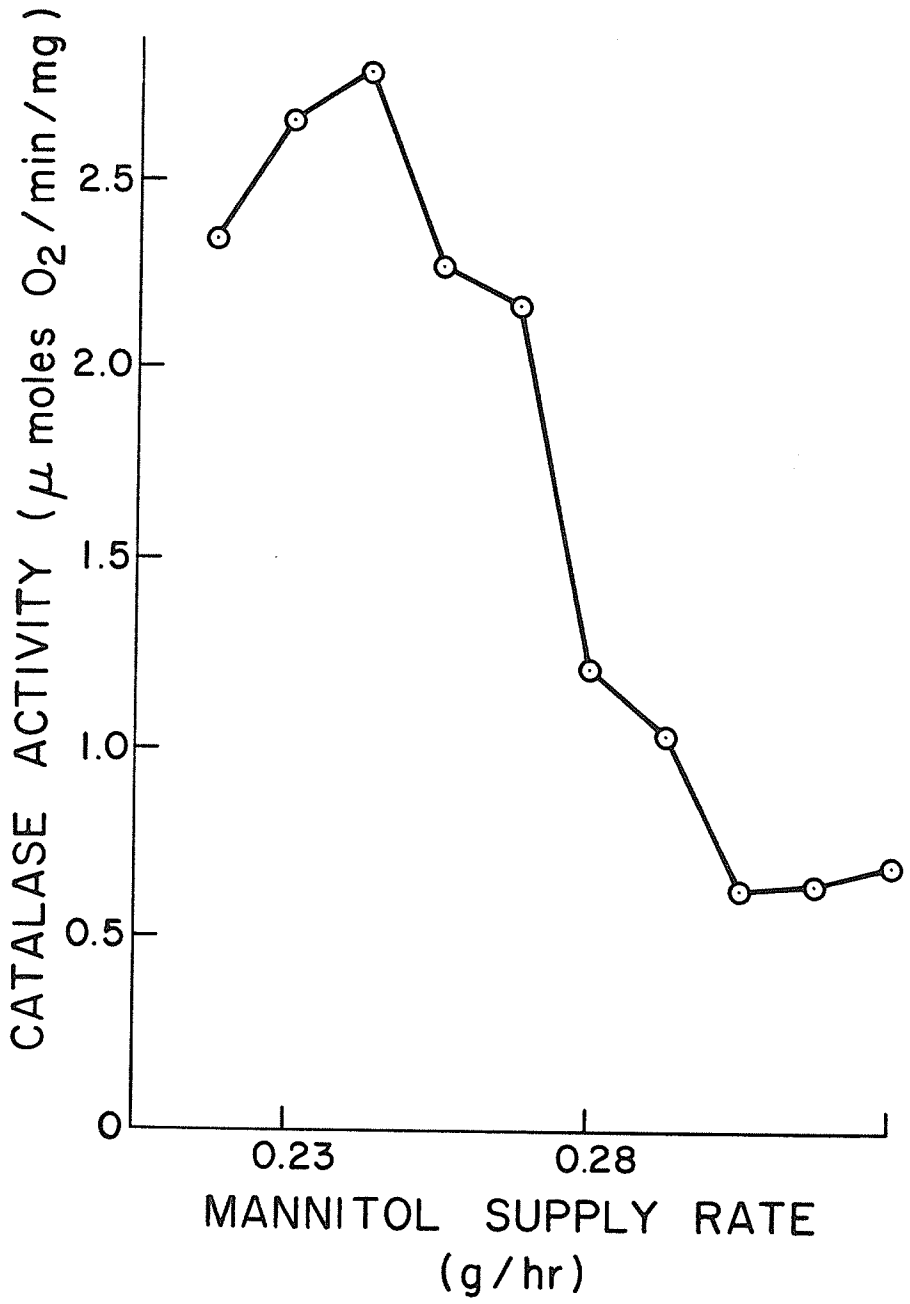

CATALASE ACTIVITY (μ moles O2 / min / mg)
2.5
2.0
1.5
1.0
0.5
0
0.23
0.28
MANNITOL SUPPLY RATE
(g / hr)

Fig. 27. Variation in the catalase activity (μ mols O_2 released x min^{-1} x mg dry $weight^{-1}$) with different dissolved oxygen concentrations in a continuous culture of A. vinelandii at 20% O_2 with 1.5 x B_6 medium, D = 0.20 hr^{-1}.

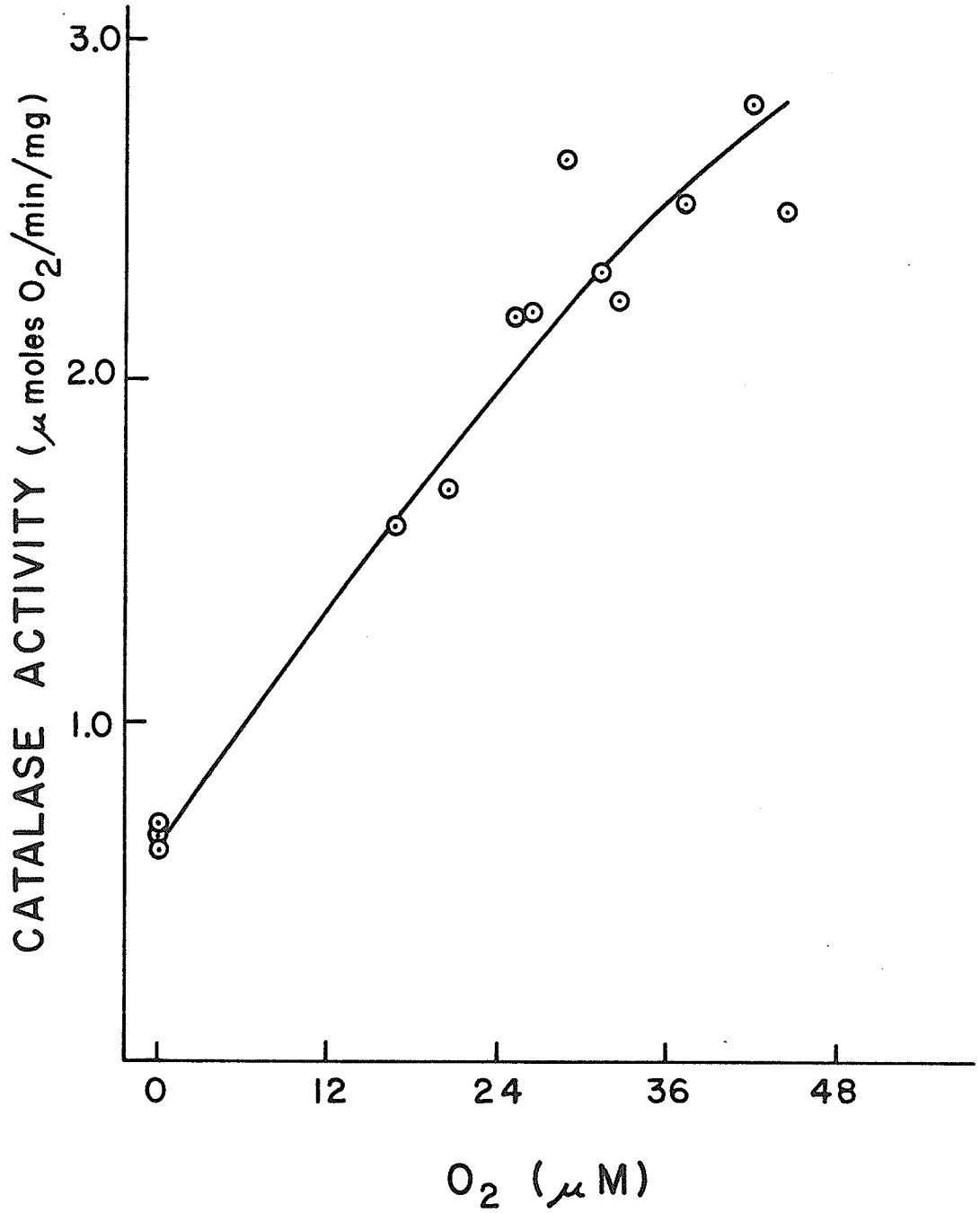

CATALASE ACTIVITY (μ moles O2/min/mg)
3.0
2.0
1.0
0
12
24
36
48
O2 (μM)

Fig. 28. Variation in the activity of isocitrate dehydrogenase with different mannitol supply rates to a continuous culture of A. vinelandii, at 20% O_2 with 1.5 x B_6 medium, D = 0.20 hr^{-1}.

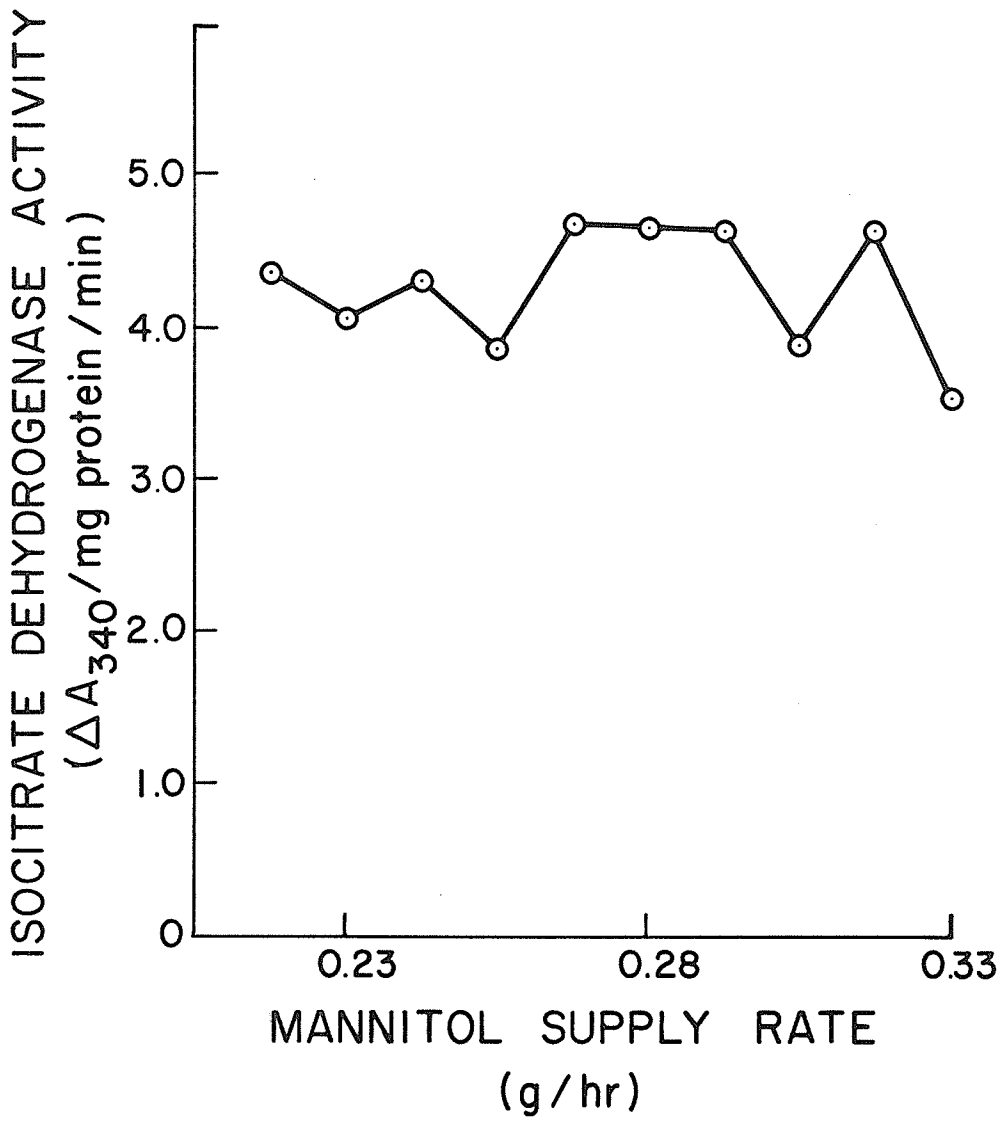
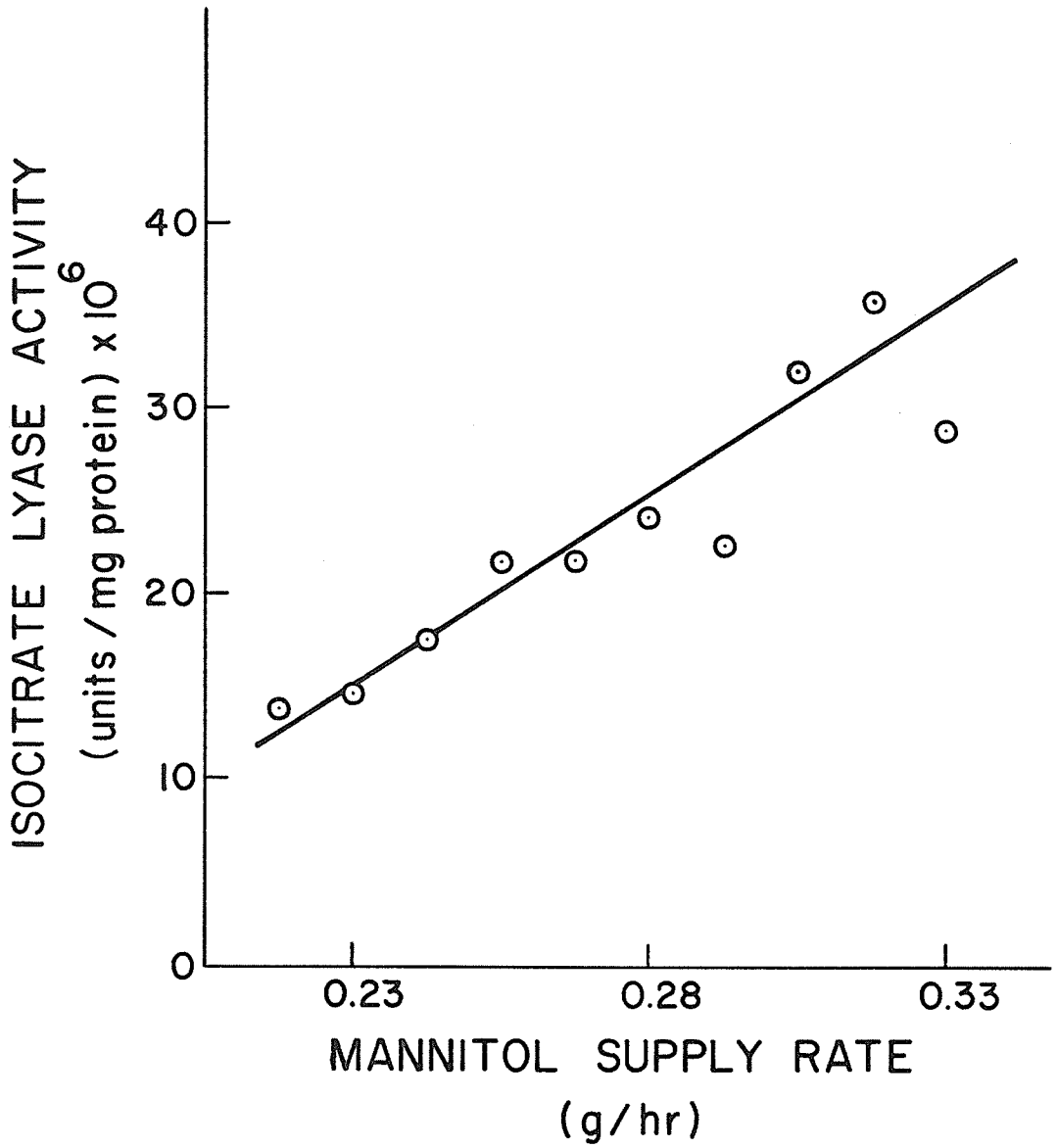

ISOCITRATE DEHYDROGENASE ACTIVITY
(ΔA_{340}/mg protein/min)
5.0
4.0
3.0
2.0
1.0
0
0.23
0.28
0.33
MANNITOL SUPPLY RATE
(g/hr)

Fig. 29. Variation in the activity of isocitrate lyase with different mannitol supply rates to a continuous culture of A. vinelandii, at 20% O_2 with 1.5 x B_6 medium, D = 0.20 hr^{-1}.


ISOCITRATE LYASE ACTIVITY
(units / mg protein) x 10^6
40
30
20
10
0
0.23
0.28
0.33
MANNITOL SUPPLY RATE
(g/hr)

The activity increased almost linearly with increasing mannitol supply rates, the activity being over three-fold greater when the cell is supplied with sufficient mannitol compared to the extremely carbon limited cells.

h) Change in cytochrome levels

Although the levels of cytochromes do not necessarily reflect their activity in the cell, they were measured to determine if excess oxygen would inhibit their production. The levels of cytochromes relative to levels at 0.33 g hr^{-1} are shown in Fig. 30.

Cytochrome d

During periods of excess oxygen this main cytochrome oxidase is produced in lower quantities compared to its production when no excess O_2 is present. The levels increase as the dissolved oxygen tension approaches zero. The maximum levels of cytochrome d occurred at a supply rate of 30.50 mg/hr and at higher rates the levels decreased.

Cytochrome o

The levels of cytochrome o were determined by CO-reduced minus reduced spectrum estimated by the difference between OD at 417 and 432 nm. Fig. 30 shows how the cytochrome levels change with mannitol supply rates. These cytochrome levels increased as the dissolved oxygen tension was reduced. When the dissolved oxygen tension approached zero the levels of this cytochrome were more erratic indicating the control of the production to be no longer dependent upon the d.o.t.

b type cytochromes

The relative amount of b type cytochromes were determined from

Fig. 30. Variation in the cytochrome levels with different mannitol supply rates to a continuous culture of A. vinelandii, at 20% O_2 with 1.5 x B_6 medium, D = 0.20 hr^{-1}.

Cytochrome d — Δ———Δ

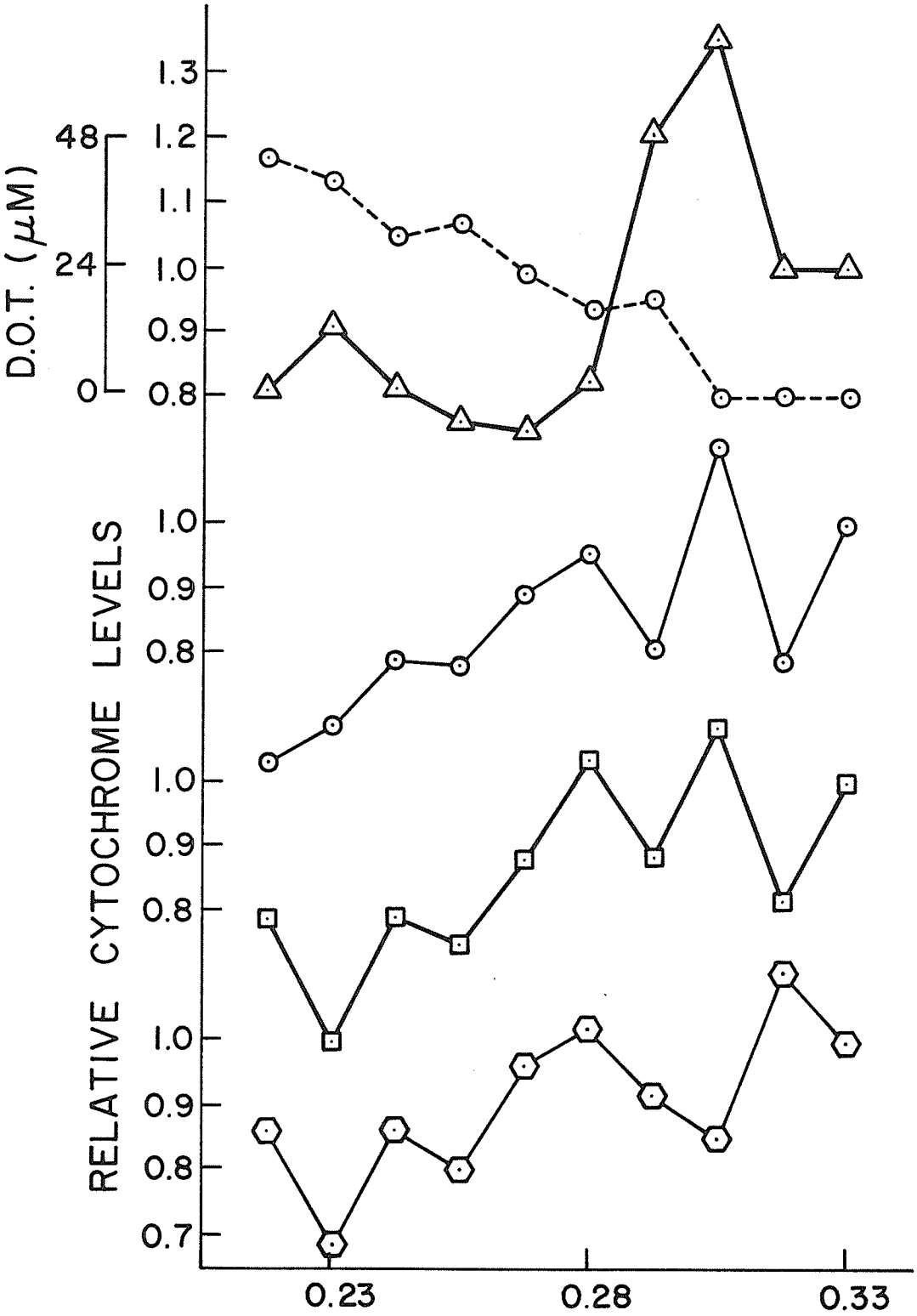
Cytochrome o — o———o

Cytochromes c_4 & c_5 — □———□

b-type cytochromes — ⬡———⬡

D.O.T. — o-----o

Cytochrome levels are expressed relative to the levels occuring in cells from cultures provided with 0.330 grams mannitol per hour.


D.O.T. (μM)
48
24
0
1.3
1.2
1.1
1.0
0.9
0.8
RELATIVE CYTOCHROME LEVELS
1.0
0.9
0.8
1.0
0.9
0.8
1.0
0.9
0.8
0.7
0.23
0.28
0.33

the shoulder occurring on the c cytochrome peak. Levels of b cytochromes, which include cytochrome b_1 and o, increase with increasing mannitol supply rates. When the d.o.t. approaches zero the levels of b type cytochromes decrease in contrast to the relative levels of cytochrome o, which shows a sharp increase when the d.o.t. reaches zero (at .305 g mannitol per hr).

Cytochrome c

The levels of the c_4 and c_5 type cytochromes were determined from the reduced spectrum by measuring the absorption difference between 551 and 540 nm. The levels of this cytochrome increase as the culture is given more mannitol and the d.o.t. is reduced. This pattern of change of the cytochrome c is similar to that described for cytochrome o levels. Such a similarity may reflect the functional relationship between these two cytochromes since both cytochromes are believed to form the minor pathway in electron transport (Jones and Redfearn 1967).

Cytochrome a_1

The levels of cytochrome a_1 were determined by the difference spectra of oxidized minus reduced at 592 nm. No significant difference was found in the levels of cytochrome a_1 throughout the various degrees of mannitol limitation. The levels of the cytochrome a_1 can also be determined by the magnitude of absorption at 428 nm in its CO-reduced minus reduced spctrum, shown for O_2 limited batch cultures in Fig. 31. However no significant absorption peak was found for the CO-reduced minus reduced spectrum at this wavelength, for cells from a chemostat in any degree of carbon limitation or oxygen limitation. Since cytochrome a_1 from chemostat grown cells does not appear to

Fig. 31. CO-reduced minus reduced spectrum.

----- oxygen limited chemostat grown cells, utilizing mannitol (cytochrome o peak at 417 nm)

——— oxygen limited batch grown cells, utilizing acetate (cytochrome o peak at 417 nm and cytochrome a_1 peak at 428 nm)

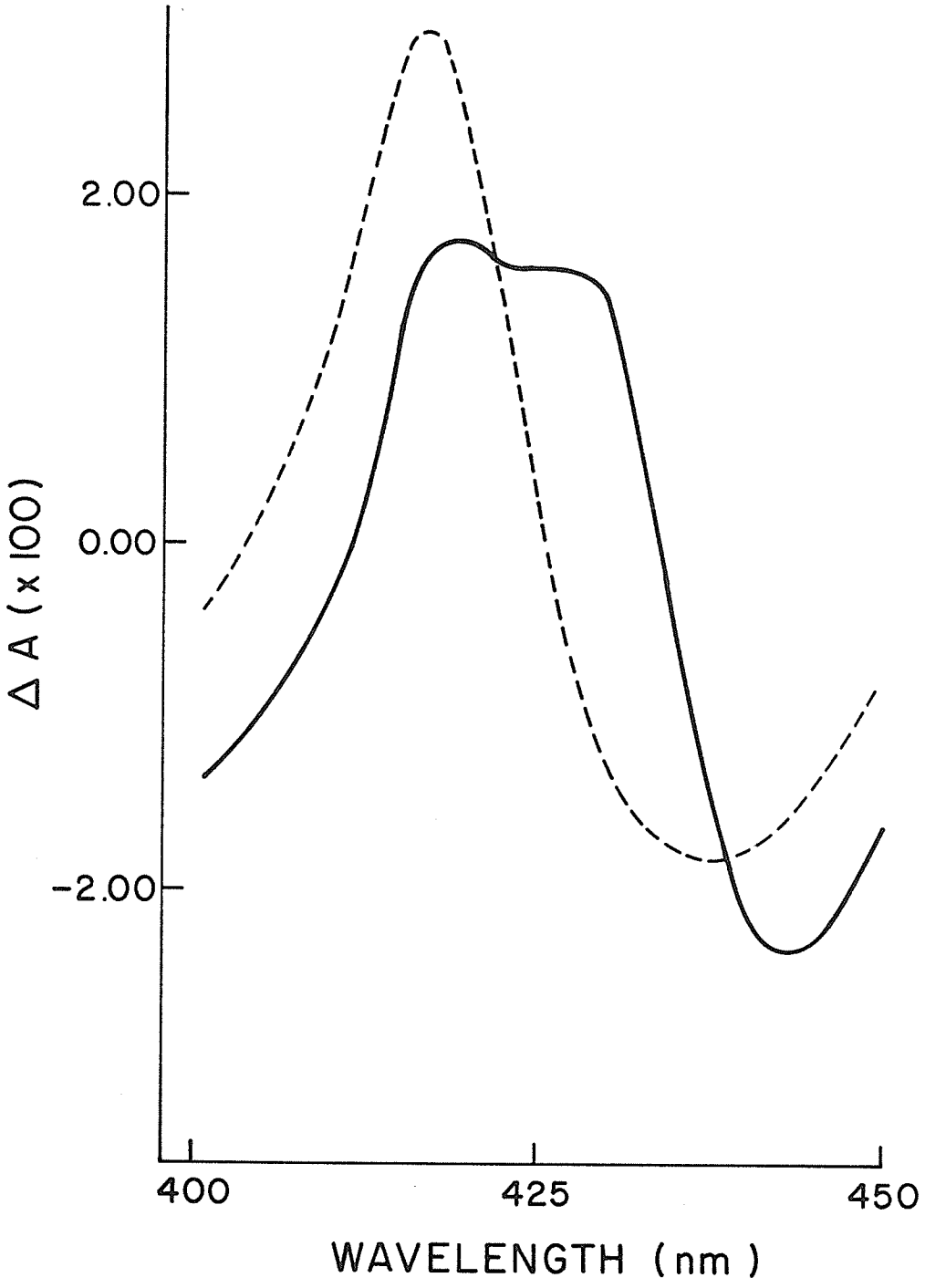
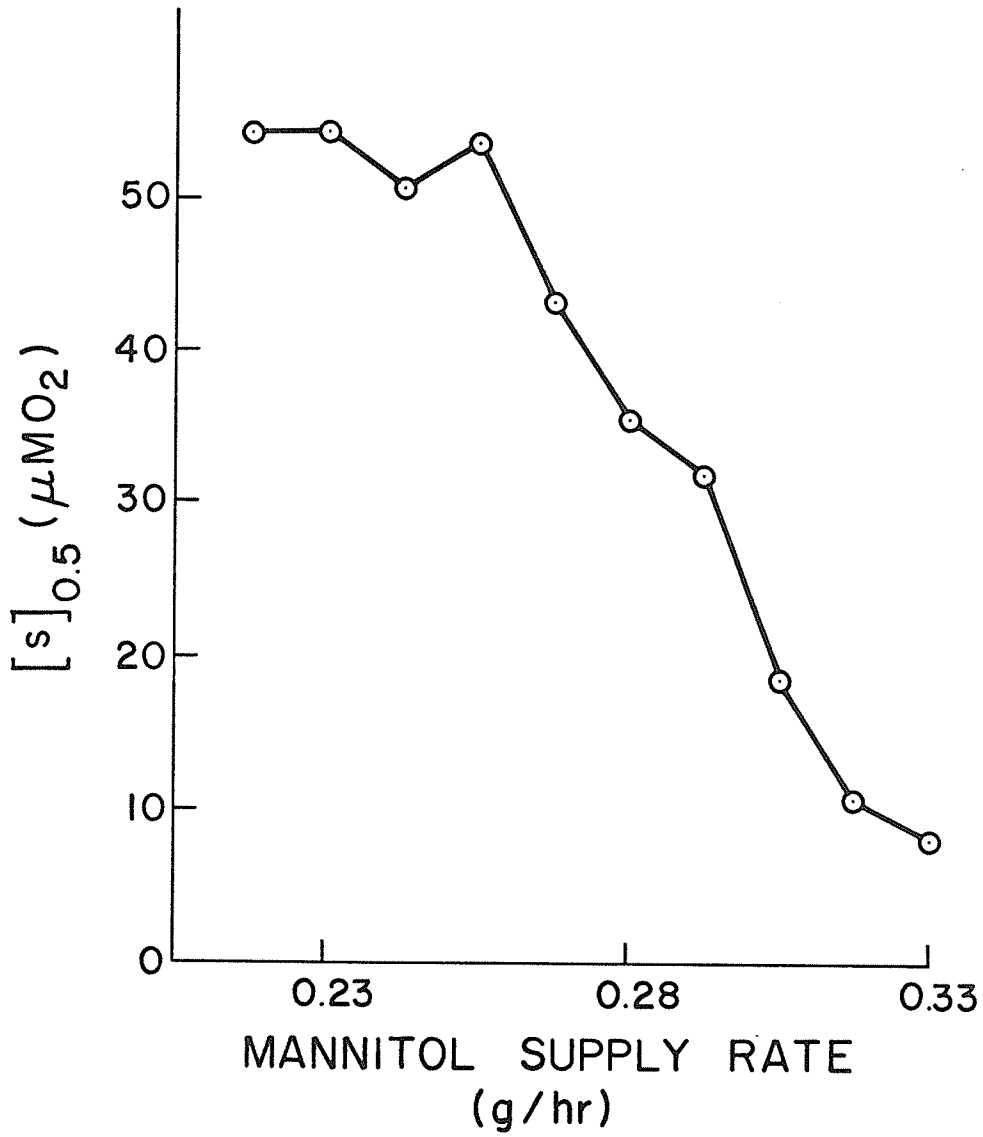

2.00
0.00
-2.00
Δ A (x 100)
400
425
450
WAVELENGTH (nm)

Fig. 32. Variations in the concentration of oxygen supporting half the observed maximum rates [S] 0.5, with different mannitol supply rates to a continuous culture of A. vinelandii, at 20% O_2 with 1.5 x B_6 medium, D = 0.20 hr^{-1}.


$[s]_{0.5}$ (μMO_2)
50
40
30
20
10
0
0.23
0.28
0.33
MANNITOL SUPPLY RATE
(g/hr)

bind CO it probably does not act as a cytochrome oxidase. Therefore, under these conditions the main cytochrome oxidase of the minor pathway would appear to be cytochrome o.

i) Affinity for oxygen

The affinity of the cytochrome oxidase for dissolved oxygen was estimated by determining the concentration of oxygen at which the rate of oxygen uptake is half the maximum. This rate was determined by drawing tangents to the O_2 uptake curve. The results are presented in Fig. 32. During extreme carbon limitation with high d.o.t. the O_2 concentration at 1/2 V_{max} is equal to 54 μM O_2. The affinity for oxygen remains low when the cell is exposed to extreme carbon limitation, that is from 0.218 to 0.255 g mannitol hr^{-1}. However it increases as the culture is provided with mannitol supply rates greater than .268 g hr^{-1}. The maximum affinity was reached at 0.33 g mannitol hr^{-1}.

Structural changes accompanying carbon starvation

The exposure to excess oxygen via carbon limitation produces drastic changes in the structure of Azotobacter cells. These include changes in cell size, pleomorphism, vesicle formation, separation of the cytoplasmic membrane from the cell wall and the appearance of cytoplasmic inclusion bodies.

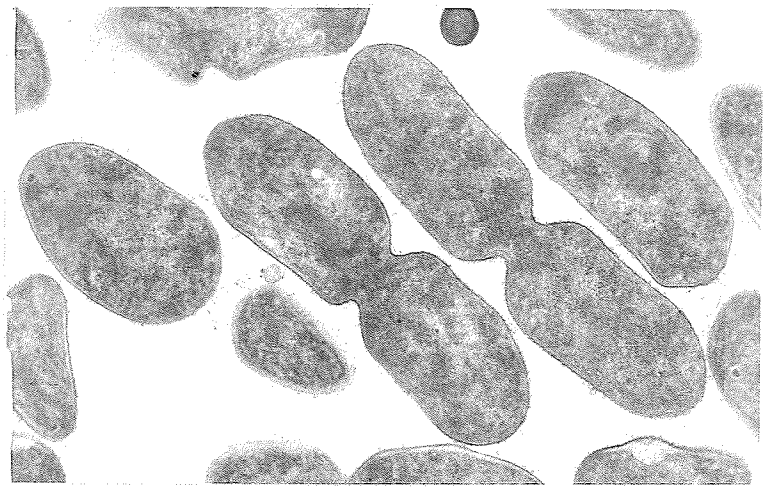
Thin sections of Azotobacter cells, cultured with sufficient oxidizable substrate to maintain the dissolved oxygen tension at zero, are shown in plate 1. The cell wall is fairly uniform with occasional localized expansion of the periplasmic space. Generally the cytoplasm appears to be of constant density, and the amount of cell wall cytoplasmic membrane separation is low.

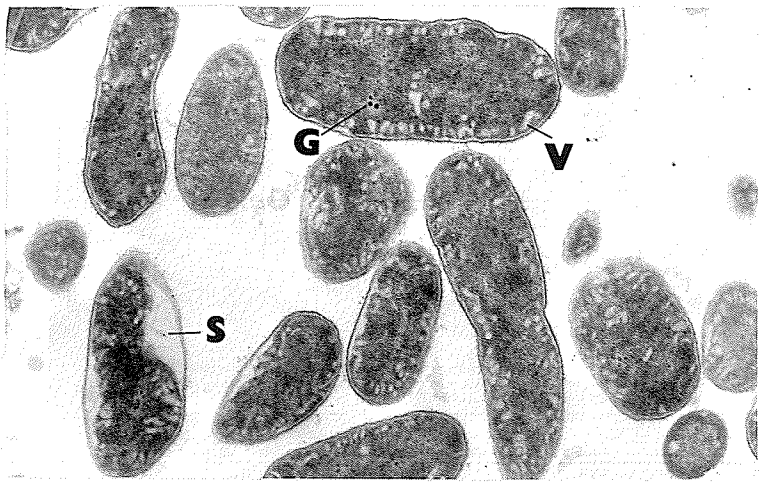
Plate 1. A. vinelandii from an oxygen limited chemostat culture with a mannitol supply rate of 0.330 grams per hour and D.O.T. of zero.

x 15,750

Plate 2. A. vinelandii from a carbon limited chemostat culture; mannitol supply rate of 0.218 grams per hour, D.O.T. of 44 μM. These cells demonstrate vesicles (V), granular inclusion particles (G), cell wall-cell membrane separation (S).

x 15,750




G
V
S

In contrast, the cells from cultures which are in extreme carbon limitation, with resultant high d.o.t. are shown in Plate 2. The dissolved oxygen tension in the culture from which these cells were taken was 26 μM. The structure of these cells differs from the oxygen limited cells by the presence of large numbers of vesicles within the periphery of the cytoplasmic membrane. The cell membrane of these cells shows much greater separation from the cell wall which may be the reason why they also show much more pleomorphism. The cytoplasmic volume of the cells grown under extreme carbon starvation appeared to be reduced, with an apparent increase in the electron density of the cytoplasm.

These results appear to be contradictory to the two suggested roles of vesicles in Azotobacter given by Oppenheim and Marcus (1970) and Pate et al. (1973). Oppenheim and Marcus (1970) suggested the vesicles are produced as a response of the cell which depends upon whether the nitrogen source is fixed nitrogen or dinitrogen. The vesicle was proposed to act either as the vehicle in which the nitrogenase is embedded or the vesicular membrane may allow the cell to cope with high oxygen tensions so as to protect the oxygen sensitive nitrogenase enzyme. However my results indicate vesicle production to be dependent upon the d.o.t. in the medium rather than on the N-sources. The increase in vesicle production in the presence of high dissolved oxygen does suggest that a respiratory protective role is played by these structures which may, as Oppenheim and Marcus suggest, protect the oxygen sensitive nitrogenase. It is therefore likely that the respiration is adequate to protect the nitrogenase when the d.o.t. is zero, however at high d.o.t. this protection is

augmented by higher membrane production. A second proposal was suggested by Pate and Brill (1973) who reported greater vesicle production when the d.o.t. is very low and therefore proposed that increased membranes would provide increased surface area enabling the cell to be able to take up more oxygen. My results demonstrating increased vesicle production in the presence of high d.o.t. are directly contradictory to the results of these authors.

Structural changes accompanying a transition from C-limitation to C-sufficiency.

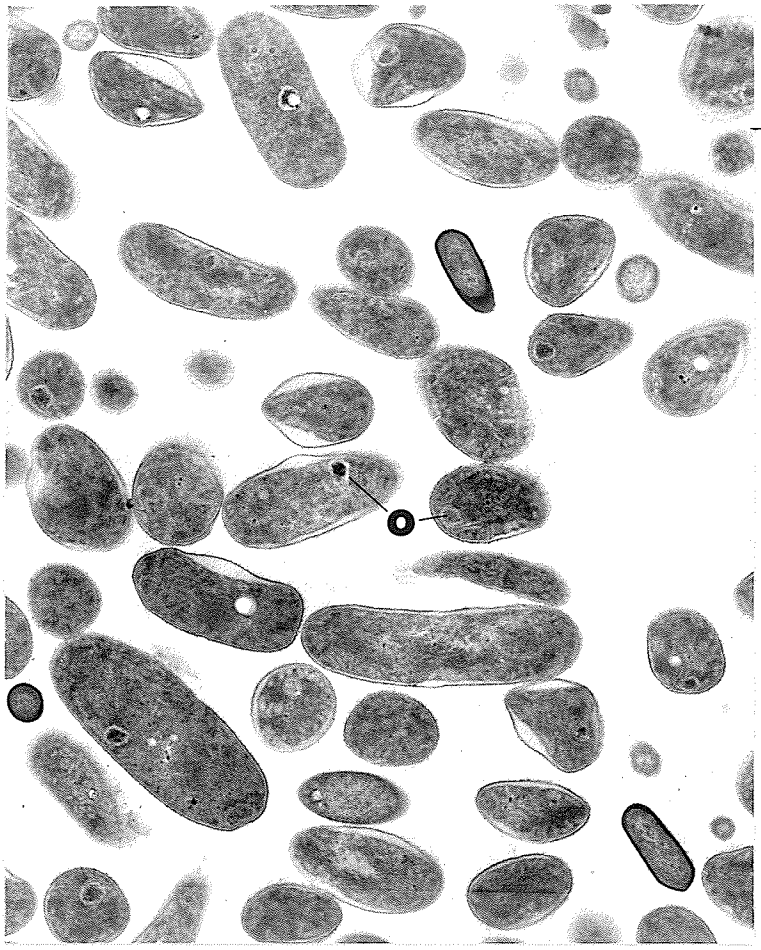
To examine the effects on structure of transition from high dissolved oxygen to zero dissolved oxygen, samples were taken and examined by electron microscopy at various degrees of carbon limitation. The most extreme state of carbon limitation was shown in plate 2. These cells produce a large number of vesicles, the cell wall and cell membrane shows separation and the electron density of the cytoplasm is high. Within these cells granular inclusion bodies occur which resemble polyphosphate granules.

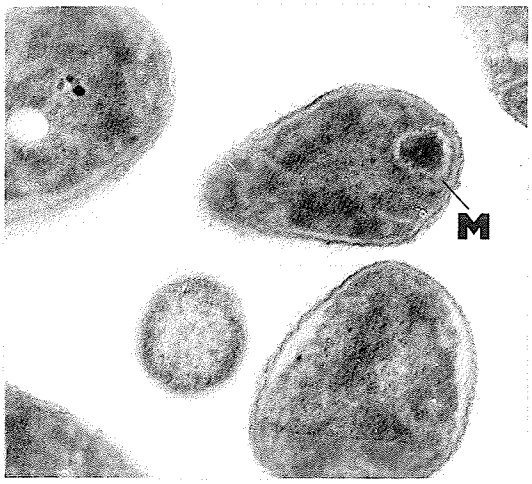
Cells grown at a mannitol supply rate of 0.243 g hr^{-1} had fewer vesicles and formed cytoplasmic organelles which had been seen neither in the extremely carbon-limited cells nor in carbon sufficient cells (Plate 3). These organelles were often of a different electron density than the cytoplasm, appearing darker or lighter than the rest of the cell. They are surrounded by a characteristic light zone which is less electron dense than either the cytoplasm or the interior of the organelle. The organelle is enclosed by a membrane (Plate 4), and often within the organelle, granular inclusion bodies can be found.

Plate 3. A. vinelandii from a carbon-limited chemostat culture; mannitol supply rate of 0.243 grams per hour, D.O.T. of 29 µM.
Cytoplasmic organelles (O).

x 12,600

Plate 4. A. vinelandii cell with cytoplasmic organelle, contained in membrane (M). Conditions of growth as for plate 3.


O


M

Cells which are provided with 0.255 g mannitol hr^{-1} (Plate 5) also form organelles, and have fewer vesicles than observed in cells grown at 0.218 g hr^{-1}. The organelles in these cells appear to be predominantly situated at the poles of the cell.

When the mannitol supply rate is increased to 0.263 g hr^{-1} (Plate 6) the organelles are still formed. The cells appear to be much more normal and have very few vesicles. Increasing the supply rate to 0.280 g hr^{-1} produced an entirely different type of cell (Plate 7), which did not appear to produce organelles, the cytoplasm contained a large number of vesicles; the cell wall and cell membrane showed much more separation.

When the culture was provided with 0.293 g mannitol hr^{-1} the cells lacked organelles, there was still a high production of vesicles but the cell wall-cytoplasmic membrane separation occurred to a lesser degree, Plate 8.

Cells from a culture with sufficient mannitol to maintain the d.o.t. at zero (0.33 g/hr) are shown in Plate 9. These cells appear to have fewer vesicles and rarely show organelle formation. The cytoplasm generally completely fills the cell wall and the cells appear to be fuller and rounder.

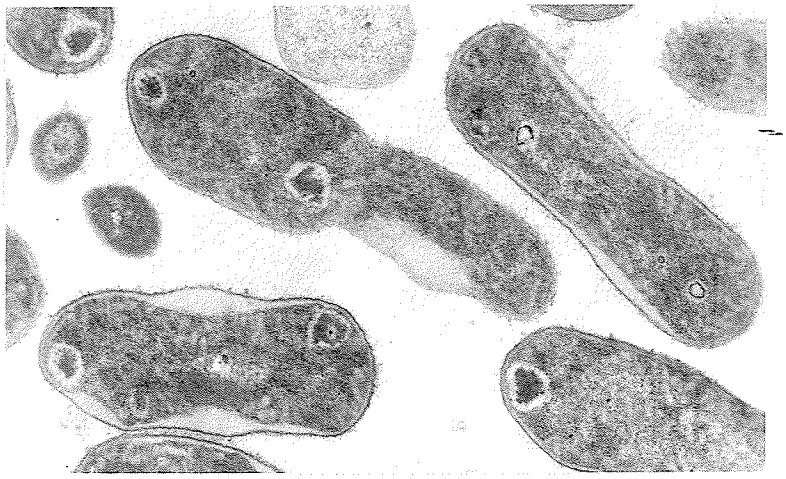
The role of the organelle has not been defined. Those cells which contain the organelles appear to have fewer vesicles. The terminal location of many but not all of these organelles raises the possibility of a mechanism existing to draw the organelles to the terminals. Often more than one organelle is present in a cell, although no organelle-to-organelle relationship was observed.

Plate 5. A. vinelandii from a carbon-limited chemostat culture with a mannitol supply rate of 0.255 grams per hour, D.O.T. of 32 μM.

x 22,000

Plate 6. A. vinelandii from a carbon-limited chemostat culture with a mannitol supply rate of 0.268 grams per hour, D.O.T. of 25 μM. Note polar location of mature cytoplasmic organelles.

x 12,600



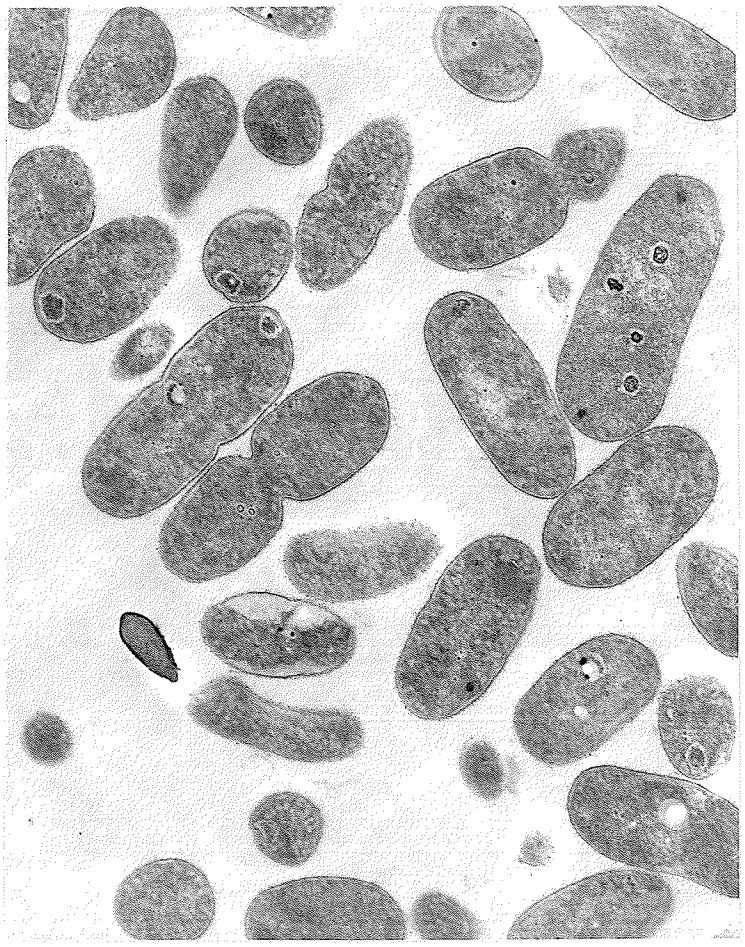
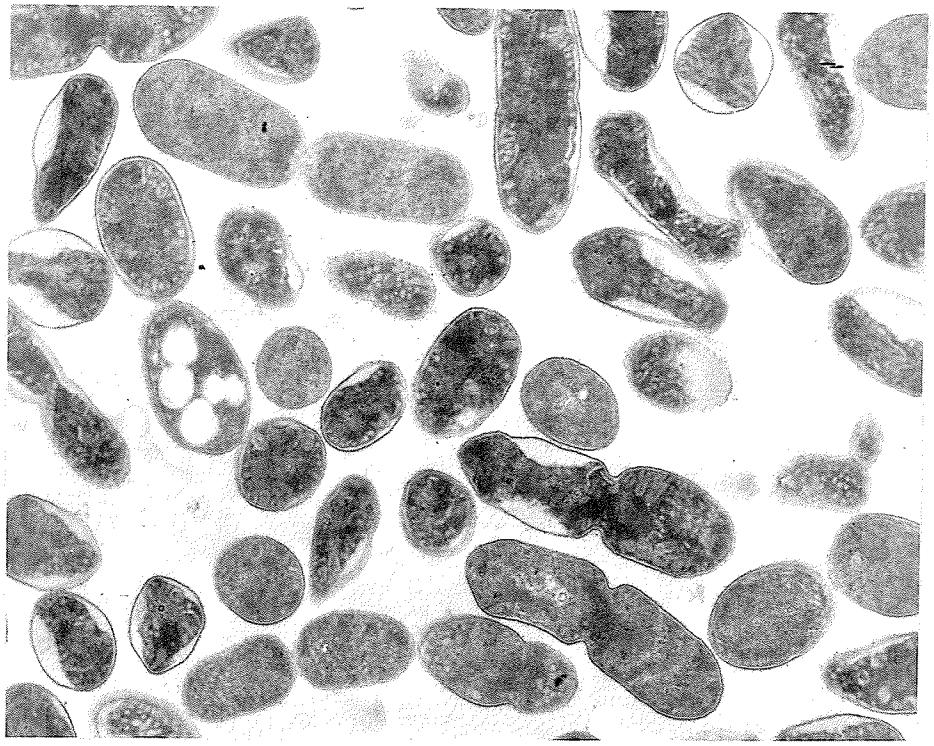


Plate 7. A. vinelandii from a carbon-limited chemostat culture with a mannitol supply rate of 0.280 grams per hour, D.O.T. of 3 μM.

x 12,600

Plate 8. A. vinelandii from a carbon-limited chemostat culture with a mannitol supply rate of 0.293 grams per hour, D.O.T. of 18 μM.

x 12,600



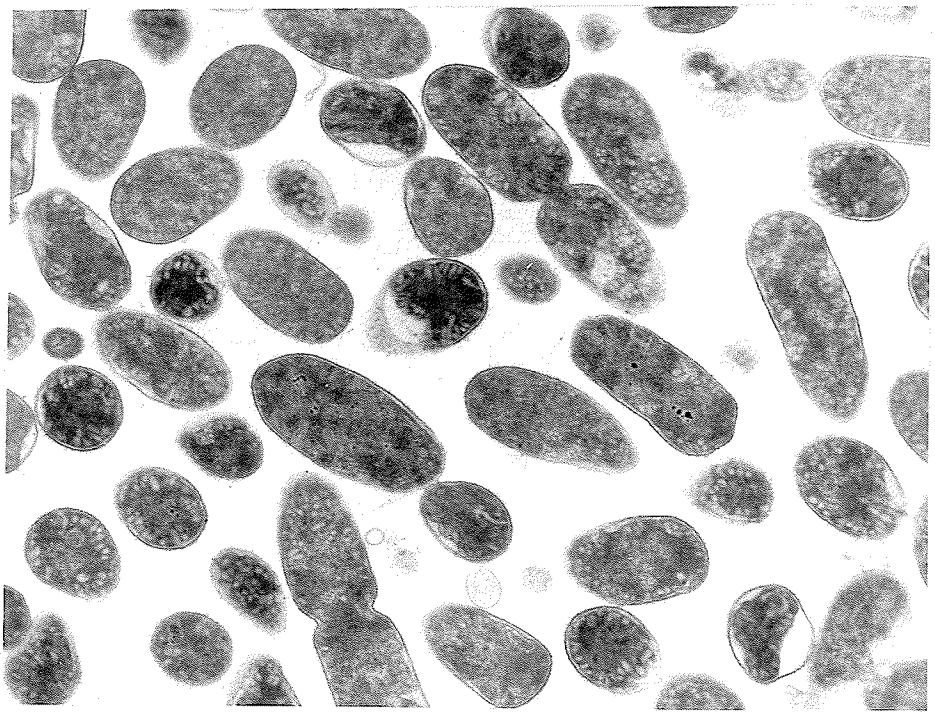
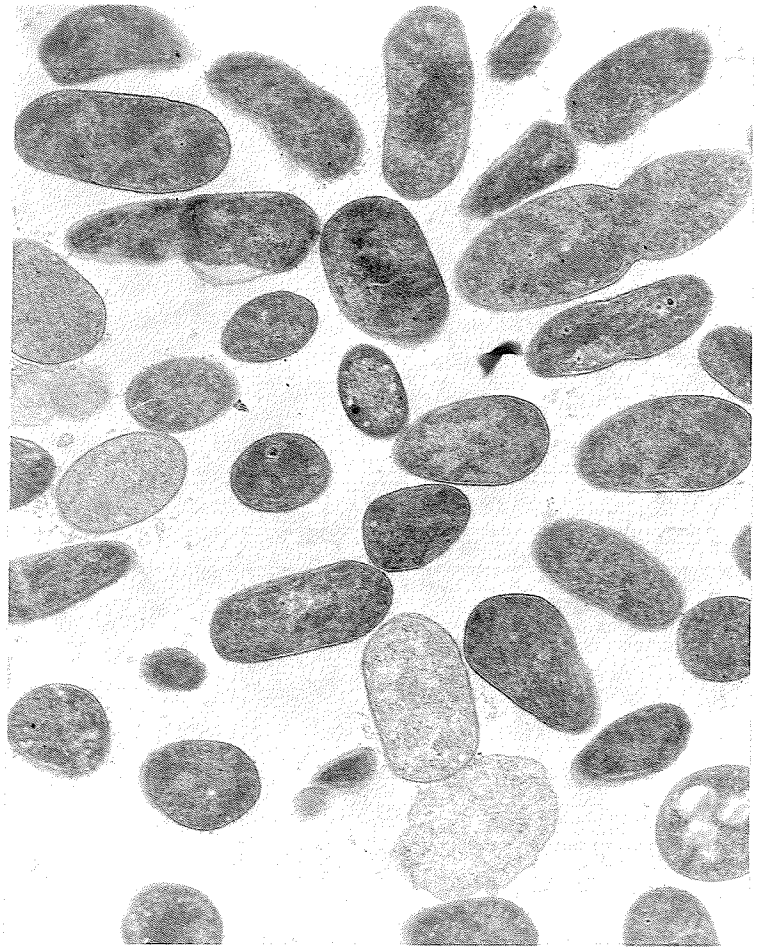


Plate 9. A. vinelandii from an oxygen-limited chemostat culture with a mannitol supply rate of 0.330 grams per hour, D.O.T. of zero.

x 12,600



Leaf blank to correct
numbering

The organelle did appear to be able to affect the cell wall when in close proximity. Plate 10 shows how the cell wall in one cell appears to have disintegrated at the point of contact with the organelle. The light periphery of the organelle has disappeared at this point of disintegration and it would appear that the interior of the organelle is in contact with the medium. A similar type of phenomenon is shown in Plate 11, whereby the organelle appears to be connected to the medium by a channel.

Cytoplasmic division

In order to provide equal opportunity to both daughter cells, the division of cytoplasm should be equal between the two newly formed cells. If this does not occur one cell may be placed at a disadvantage whereby it would be required to produce much more cytoplasmic material before it too can divide.

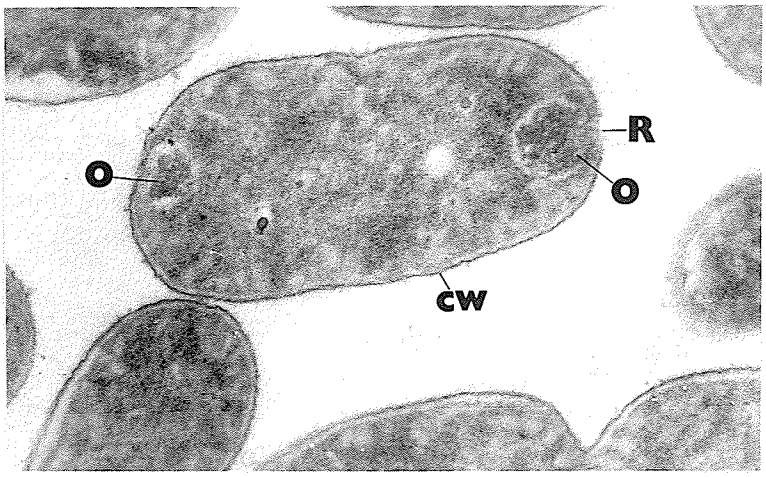
Normally in a cell which has the volume, contained by the cell wall, filled to capacity with cytoplasm, the separation into equal cytoplasmic volumes would not pose a problem. But A. vinelandii grown under conditions of high d.o.t. have greatly increased volumes in the periplasmic space similar to cells in a state of plasmolysis. With a great deal of volume within the cell wall being essentially empty, cell division by the pinching off by the cell wall may produce an unequal sharing of cytoplasm between cells. To take an extreme example cell division could occur with all the cytoplasm in one daughter cell, the other being an empty cell. Several observations lead to a possible mechanism whereby Azotobacter would overcome the problem. The separation of cytoplasm membrane from cell wall occurs generally in the mid section of the cell (Plate 12). The cytoplasm rarely separates at the terminals indicating a tighter binding at these points. This would provide a

Plate 10. Rupturing of the cell wall in A. vinelandii from a carbon-limited chemostat culture with a mannitol supply rate of 0.268 grams per hour, D.O.T. of 25 μM. Cell wall is indicated by (CW), area of rupture by (R), the organelles (O).

x 31,500

Plate 11. A. vinelandii cell from same chemostat described in plate 10, showing desintegration of cell wall at (R), with apparent channel formation (C), organelle (O).

x 31,500


R
O
O
CW

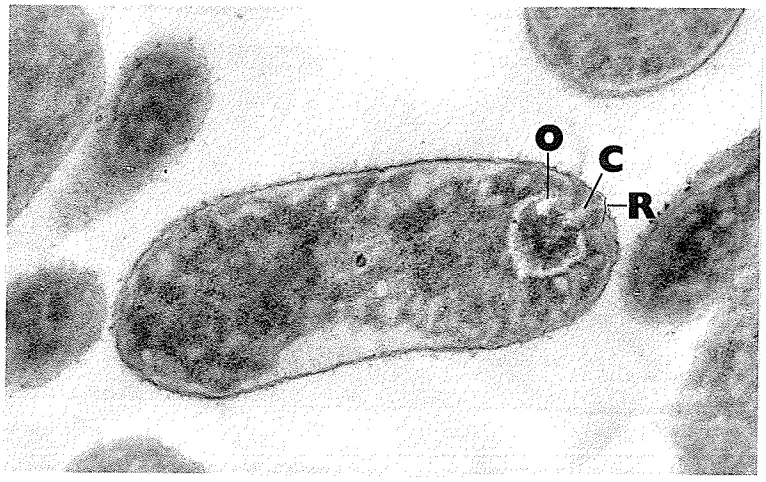
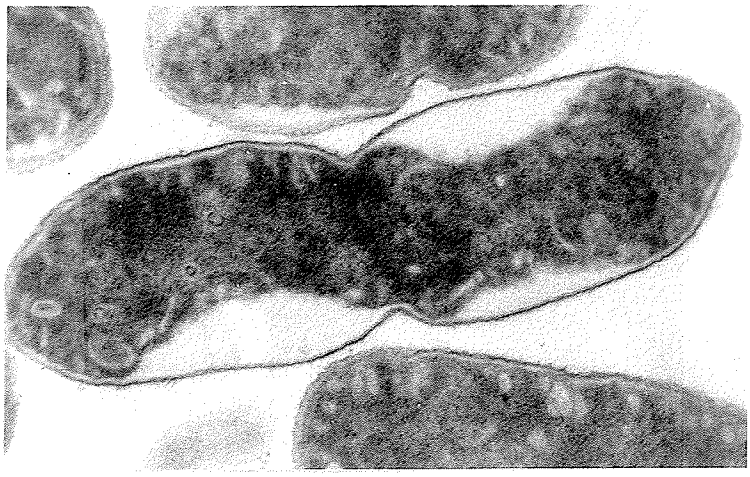

O
C
R

Plate 12. Longitudinal-section of carbon-starved A. vinelandii dividing cell demonstrating separation of the cell wall from cell membrane. Mannitol supply rate was 0.280 grams per hour, D.O.T. of 3 μM.

x 31,500

Plate 13. Cross-section of carbon starved A. vinelandii cell demonstrating triangular binding of cell membrane to the cell wall at sites indicated as (B). Mannitol supply rate was 0.280 grams per hour, D.O.T. of 3 μM.

x 31,500



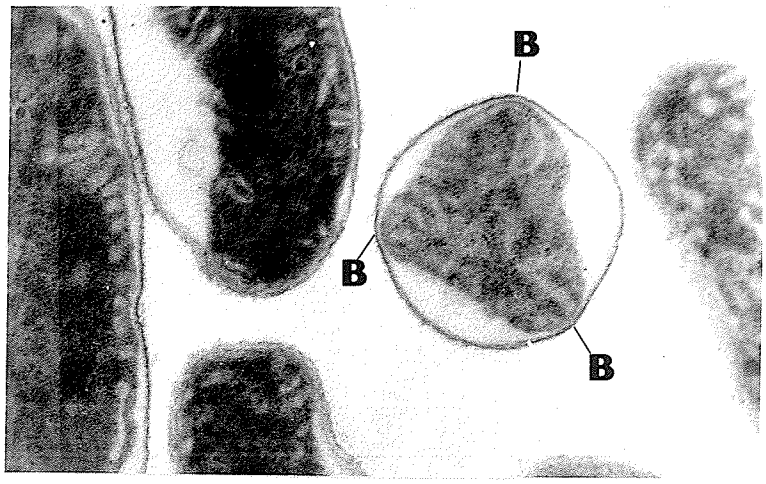
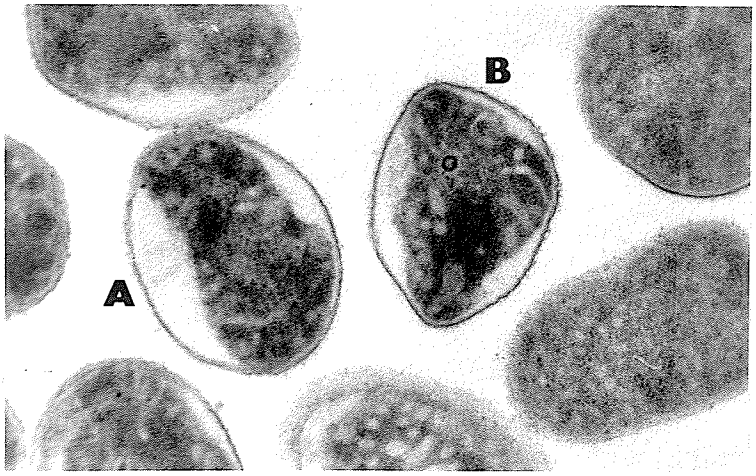
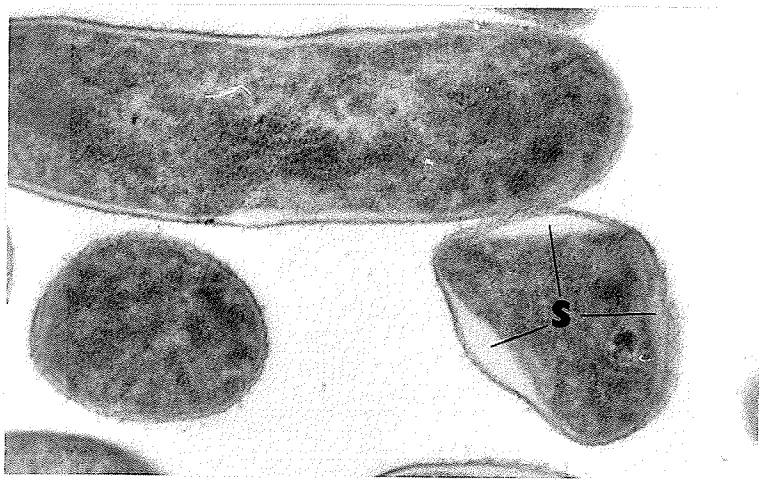

B
B
B

Plate 14. Cross-section of carbon starved A. vinelandii cell, cell A showing cell membrane separating from the cell wall on only two sides, whereas cell B shows separation at three sides. Condition for growth same as described in Plate 13.

Plate 15. Cross section of a carbon starved A. vinelandii cell demonstrating cell wall-cell membrane separation at three sides. Mannitol supply rate was 0.243 grams per hour, D.O.T. of 29 μM.

x 31,500


B
A


S

means whereby the growing cell wall would draw out cytoplasm between two daughter cells. Along with tight, terminal binding three lines of logitudinal binding may also occur between the cell membrane and cell wall. The resultant triangular cross sections can be seen in Plates 13, 14 and 15.

Role of the granular inclusion bodies

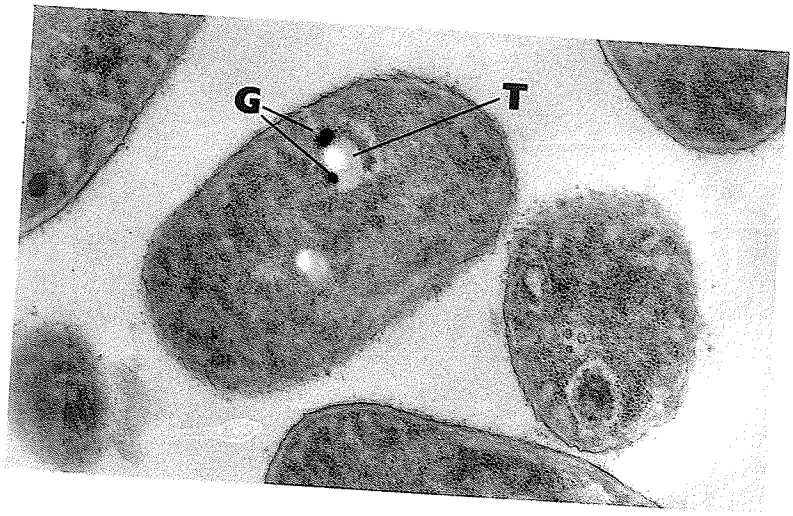
The apparent intimate nature of the granular inclusion (GI) particles and the cytoplasmic organelles suggest a functional relationship between the two. The GI particles were observed in cells prior to the appearance of the organelle and since GI particles appear in apparently immature organelles it is tempting to speculate that the organelle is produced from material within the GI particle although evidence is as yet insufficient. Plates 16 and 17 show the formation of a light zone within the cytoplasm possibly formed by the granular inclusion bodies. In plates 16, 18 and 19, the GI particle appears on the interior edge of an electron transparent zone with the possible concurrent unwinding of electron dense material (Plate 19, cell A). The electron transparent area may be poly-β-hydroxybutyrate (Leung 1979) however the carbon limited nature of growth makes this unlikely. The unwinding of the material appears to be more complete in the

Plate 16. Formation of electron transparent zone (T) within the cytoplasm of A. vinelandii from a carbon-limited chemostat. Mannitol supply rate was 0.263 grams per hour, D.O.T. of 25 μM. Granular inclusion particles (G).

x 31,500

Plate 17. Cross-section of a carbon-starved A. vinelandii cell demonstrating involvement of the G.I. particles (G) in formation of two cytoplasmic organelles. Mannitol supply rate was 0.255 grams per hour, D.O.T. of 32 μM.

x 31,500


G
T

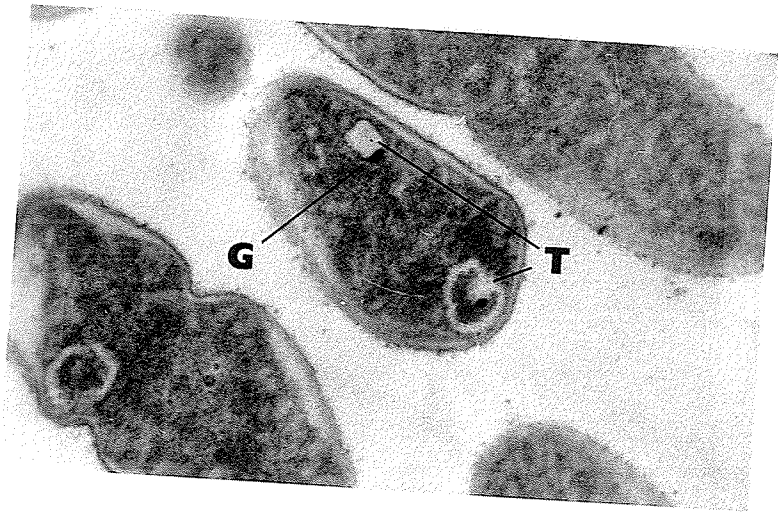
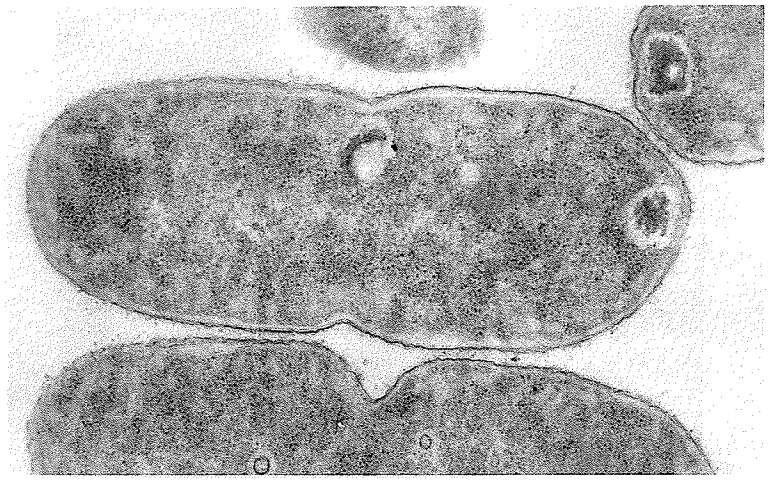

G
T

Plate 18. Longitudinal section of a carbon-starved A. vinelandii cell demonstrating involvement of the G.I. particles in formation of the cytoplasmic organelle. Mannitol supply rate was 0.263 grams per hour, D.O.T. of 25 µM.

x 31,500

Plate 19. A. vinelandii cells from a mannitol-limited chemostat culture, demonstrating in cell A apparent unravelling of the G.I. particle during formation of the cytoplasmic organelle. In cell B at least three G.I. particles are involved at an electron transparent zone. Mannitol supply rate was 0.243 grams per hour, D.O.T. of 29 µM.

x 31,500



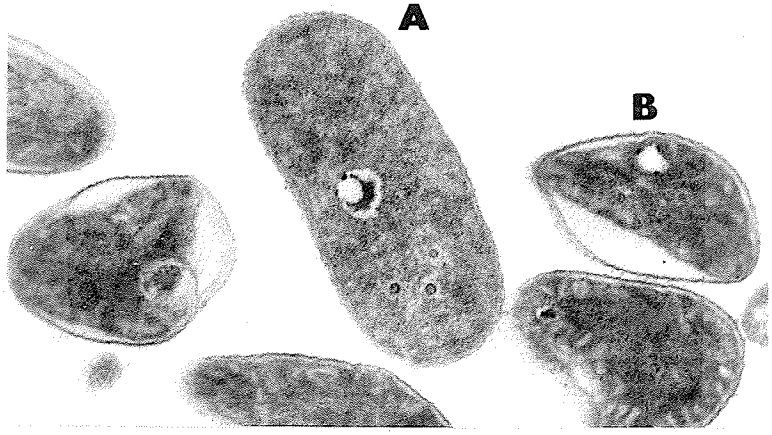
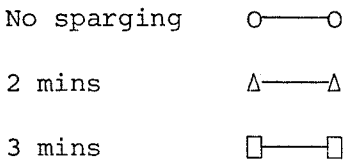

A
B

Fig. 33. Variation in the rate of oxygen uptake, at different dissolved oxygen concentrations, and the effect of sparging with pure oxygen for various times on cells from an oxygen-limited-chemostat culture.



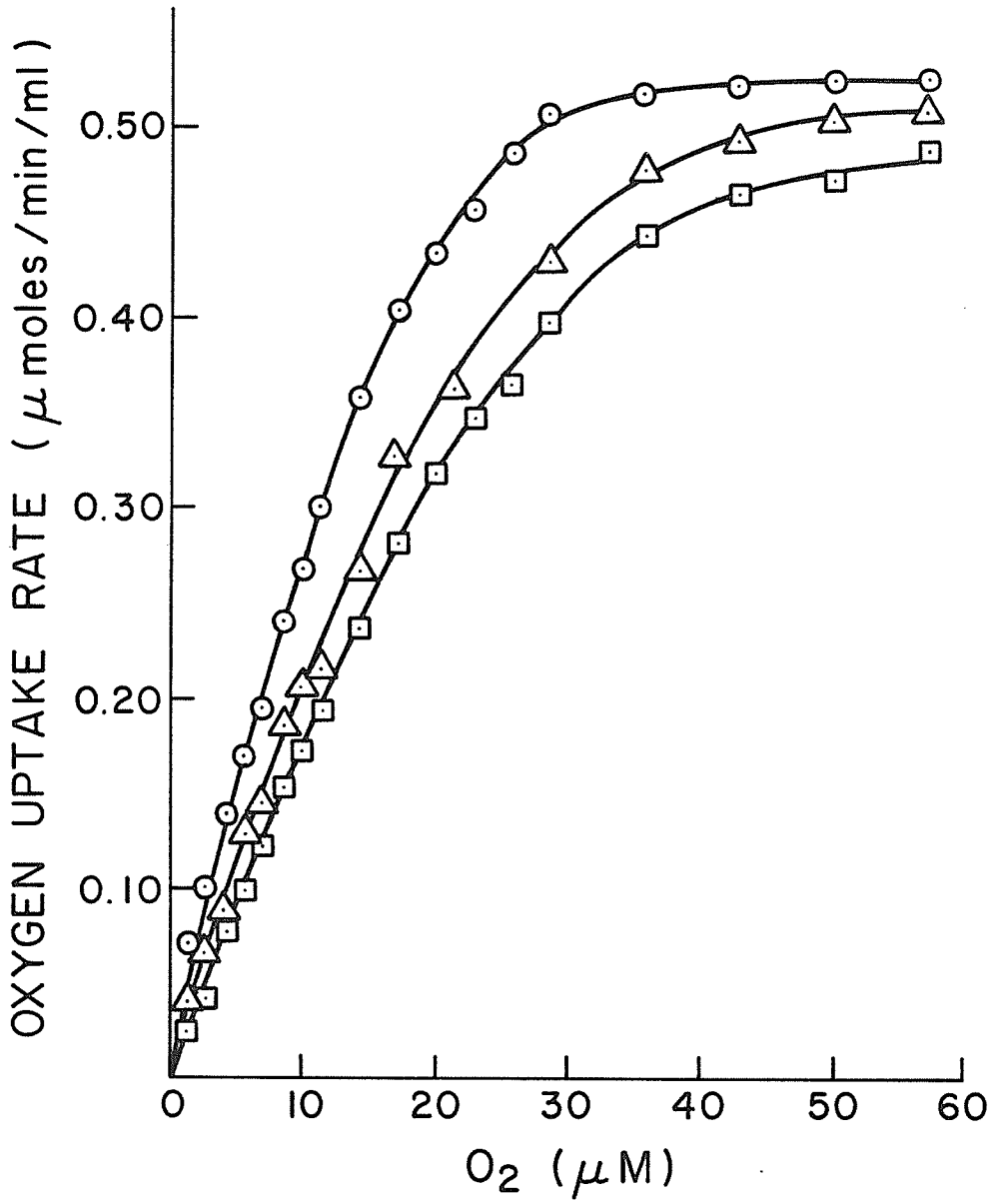
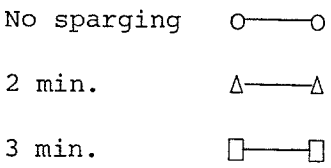
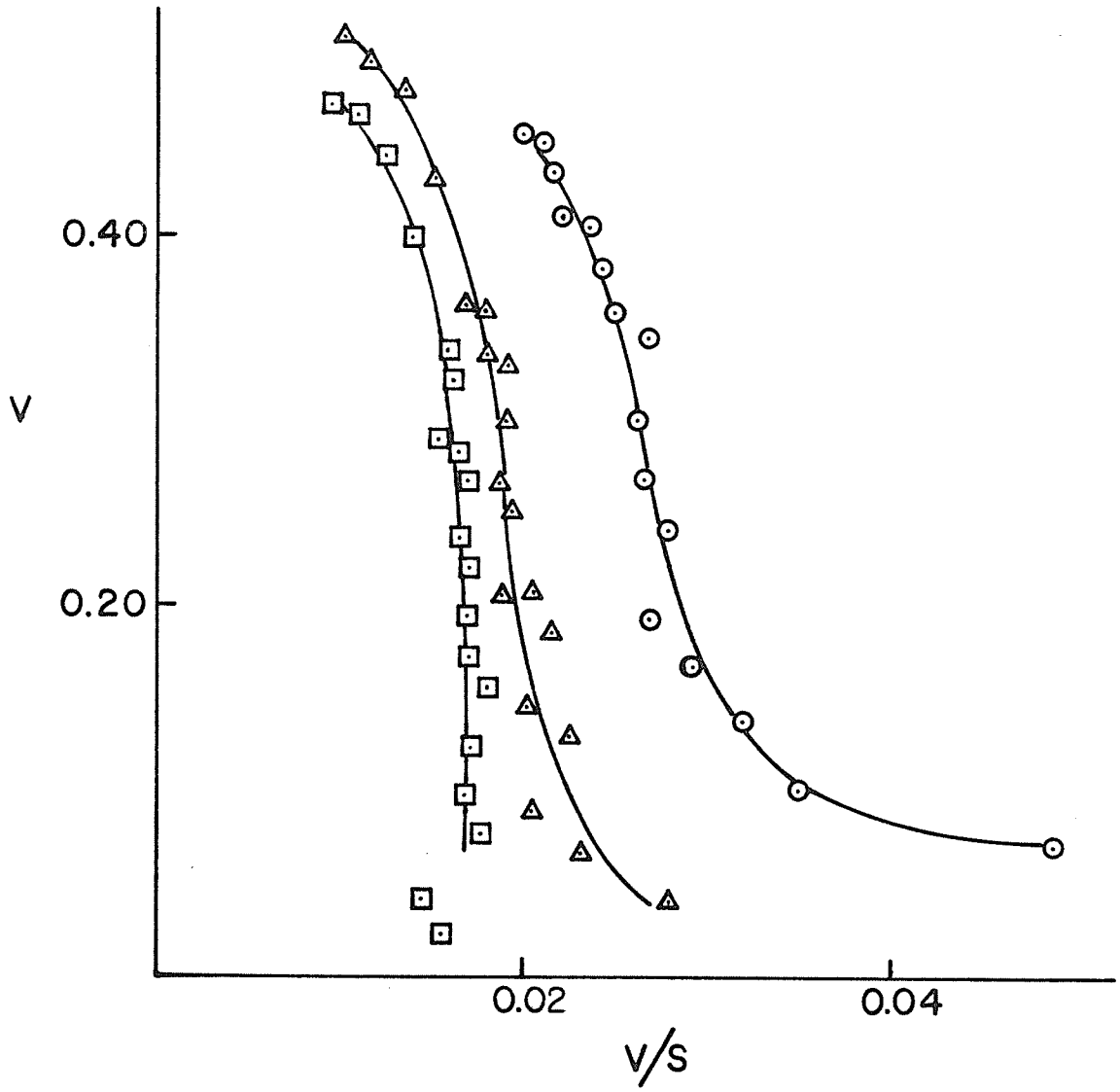

OXYGEN UPTAKE RATE (μ moles/min/ml)
0.50
0.40
0.30
0.20
0.10
0
10
20
30
40
50
60
O2 (μM)

Fig. 34. Eadie-Hofstee plot of the data presented in Fig. 33.




0.40
0.20
V
0.02
0.04
V/S

lower organelle of the cell indicated in Plate 17. Plate 18 shows completed organelles in which the granular inclusion body appears to have essentially disappeared, possibly having completely unwound, filling the organelle with electron dense material.

2.3 Effects of 100% oxygen upon the oxygen uptake rate of A. vinelandii

Among the effects noted for A. vinelandii in extreme carbon limitation with excess oxygen present in the medium was a reduced affinity of the cytochrome oxidase for oxygen. This was reflected in higher apparent $[S]_{0.5}$ values when the rate of oxygen uptake was determined by measuring dissolved oxygen tension in solution. Oxygen limited cells from a chemostat also exhibited this reduction in affinity for oxygen after sparging with pure oxygen. Fig. 33 shows how the rate of oxygen uptake depends upon the dissolved oxygen tension, and how the affinity for the oxygen decreased after two and three min of sparging with oxygen. Rates were determined by drawing tangents at specific dissolved oxygen tensions as the d.o.t. was reduced by respiration. These results were then expressed in an Eadie-Hofstee plot shown in Fig. 34. From this plot at least two cytochrome oxidases appear to be operative in cells which have not been exposed to pure oxygen. One cytochrome oxidase appears to be operative at low oxygen tension and has a low V_{max} and Km. The second oxidase has a higher V_{max} and Km. The exposure to excess oxygen for two min results in the reduction of the activity of the low Km and V_{max} oxidase. A three min oxygen treatment resulted in complete suppression of this oxidase.

Oxygen-limited mannitol or acetate oxidizing cells from batch cultures do not exhibit this loss of affinity for oxygen after sparging with pure oxygen for 10 min. It appears that the cytochrome oxidase responsible for reduction of oxygen at very low pO_2 is inhibited by pure oxygen. However inherent limitations in the measurement of rate and d.o.t. at such low oxygen concentrations must be taken into account, therefore it would be only reasonable to conclude that excess oxygen rapidly changes the cytochrome oxidases with a resultant apparent loss of affinity for oxygen.

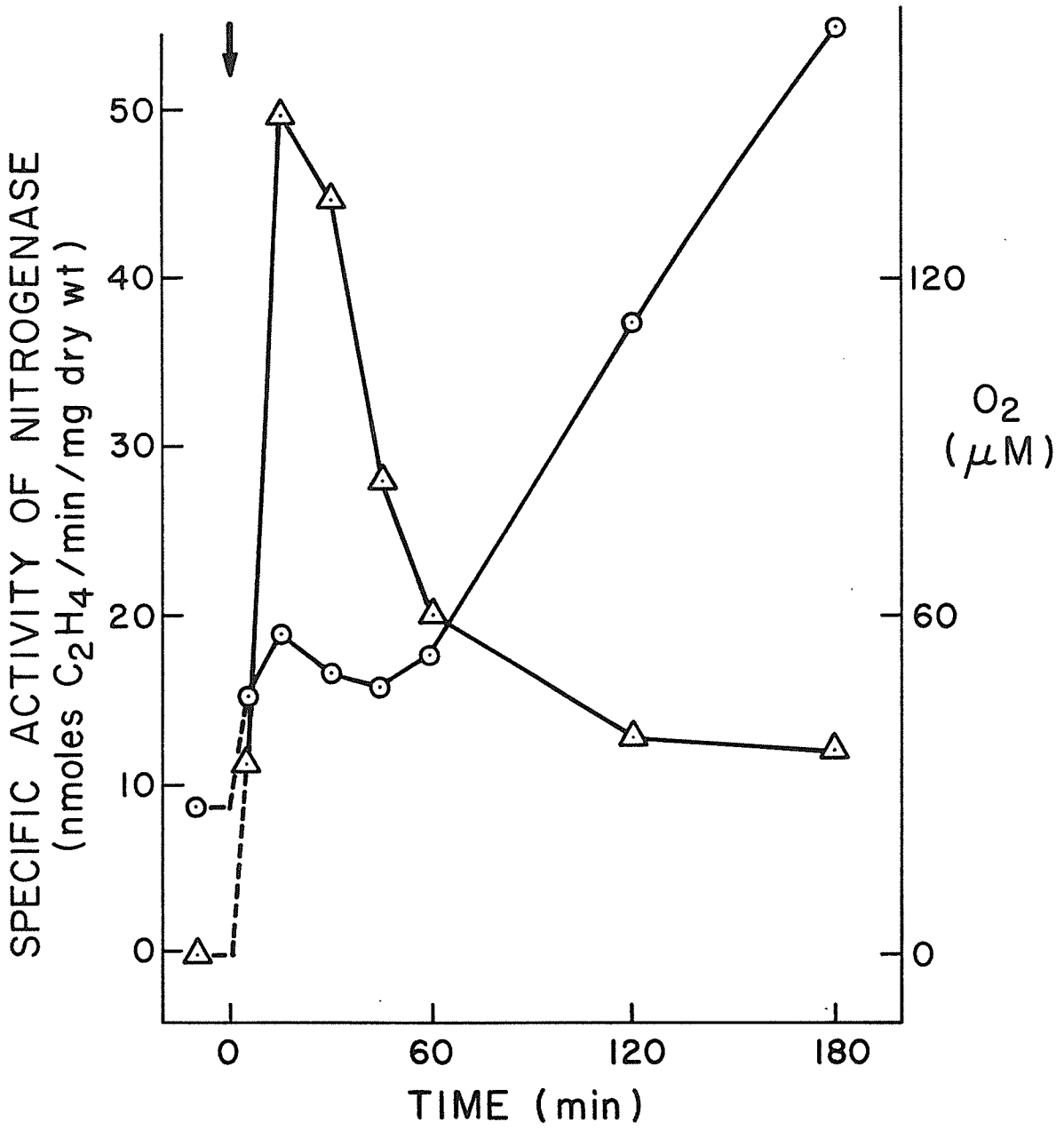
2.4 Effects of High Oxygen Tension on the Nitrogenase Activity of A. chroococum.

The effects of oxygen on nitrogenase have been reported as producing complete loss of activity in purified nitrogenase preparations (Bulen and LeComte 1966). Dalton and Postgate (1969) have also explained the loss of viability of A. chroococum placed in a medium with high d.o.t., as being due to the toxic effects of oxygen on the nitrogenase enzyme. Contrary to these results, Buchanan (1978) demonstrated an increase in nitrogenase activity after a chemostat culture of A. chroococum has been exposed to high partial pressures of oxygen in the gas phase. I repeated the work of Buchanan using the same organism in a chemostat under an initial atmosphere of 10% O_2 which was subsequently increased to 30%. With the increase to 30% O_2, nitrogenase activity rose rapidly and stabilized for approximately one hour. Simultaneously within the first 15 min of this period, dissolved oxygen tension increased sharply,(Fig. 35). Commensurate with this rapid rise in d.o.t., the respiration rate of the culture increased sharply about 30 min after the atmospheric O_2 level had been

Fig. 35. Variation in the dissolved oxygen concentration and nitrogenase activity of A. chroococcum with time after increasing oxygen concentration from 10 to 30% in the gas phase above a chemostat culture. $D = 0.20\ hr^{-1}$.

Nitrogenase activity o———o

Dissolved oxygen concentration Δ———Δ


SPECIFIC ACTIVITY OF NITROGENASE
(nmoles C_2H_4/min/mg dry wt)
50
40
30
20
10
0
120
60
0
O_2
(μM)
0
60
120
180
TIME (min)

adjusted to 30%. The increase in respiration produced a rapid drop in d.o.t. of about 80 μM within a further 30 min period. During the period of high oxygen uptake nitrogenase activity remained constant, but then increased rapidly after the respiration rate had stabilized reaching an activity five-fold greater than that observed before the oxygen tension was increased. This experiment was repeated, with similar results but one such attempt to demonstrate this increase in nitrogenase activity with an increase in atmospheric oxygen was not successful. The culture, under identical conditions to those described above, was apparently unable to decrease the oxygen tension of the medium once it had been raised. Under these conditions the nitrogenase activity did not increase; rather, the activity diminished and the culture washed out.

In the first case, it appears that the nitrogenase activity was not inhibited when excess oxygen was present in the culture. On the contrary these cells appeared to exhibit greater nitrogenase activity after continued exposure to high dissolved oxygen tensions. Since the activity of nitrogenase increases only after the respiration rate has stabilized, the plateau of nitrogenase activity occurring within the first hour after the atmospheric oxygen concentration had been increased may be due to competition for reducing power between the terminal oxidase and biosynthetic processes.

The loss of nitrogenase activity in cells from a culture which is unable to remove excess oxygen may be used as evidence for the inactivation of nitrogenase by oxygen. But that high nitrogenase activity in cells capable of increasing respiration, promotes the hypothesis that the loss of activity may be due to the above mentioned

competition. The main requirement for cells to increase respirative rates to adapt to high oxygen solution rates (and increased biosynthesis) would be the capability to rapidly increase the carbon substrate uptake rate. If the cell is unable to increase this uptake rate, the respiration rate will increase only at the expense of intracellular carbon reserves and biosynthesis, and thus because of the lack of sufficient readily oxidizable carbon substrate the cell may be unable to reduce the dissolved oxygen tension significantly. In the presence of excess oxygen biosynthesis would continue to lose reducing power to respiration with eventual washout of the culture. A prime factor which may determine the electron flow, whether to respiration or nitrogenase may be the binding of the Shethna protein (Fe-S protein II). This protein has been shown to render the oxygen sensitive purified nitrogenase oxygen-tolerant (Robson 1979), but upon binding the enzyme cannot fix N_2. Nitrogenase activity is reinitiated after sufficient electron flow is restored, with concurrent dissociation of the Shethna protein-nitrogenase complex (Brill 1980).

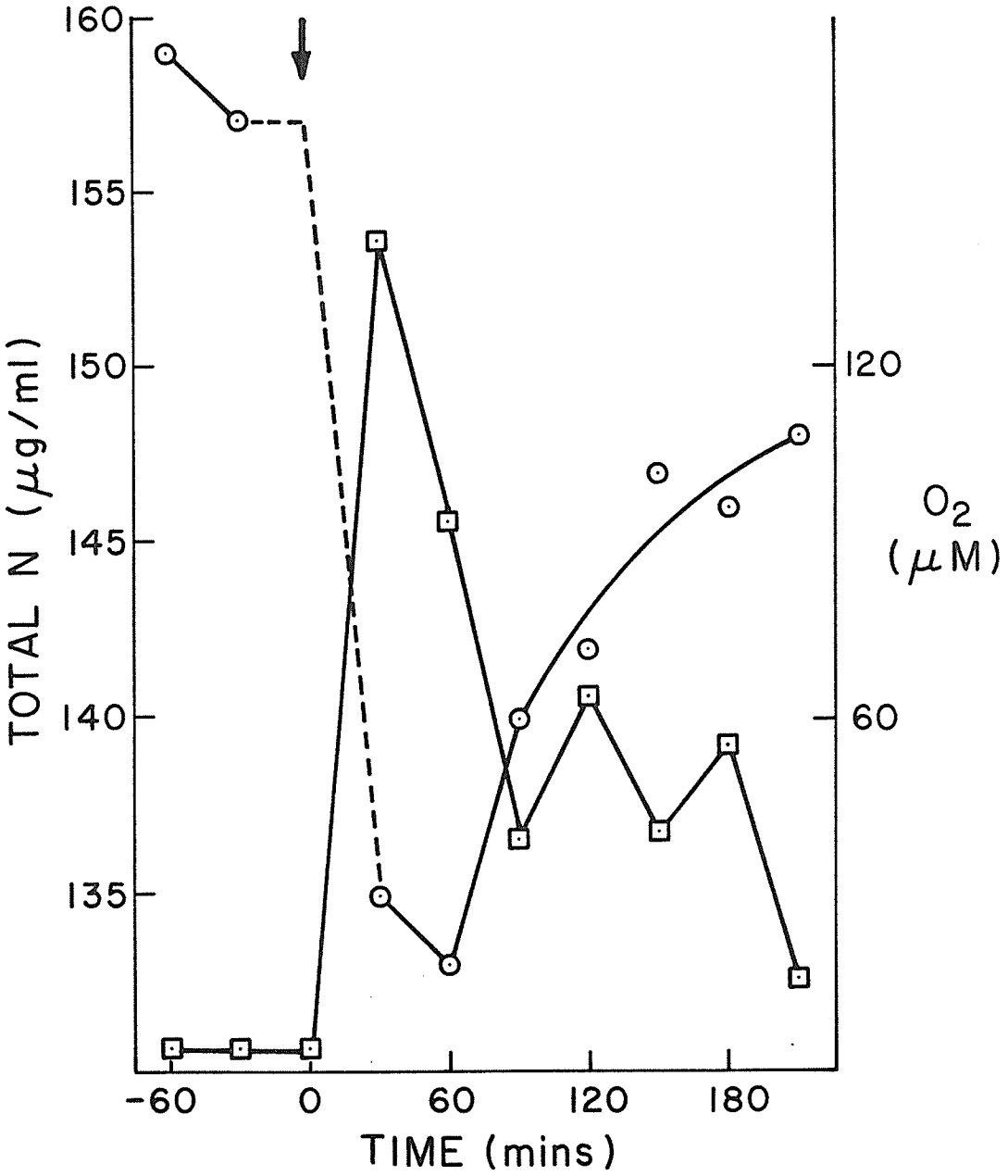
The effect of excess oxygen on nitrogenase was assayed by measuring the acetylene reduction capability of these cells. Unfortunately this assay requires that the sample be removed from the chemostat and the activity is determined under conditions of much lower aeration rates. To determine the effects of an increase in oxygen from ten to thirty percent on the nitrogenase activity *in situ*, samples were taken at various time intervals and assayed for total nitrogen. Fig. 36 illustrates how the nitrogenase activity in the chemostat appears to be completely inhibited within the first half-

Fig. 36. Variation in the total nitrogen concentration and the dissolved oxygen concentration with time after increasing the oxygen concentration from 10 to 30% in the gas phase above a chemostat culture of A. chroococcum. D = 0.20 hr^{-1}. Arrow indicates time of change in atmospheric oxygen concentration.

Total Nitrogen ○——○

Dissolved oxygen concentration □——□

Dashed line indicates transition period


160
155
150
145
140
135
TOTAL N (μg/ml)
120
60
O2
(μM)
-60
0
60
120
180
TIME (mins)

hour after an increase in oxygen tension. The amount of total nitrogen appears to decrease as would occur if the nitrogen was being simply washed out of the culture, no additional fixed nitrogen appears in the culture until the respiration rate stabilizes as indicated by the drop in d.o.t. This absolute loss of nitrogenase activity during conditions when the culture is exposed to high O_2 tensions was not expected since the acetylene reduction capabilities of cells under similar conditions was fairly high. Therefore it appears that a competition for reducing power does occur in these cells in situ, the direction of electron flow depending upon the presence and quantity of excess oxygen.

2.5 Use of mannitol-limited cultures for substrate oxidation studies.

Under conditions of excess dissolved oxygen brought about by carbon-limitation, the rate of oxygen uptake in situ is dependent on the rate of carbon uptake (Haaker and Veeger 1976). If a new carbon source (i.e. one to which the cell has not been previously exposed) is provided in excess after the supply of the initial substrate has been terminated, the rate of oxygen uptake will therefore depend on either the rate of substrate uptake or on the rate of catabolism. A vortex-stirred carbon limited chemostat is ideal for studies of uptake rates of intermediates already involved in catabolism and would be useful for studies of adaptation to a foreign carbon source. Such a system has several advantages: first, the carbon starved nature of the culture encourages a rapid uptake of extracellular carbon sources when the inflow of medium carbon has been stopped, secondly, very little intracellular carbon resources would be

expected to be present because of the prior carbon limitation; thirdly, an easily measurable parameter, the dissolved oxygen tension, is used to monitor the respiration rate and fourthly, the rate of respiration can be determined over a long period of time to permit studies of the adaptation of the cells to a foreign carbon source.

The response of carbon-limited Azotobacter vinelandii respiration to a pulse of individual carbon substrates was determined. The culture was grown at a low dilution rate of 0.11 hr^{-1} with 1% mannitol in B_6 medium. During incubation the culture became carbon limited to an extreme degree. The low rate of carbon supply limited respiration rates to below the oxygen solution rate with a resultant high d.o.t. To remove all excess mannitol the flow of medium to the culture was stopped, within seconds the d.o.t. began to rapidly increase indicating essentially a zero concentration of mannitol in the medium. When the d.o.t. started to increase a pulse of the new carbon source was added to give a final concentration of 0.5% substrate.

2.5.1 Change in respiration accompanying the addition of new carbon sources.

a) Glucose

The change in dissolved oxygen tension after the addition of glucose to a final concentration of 0.5% is shown in Fig. 37. The time between the addition of glucose and the commencement of respiration is approximately ten minutes.

To determine the rate of respiration, the rate of oxygen solution into a culture must be determined. This latter rate is expressed by the equation

$$R_S = K_L a\ (C_s - C) \qquad \text{(Pirt 1975)} \qquad (1)$$

where

R_s = oxygen solution rate of the culture

$K_L a$ = oxygen transfer coefficient

C_s = oxygen partial pressure in gas phase

C = dissolved oxygen tension.

If a culture is consuming oxygen at a constant rate xq_{O_2}, the rate of oxygen supply minus the rate of oxygen consumption will equal the rate of change in the dissolved oxygen tension.

$$\frac{dc}{dt} = R_s - xq_{O_2} \qquad (2)$$

$$= K_L a(C_s - C) - xq_{O_2} \qquad (3)$$

Plotting $\frac{dc}{dt}$ against $(C_s - C)$ will produce a straight line of slope $K_L a$ and y intercept of $-xq_{O_2}$. This relationship is shown for the culture after addition of glucose in Fig. 38,. Since the consumption of O_2 by glucose-oxidizing cells (xq_{O_2}) is a constant 141.6 µM/hr the oxygen transfer coefficient can be determined to be 0.590 hr^{-1}.

An interesting point to be made here is that even in the presence of excess oxygen the rate of change in the dissolved oxygen tension extrapolates to zero, when the d.o.t. is zero (i.e. $C_S - C$ = 240 µM). This method of determining the oxygen solution rates and the oxygen consumption rates of a culture of A. vinelandii is much more convenient than by either steady state kinetics or non-steady state kinetics described by Pirt, 1975. Steady state kinetics (see historical section 9.1) requires separate determination of xq_{O_2}, the value of which may not represent the actual "in situ" rate . Non-steady

state kinetics, which is similar to the described method, measures the rate of decline of the oxygen concentration in the culture when the oxygen supply is stopped, i.e. $K_L a(C_s - C)$ is presumed to equal zero and $\frac{dc}{dt} = xq_{o_2}$. When the oxygen supply is resumed the increase in d.o.t. is presumed to be linear and the slope of C versus time is equal to $\frac{dc}{dt}$.

Rewriting equation (3)

$$c = \frac{-1}{K_L a} \left(\frac{dc}{dt} + q_{o_2} x\right) + c_s$$

Plotting c versus $\left(\frac{dc}{dt} + q_{o_2}\right)$ will have a slope $-\frac{1}{K_L a}$.

Pirt (1975) had presumed the rate of change in d.o.t. would be constant and c versus time would equal $\frac{dc}{dt}$, and so suggested varying the term $\left(\frac{dc}{dt} + q_{o_2} x\right)$ by varying either biomass value or the partial pressure of oxygen in the gas phase. But this is not correct since the original presumption that $\frac{dc}{dt}$ would be constant is contradictory to the equation (3), that is to say $\frac{dc}{dt}$ would decrease as the d.o.t. increased. The change in $\frac{dc}{dt}$ could therefore be used to determine the constants at specific oxygen concentrations without having to change biomass or oxygen concentration.

This last method has other basic assumptions. The first one being that the rate of decrease in d.o.t. when the O_2 supply is stopped is strictly dependent upon microbial respiration. The rate of diffusion of oxygen from a culture to an anaerobic gas is ignored, but should also be taken into account for determination of xq_{o_2}. This is especially important if working with either a vortex-stirred culture or bacterial culture with low oxygen consumption rates. Secondly it is assumed that an immediate change occurs in the atmosphere above a culture when the oxygen supply is either stopped or recommenced.

Fig. 37. Variation in the dissolved oxygen concentration with time after addition of glucose to a mannitol limited culture. The mannitol supply was stopped upon addition of glucose at zero hour. Curve shown is a recorder tracing.

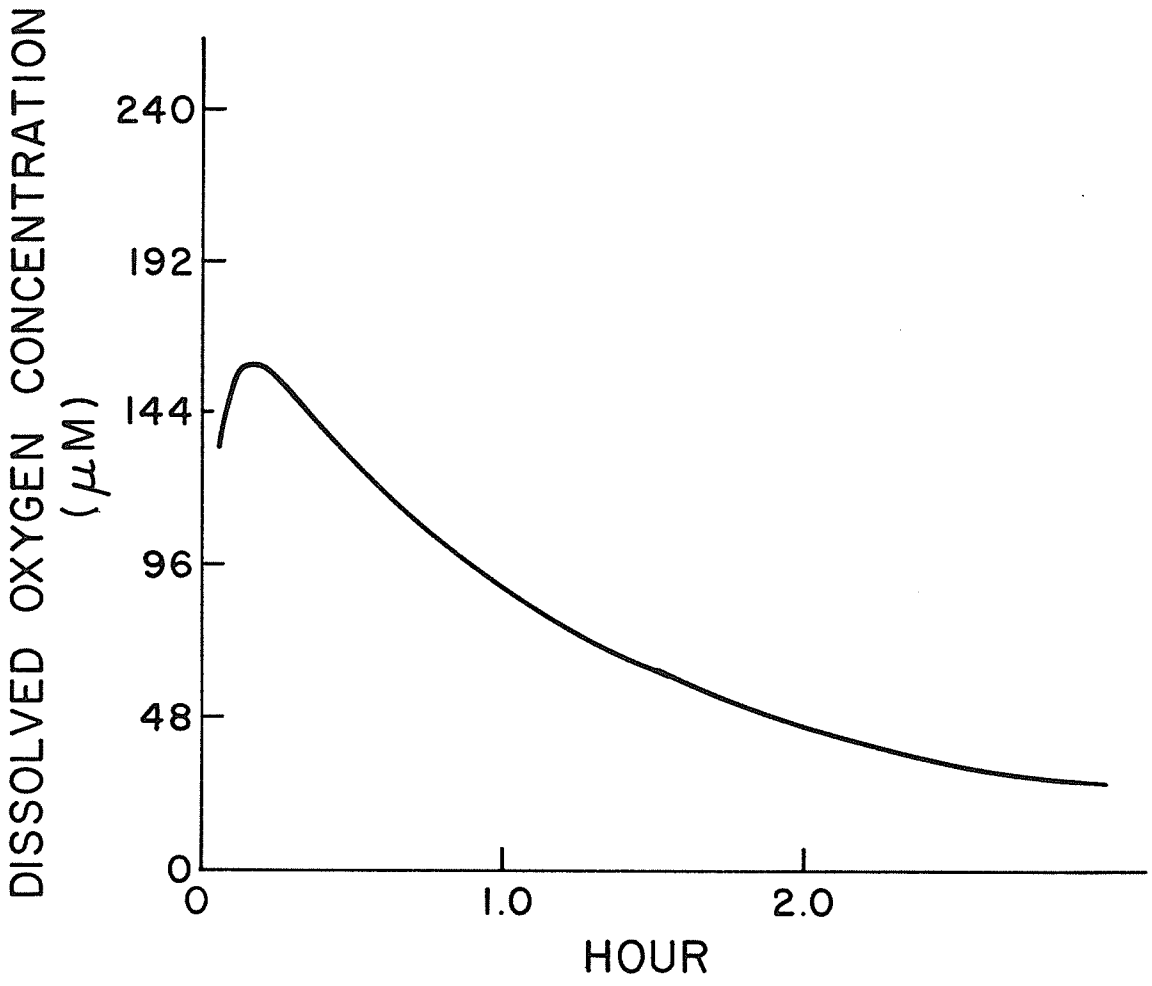
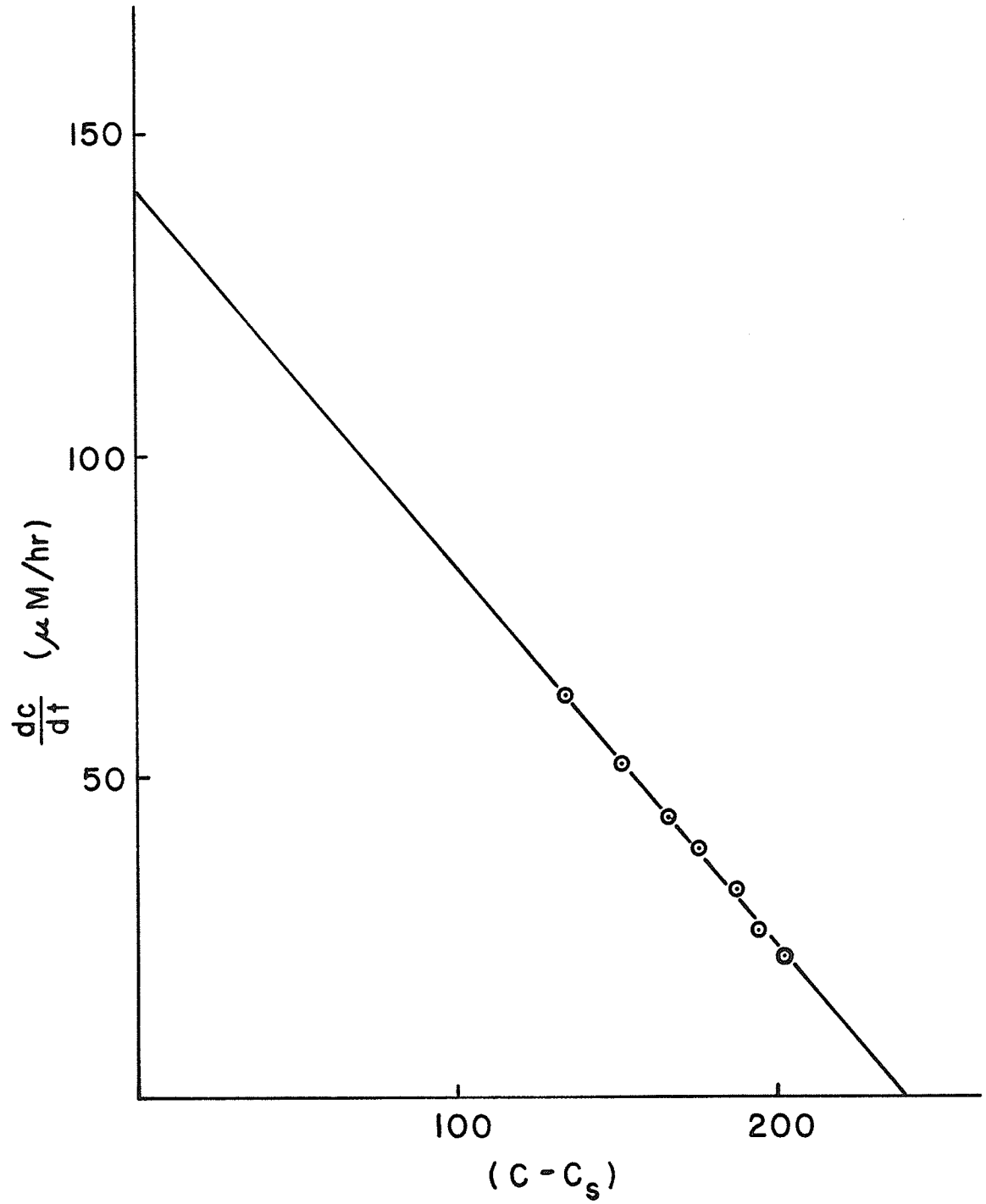

DISSOLVED OXYGEN CONCENTRATION
(μM)
240
192
144
96
48
0
0
1.0
2.0
HOUR

Fig. 38. Plot of the rate of change in dissolved oxygen concentration $(\frac{dc}{dt})$ versus the difference between oxygen concentration at the gas liquid interface and the dissolved oxygen concentration $(C-C_s)$.


150
100
50
$\frac{dc}{dt}$ (μM/hr)
100
200
(C - C_s)

Unless the atmosphere is very rapidly replaced, residual oxygen may influence the apparent oxygen uptake rate of the culture. Both the steady state kinetics and the non-steady state kinetics method described by Pirt (1975), require separate determination of xq_{O_2}. Utilizing the method described in this text does not require separate measurements of xq_{O_2} to determine $K_L a$, rather the value of both may be determined "in situ" under identical conditions.

b) Malate

The change in d.o.t. after the addition of malate to carbon-starved cells is shown in Fig. 39. Unlike glucose the oxidation rate is not constant. Rewriting the equation (3)

$$xq_{O_2} = K_L a(C_s - C) - \frac{dc}{dt} \qquad (4)$$

$K_L a$ was assumed to be identical to what it had been for the addition of glucose. Constitutive levels of malate translocase appear to be very low, i.e. capable of supporting respiration of approximately 18 μM O_2/hr. The rate of malate oxidation increases linearly with time after the addition of malate (Fig. 40). When the d.o.t. has been reduced (at approximately 1.5 hours) the rate of malate oxidation decreases to a rate similar to glucose oxidation.

c) Acetate

The change in the dissolved oxygen tension after addition of acetate is shown in Fig. 41. The enzymes required by A. vinelandii to use acetate as a carbon source appear to be constitutive. The rate of increase in dissolved oxygen is initially high after addition of acetate. The respiration then appears to increase for a short period of time, (ie. from 4 to 7 minutes), followed by a period of

Fig. 39 Variation in dissolved oxygen concentration with time after addition of malate to a mannitol limited chemostat culture. Mannitol supply was stopped and malate was added at time indicated by arrow.

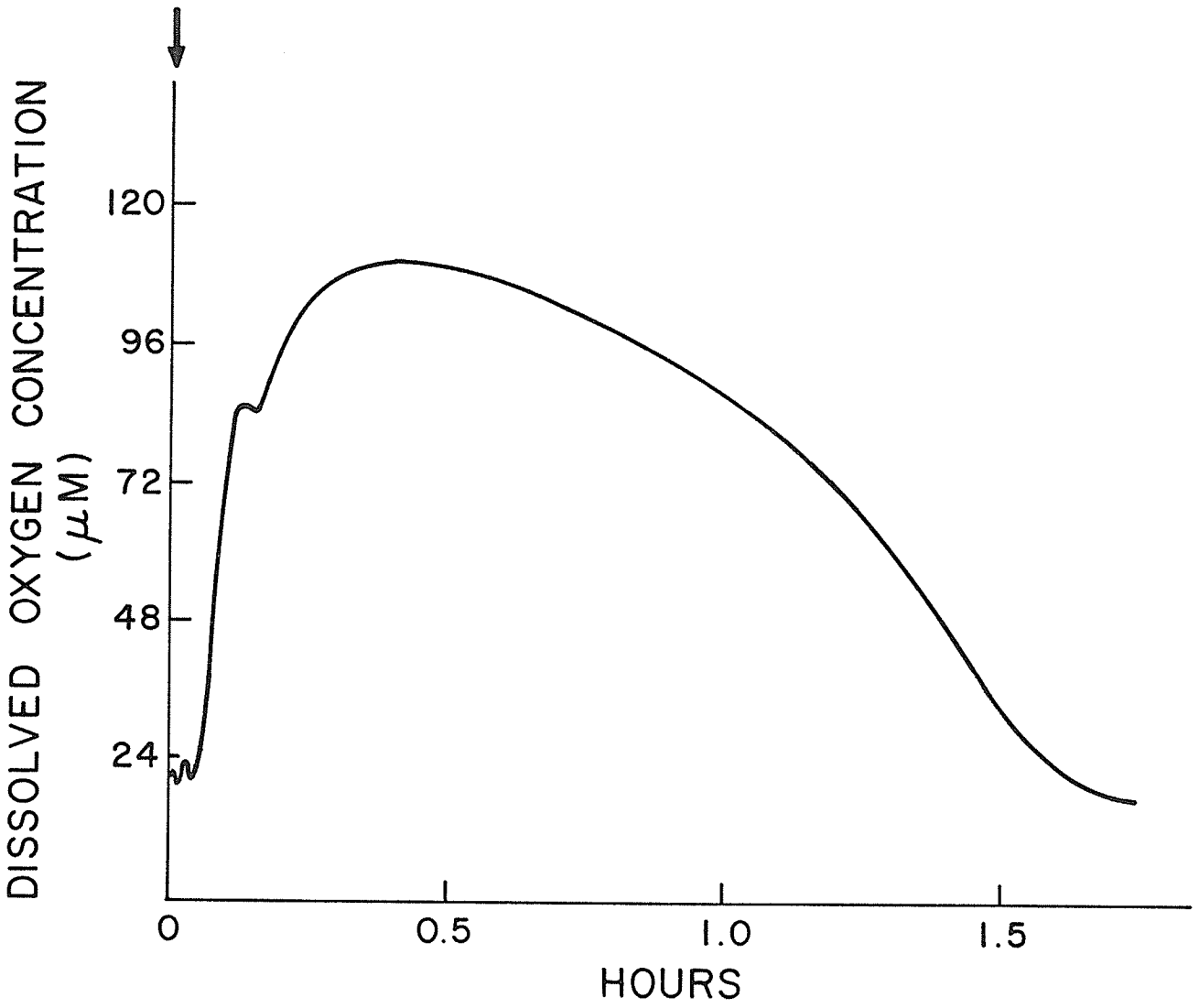

DISSOLVED OXYGEN CONCENTRATION
(μM)
120
96
72
48
24
0
0.5
1.0
1.5
HOURS

Fig. 40 Variation in the oxygen uptake rate with time after addition of malate to a mannitol limited culture.

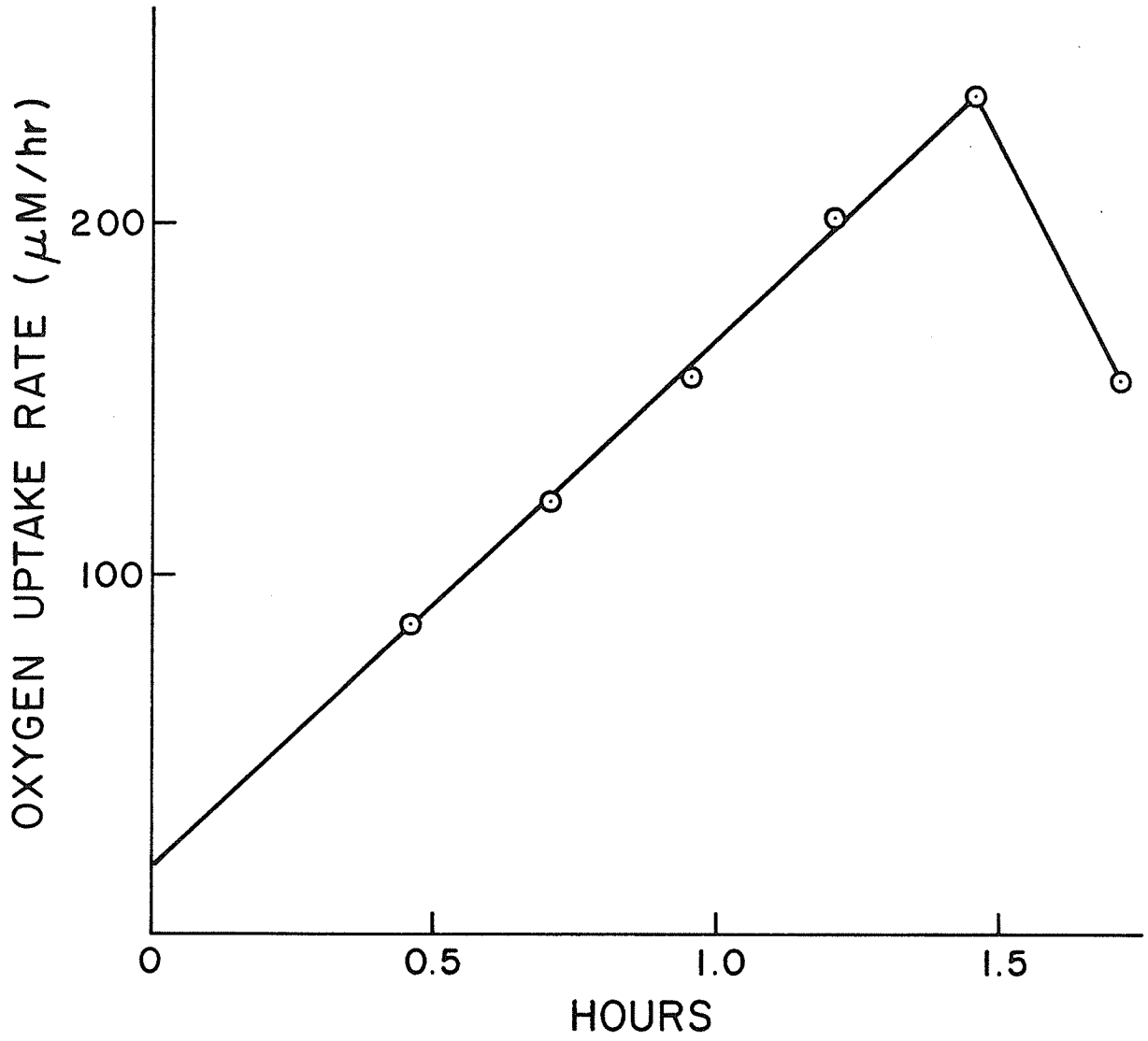

OXYGEN UPTAKE RATE (μM/hr)
200
100
0
0.5
1.0
1.5
HOURS

Fig. 41 Variation in the dissolved oxygen tension with time after addition of acetate to mannitol limited culture. Mannitol supply was stopped, and acetate was added at the arrow.

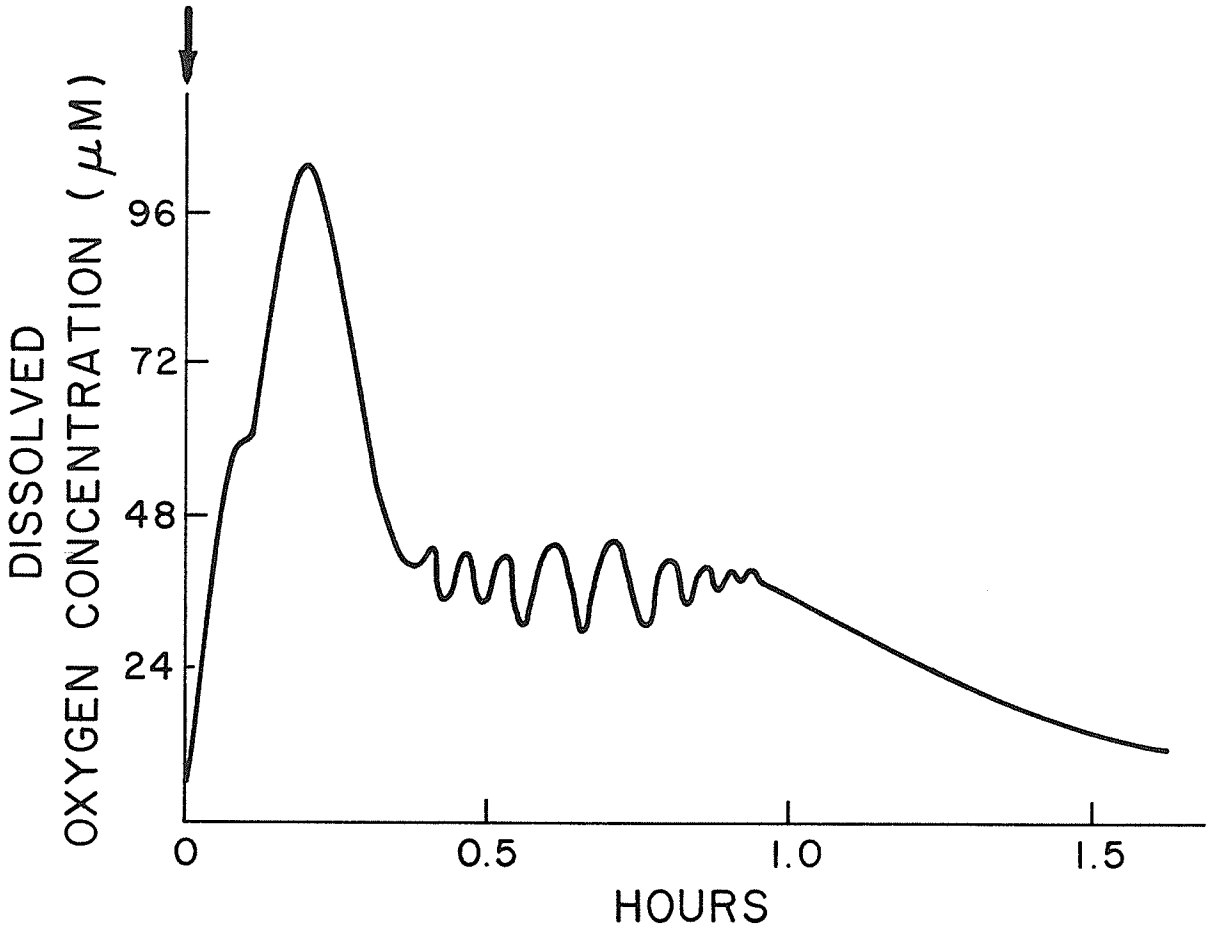
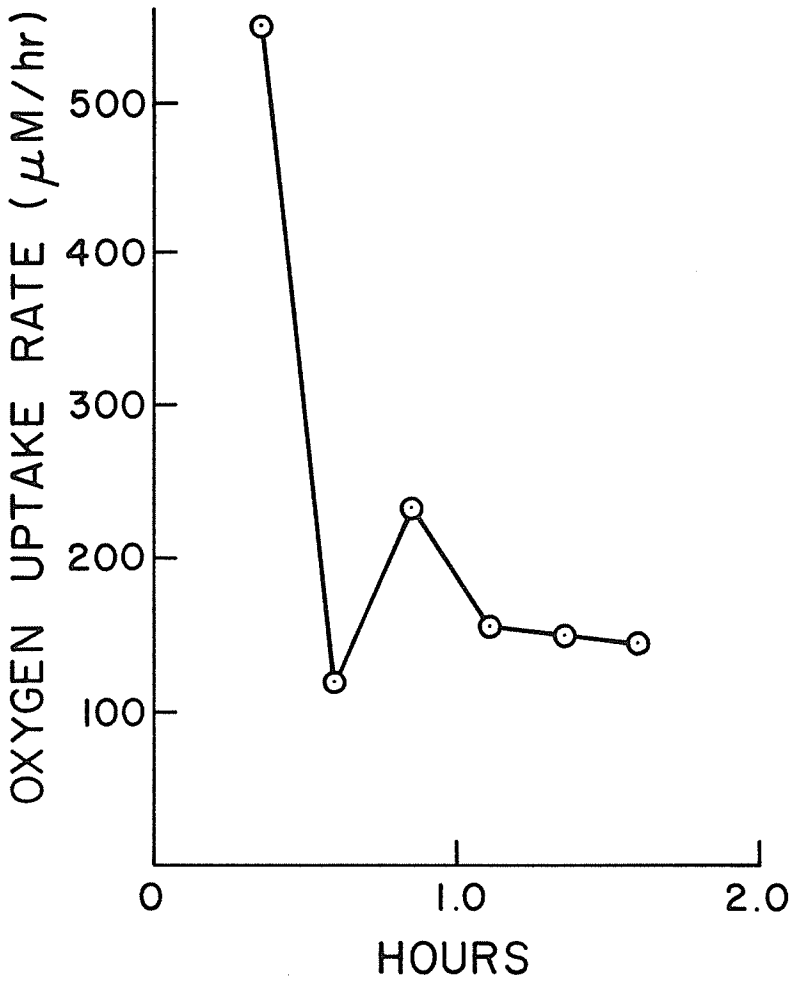

DISSOLVED
OXYGEN CONCENTRATION (μM)
96
72
48
24
0
0.5
1.0
1.5
HOURS

Fig. 42. Variation in the oxygen uptake rate with time after the addition of acetate to a mannitol limited culture.


OXYGEN UPTAKE RATE (μM/hr)
500
400
300
200
100
0
1.0
2.0
HOURS

respiration (from 7 to 12 minutes). After 12 minutes, the respiration rate is extremely high but inhibition of respiration occurs when the dissolved oxygen tension is reduced to about 36 μM O_2. The d.o.t. then undergoes a series of oscillations in oxygen tension. The respiration settled to a constant rate about sixty minutes after addition of acetate. The rate of oxygen consumption is shown at various time intervals after addition of acetate in Fig. 42. The rate at low oxygen concentration (150 μM O_2/hr) is remarkably similar to that of malate at low d.o.t. and glucose.

d) Citrate

The addition of citrate (0.5%) to a mannitol-limited cultures produced no respiration. Subsequent mannitol addition did not support respiration and the culture eventually washed out.

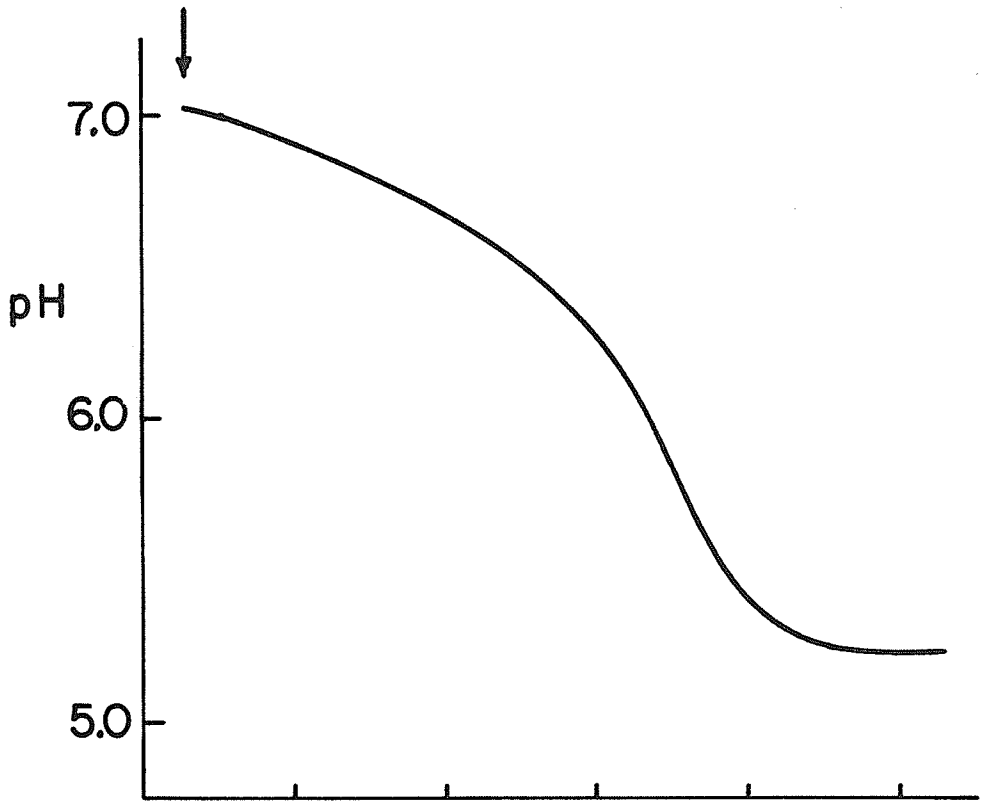
e) Mannose

The addition of mannose to a mannitol-limited culture did not result in its use as a carbon source for respiration or growth. Upon recommencement of the flow of mannitol the d.o.t. returned to what it had been prior to turning off the mannitol supply. This indicates that mannose does not even augment mannitol-supported respiration.

f) Addition of readily metabolizable substrates to mannitol starved cells

The addition of mannitol or fructose to carbon starved cells resulted in an immediate commencement of respiration, with the dissolved oxygen tension in the culture being rapidly reduced to zero. Interestingly, after addition of these carbon sources the pH of the culture

Fig. 43. Change in pH after addition of mannitol (final concentration 0.5%) to a mannitol-limited chemostat culture of A. vinelandii D = 0.13 hr^{-1}. Curve shown is a recorder tracing.


7.0
pH
6.0
5.0
TIME
(5 minute intervals)

started to decrease and fell from pH 7.0 to below 5.0 within 2 hours (Fig. 43). The amount of acid produced was 2.62 meq/l as determined by titration of the B_6 medium. When the acidity of the medium increased to a pH value of 5.2, respiration stopped and shortly after the production of acid apparently ceased.

The acid was determined to be a carbonyl compound by its reaction with 2,4 dinitrophenylhydrazine. Further identification was carried out as described in Materials and Methods section 1.6. The melting point of the derivative was determined to be 217°C $\pm$ 1°C corresponding to the melting point of the pyruvate derivative of 2,4 dinitrophenylhydrazine. The derivative was also identified as pyruvate by paper chromotography using the solvent systems described by Dilworth (1961). The possibility of other acids being present which are not carbonyl compounds was tested by paper chromatographic analysis with ether, formic acid and water (5:2:1) and detection by spraying with 0.1% chlorophenol red in ethanol. No other acids were detected.

2.6 Pyruvate Production

2.6.1 Role of excess oxygen in pyruvate production

The presence of excess oxygen may have a detrimental effect on the pyruvate dehydrogenase complex thus resulting in release of pyruvic acid (Dilworth, 1961). To test this hypothesis cultures were maintained in three states prior to introducing the pulse of mannitol. These were extreme sulfur limitation, extreme carbon limitation with reduced oxygen and thirdly moderate carbon limitation with no detectable oxygen in solution. Cultures grown under extreme sulfur limitation had reduced cell production with resultant accumulation of excess dissolved oxygen. Unlike mannitol limited cultures, when

exposed to a pulse of mannitol no accumulation of acid was observed. Cultures which were grown in carbon-limited conditions at 20% oxygen and subsequently reduced to 5% so as to permit a zero d.o.t., produced pyruvate when supplied with a pulse of mannitol. Therefore, it appears that the production of pyruvate by Azotobacter is not dependent upon the presence of oxygen, rather it may be a consequence of overflow metabolism (Neijssel and Tempest, 1975). Because of carbon starvation the mannitol uptake mechanism of the cell may be over active, resulting in an overaccumulation of carbon within the cell when suddenly exposed to high levels of mannitol. The importance of carbon limitation rather than excess oxygen for pyruvate excretion is best demonstrated by a culture which had been growing under carbon-limited conditions but was supplied with sufficient mannitol to reduce the d.o.t. to zero and maintain it so for several days. Upon exposure to high mannitol concentrations, this culture still produced acid, which accumulated in the medium.

2.6.2 Effect of uncouplers

The effects of uncouplers also were investigated to determine if an energy activated cell membrane is required for the cell to produce acid. Cells were grown as previously described to produce a carbon limited condition. To this culture 2,4-dinitrophenol was added to a final concentration of 3.0 mM. The respiration of the culture decreased three minutes after the addition of the uncoupler, this inhibition was slightly relieved by the addition of the mannitol (0.5%). Fifteen minutes after the addition of the excess mannitol, the pH of culture medium started to decrease. The production of acid was, however, at a much slower rate than had occured with no uncoupler and to a lesser

extent, i.e. to pH 5.9 from 6.5. In order to reduce the concentration of dissolved oxygen, which was approaching 20% due to inhibition of respiration the concentration of oxygen above the culture was reduced to 5%.

The addition of mannitol in the presence of the uncoupler also allowed the cells to produce a large amount of carbohydrate (Table 3). This was probably fructose, since fructose is a direct oxidation product of mannitol and the levels of ketohexose increased in a similar pattern to the carbohydrate. The apparent difference between the carbohydrate and ketohexose concentrations observed in the first two days is due to the fact that the carbohydrate analysis was done on the cell culture whereas the ketohexose assay was done on the culture supernatant. On continued incubation over a period of eight days, at a low dilution rate (0.05 hr^{-1}) and under low oxygen (5%) the levels of carbohydrate and ketohexose initially increased for a two day period then drastically decreased on the third day, in the case of the ketohexose to zero. By day four, levels of carbohydrate again began to increase, but this was not accompanied by an increase in the ketohexose levels. This increase in carbohydrate was accompanied by an increase in viscosity of the culture indicating polysaccharide production (Table 3). The relative viscosity was determined by the flow of culture though a one milliter pipet and by the volume of the culture. For example, highly viscous solutions would have a reduced vortex (i.e. the liquid in the culture would not rise as much up the wall of the vessel) thus the volume of the chemostat would increase. The culture, by the fifth day, had become very viscous and had the consistency of loose jelly. The experiment was repeated

Table 3. Analysis of samples from a mannitol limited A. vinelandii culture after exposure to a pulse of mannitol in the presence of dinitrophenol.

Day	Total Nitrogen (µg/ml)	Total Carbohydrate (µg/ml)	Pentoses (µg/ml)	Uronic Acids (µg/ml)	Ketohexoses (µg/ml)	Residual Mannitol (mg/l)	Relative viscosity[a] (secs)	(ml)
1	115	130	18	153	0	0.0	-	235
1*	100	355	22	114	290	5.5	-	235
2	105	720	46	214	440	4.4	6	230
3	169	240	36	245	0	2.3	7	230
4	152	283	30	270	0	1.8	8	230
5	137	445	23	194	0	2.9	11	245
6	127	537	19	259	0	3.1	36	255
7	112	575	18	264	0	3.2	16	260
8	100	597	20	285	0	3.3	18	260

[a]Viscosity is described as time for medium to flow through a 1 ml pipet (secs) or the volume of the culture (ml).

*8 hours after addition of mannitol.

Control cultures without 2,4 DNP addition accumulate pyruvate, see text.

using identical conditions but with no addition of 2,4-dinitrophenol. There was no production of ketohexose or polysaccharide under these conditions.

In an attempt to induce production of the polysaccharide using another uncoupler, Na-arsenate (10^{-2} M), under otherwise identical conditions, the culture again produced a large amount of carbohydrate within one hour after addition of mannitol. However these cells do not produce copious amounts of polysaccharide as had been demonstrated with DNP.

3.0 A Comparison of the Kinetics of Mannitol and Fructose Oxidation

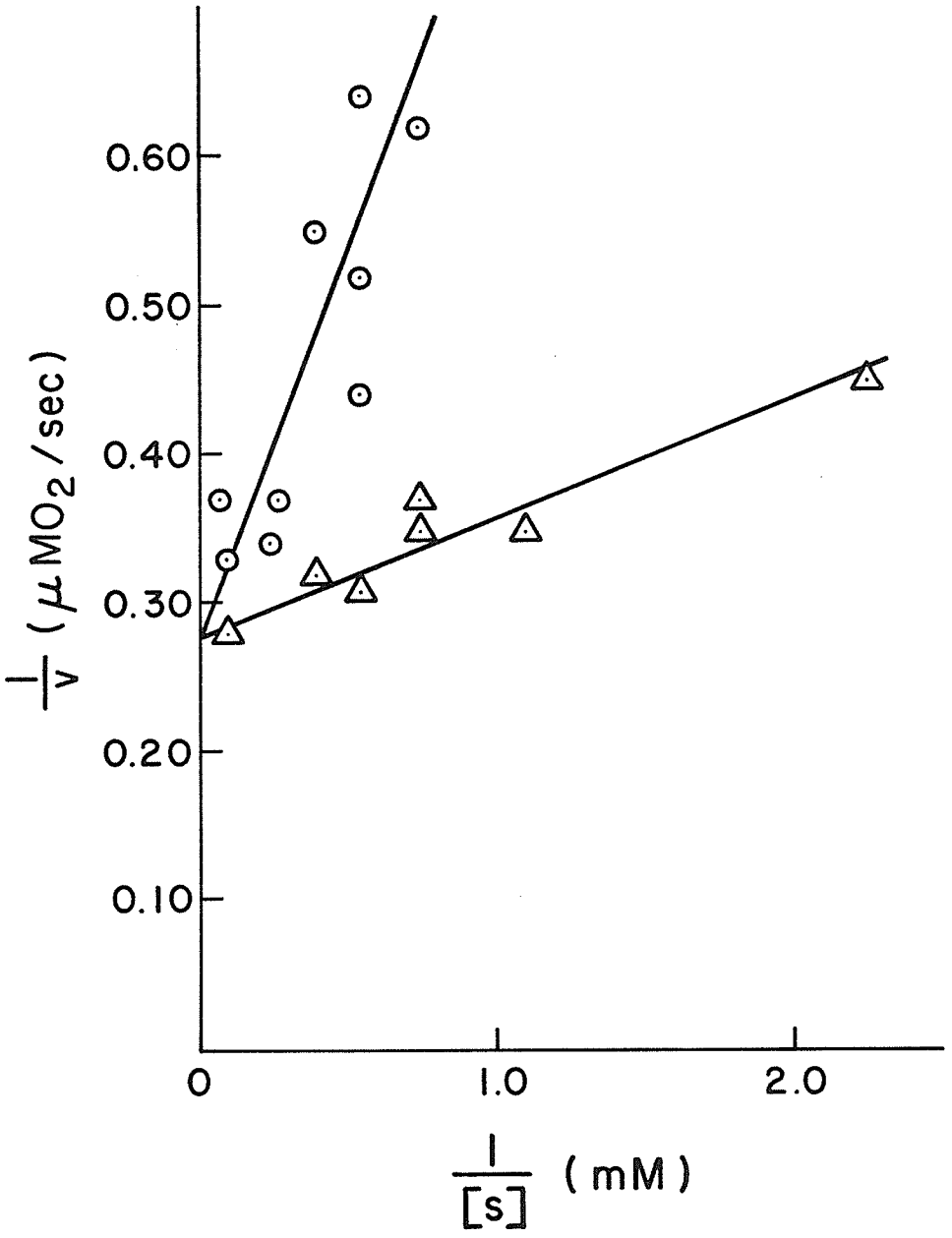
The high oxygen uptake rate of mannitol starved cells exposed to either fructose or mannitol, and the production of pyruvate by mannitol limited cells exposed to fructose, along with the production of ketohexose after addition of mannitol to carbon starved cells in the presence of an uncoupler, raises the possibility that mannitol is converted to fructose at the cell membrane, with little expenditure of energy. The fructose so formed may be taken across the membrane by an energy dependent translocator enzyme.

In order to examine the relationship between those two substrates and their translocases the maximum rate of oxygen consumption by carbon-starved A. vinelandii was determined at various substrate concentrations. The relationship between the oxygen consumption rate and the substrate concentration is shown as an inverse plot in Fig. 44. The maximum velocity for the oxidation of both substrates appear to be quite similar. However the affinity of the uptake system is much greater for mannitol. It is likely that mannitol is oxidized to fructose however the uptake of the fructose so formed may depend upon

Fig. 44. Inverse plot of oxygen uptake rate (μM O_2/sec) versus carbon substrate concentration (mm).

Fructose o———o

Mannitol Δ———Δ


0.60
0.50
0.40
0.30
0.20
0.10
1/v (μMO2/sec)
0
1.0
2.0
1/[S] (mM)

a transfer of fructose from the mannitol dehydrogenase to the energy dependent translocase; the unbound fructose may also be taken up by the translocase but with less affinity than the mannitol dehydrogenase enzyme has for mannitol.

4.0 Effects of Krebs Cycle Intermediates on Respiration

The inhibition of respiration by citrate raised an important question as to why a Krebs Cycle intermediate apparently acted as an inhibitor. To determine if other intermediates in the catabolism of mannitol could also act as inhibitors, the rate of oxygen uptake was determined for unwashed cells from an oxygen-limited chemostat in the presence of a number of these intermediates. Table 4 lists the carbon sources tested and their effects on respiration. All Krebs cycle intermediates tested plus lactate and glycollic acid at concentrations of 1% completely inhibited the respiration of A. vinelandii. Acetate also showed some inhibitory effect but its inhibition was incomplete.

The effects of KCN on the respiration in the presence of the various carbon sources was also determined. This inhibitor, at very low concentrations of 10 μM, will slightly suppress the oxygen uptake rate of cells in the presence of mannitol, glucose and pyruvate. However, the rate of oxygen uptake in the presence of acetate and KCN is greater than in the presence of acetate alone.

The effect on oxygen uptake of sparging the sample with pure oxygen for ten minutes prior to the measurement of oxygen uptake in the presence of these substrates is also shown in table 4. Such treatment causes a small increase in oxygen uptake rate in the presence of mannitol, glucose and pyruvate. The inhibition of

Table 4. Effect of various substrates (10 mg/ml), KCN (10 µM), and exposure to pure oxygen for 10 mins on the rate of oxygen uptake (µmole $O_2 \cdot min^{-1} \cdot ml^{-1}$) of chemostat grown A. vinelandii

Substrate	Effector			
	None	O_2	KCN	KCN + O_2
Endogenous	0.63	0.65	0.62	0.49
Mannitol	0.82	0.87	0.78	0.83
Acetate	0.16	0.41	0.25	0.38
Glucose	0.78	0.94	0.67	0.72
Pyruvate	0.77	0.94	0.71	0.88
Succinate	0.00	0.00	0.00	0.00
Malate	0.00	0.00	0.00	0.00
Lactate	0.00	0.00	0.00	0.00
Citrate	0.00	0.00	0.00	0.00
Fumarate	0.00	0.00	0.00	0.00
Glycollate	0.00	0.00	0.00	0.00

Organic acids were added as the sodium salts.
The initial maximal rate of oxygen uptake rate was recorded

respiration by acetate is relieved to a large extent by the exposure of the cells to pure oxygen prior to measuring oxygen uptake, but the complete inhibition by Krebs cycle intermediates, lactate or glycollic acid was not relieved by such treatment.

Treating the cells with pure oxygen and measuring the oxygen uptake rate in the presence of KCN did not result in a very significant change in oxygen uptake in the presence of mannitol, glucose or pyruvate compared to cells which have not been treated with pure O_2. The relief of acetate inhibition by treating the sample with excess oxygen and KCN is not cummulative, since combined treatments result in oxygen uptake rates similar to that of the oxygenated culture alone.

5.0 Effect of NADH on Respiration

The complete inhibition of respiration in the presence of Krebs cycle intermediates, lactate or glycollic acid suggests an inhibition of the NADH dehydrogenase activity by NADH. A similar type of inhibition was reported by Hatefie and Stempel (1969) with NADH dehydrogenase from membrane bound mitochondrial NADH dehydrogenase.

5.1 Inhibition of NADH dehydrogenase by NADH

The response of A. vinelandii NADH:ferricyanide oxidoreductase (measured by a decrease in NADH levels) to various concentrations of NADH was determined (Fig. 45). The rate of NADH oxidation increases with increasing NADH concentration to a maximum at 0.6 mM NADH. But the activity of this enzyme was reduced considerably when the concentration of NADH in the assay preparation was greater than this value. Similarly NADH dependent oxygen reduction by cell free extracts also demonstrated inhibition of oxygen uptake in the presence of high NADH levels.

Fig. 45. Variation in the NADH-ferricyanide oxidoreductase activity with different NADH concentration. Assay performed on cell-free extract of mannitol-limited A. vinelandii from a continuous culture. D = 0.20 hr^{-1}.

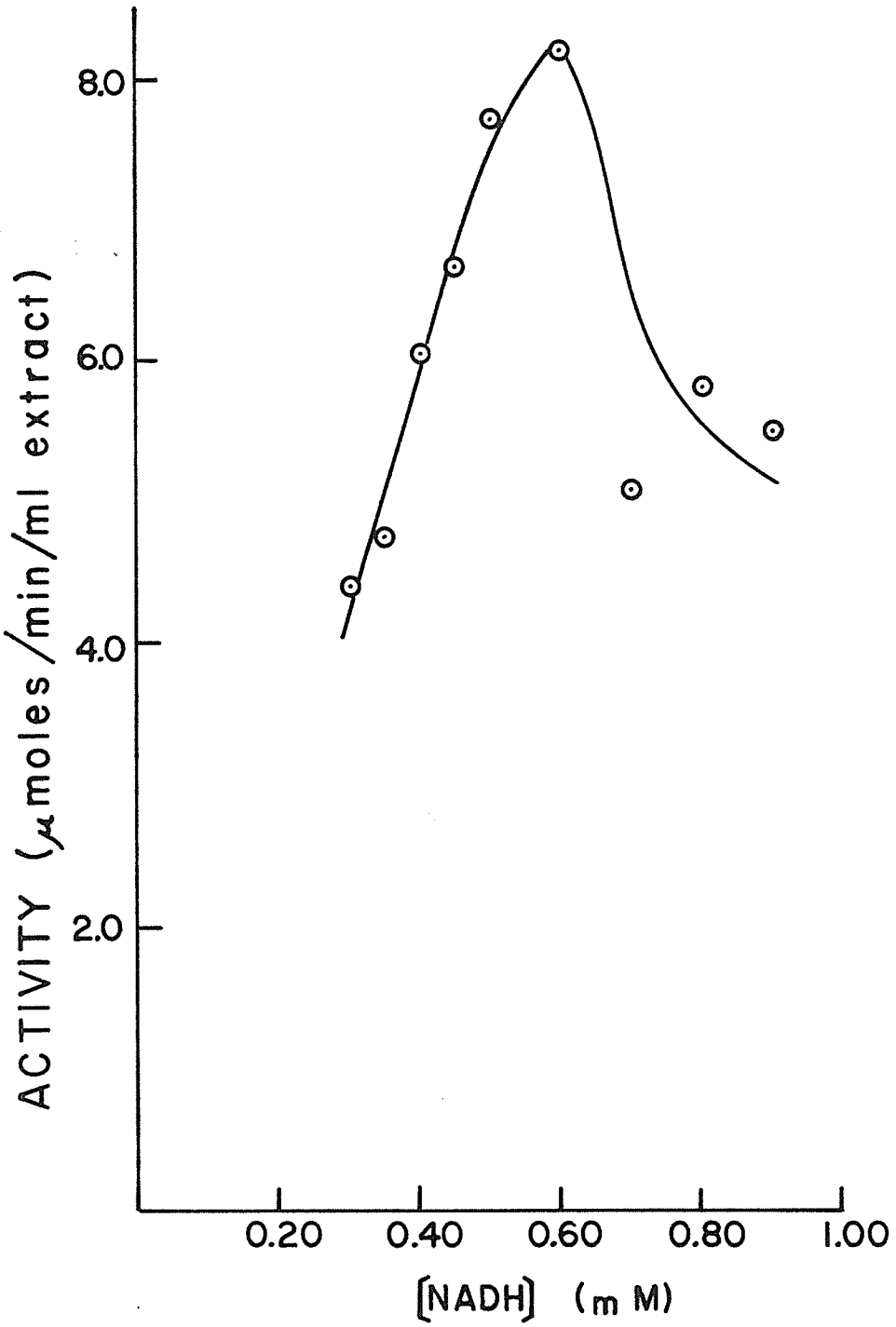

8.0
6.0
4.0
2.0
ACTIVITY (μmoles/min/ml extract)
0.20
0.40
0.60
0.80
1.00
[NADH] (m M)

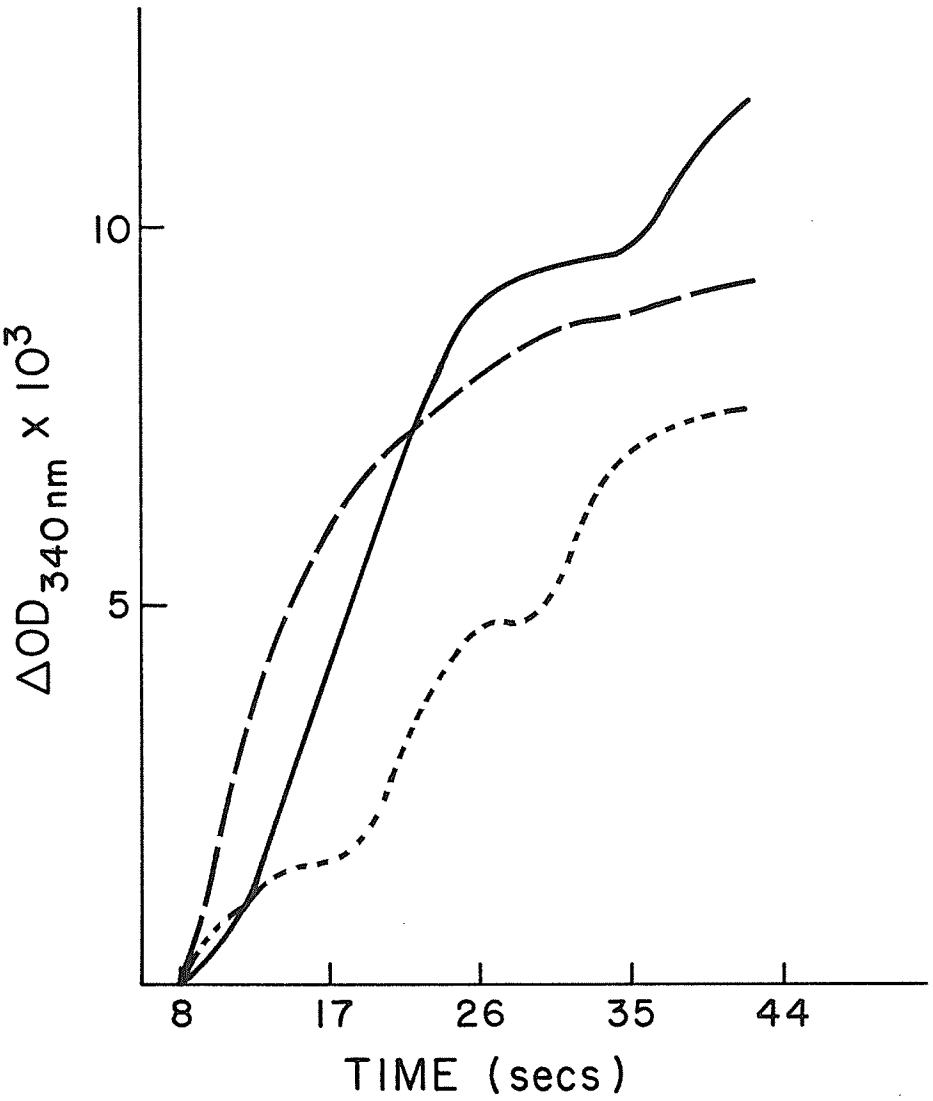
Fig. 46. Effect of different substrates upon the NAD(P)H levels in anaerobic batch-grown mannitol-oxidizing A. vinelandii.

mannitol--------

lactate — — —

citrate ————————

Curves shown are recorder tracings.


10
5
$\Delta OD_{340nm} \times 10^3$
8
17
26
35
44
TIME (secs)

5.2 Effect of Citrate, Lactate and Mannitol upon the intracellular NADH level.

The effect of citrate, lactate and mannitol on the intracellular level of NADH in A. vinelandii grown in batch culture oxidizing mannitol was determined by addition of each substrate to anaerobic cells and following the increase in O.D. at 340 nm (Fig. 46). All three substrates produce an increase in the NADH levels within the cell, however the rate of the increase in NADH levels in cells given citrate or lactate is greater than for the cells provided with mannitol. The extent of NAD(P)H accumultation was determined 2 minutes after the addition of substrate by measurement of the difference between the absorbance at 340 and 310 nm (Table 5). Citrate produced the largest increase of NADPH levels followed by succinate; acetate and malate had equal effect. Mannitol addition to these anaerobic cells induced only a small increase in the level of the reduced pyridine nucleotides.

6.0 Effects of Cyanide on the Respiration of Azotobacter

The cytochrome oxidases of Azotobacter are believed to have characteristically different susceptibilities to CN^-. Cytochrome o being extremely sensitive with a Ki of 0.5 μM KCN whereas cytochrome d is believed to be very tolerant with a Ki of 15-115 μM and cytochrome a_1, may not be affected at all.

To examine the effect of KCN on oxygen uptake, a sample of cells grown under oxygen limited conditions in 1.5 x B_6 medium with 1.5% mannitol at a dilution rate of 0.20 hr^{-1} were removed from the chemostat. Aliquots (0.20 ml) were added to a solution of B_6 medium plus 1% mannitol with varying concentrations of KCN in the well of a Gilson oxygraph.

Table 5. $A_{340} - A_{310}$ 3 minutes after addition of substrate to a suspension of A. vinelandii cells grown in batch culture.

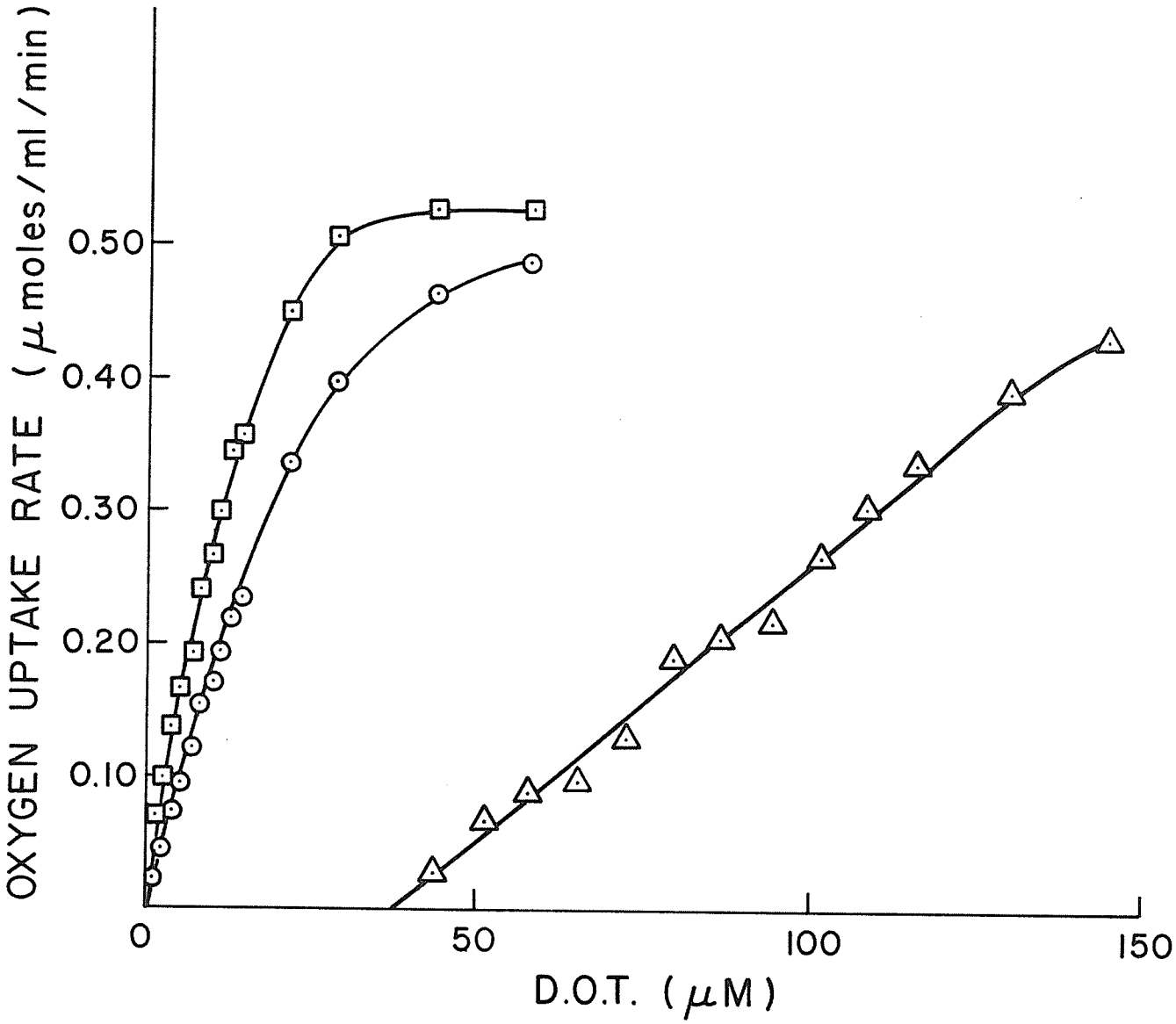
Substrate (25 mM)	$A_{340} - A_{310} \times 10^2$
Mannitol	0.3
Na^+-citrate	1.8
Malic acid	0.9
Na^+-succinate	1.3
Na^+-acetate	0.9

Fig. 47. The rates of oxygen uptake at different dissolved oxygen tensions for oxygen-limited chemostat grown A. vinelandii, in the presence of

No KCN □——□

15 μM KCN o——o

20 μM KCN Δ——Δ


OXYGEN UPTAKE RATE (μ moles/ml/min)
0.50
0.40
0.30
0.20
0.10
0
50
100
150
D.O.T. (μM)

With regard to oxygen uptake measurement, a lag period that followed the addition of the cells to the medium made it virtually impossible to measure the initial velocity at various oxygen tensions. Therefore the rate of oxygen uptake at each oxygen tension was determined by drawing tangents to the oxygen uptake curve.

Fig. 47 demonstrates the relationship between oxygen uptake and oxygen concentration at various concentrations of KCN. The maximum oxygen uptake rate appears to be only slightly affected by 15.0 μM KCN. Yet, 20.0 μM KCN reduces the affinity for oxygen considerably producing a linear decrease in activity with decreasing oxygen concentration. Such a large effect over a small concentration range of KCN was also seen when the rate of oxygen uptake was determined at specific oxygen tensions with various KCN concentrations (Figs. 48 and 49). At high oxygen tensions (72 μM O_2) 15.0 μM KCN reduces the oxygen uptake rate by 41%, this same concentration of KCN reduces the oxygen uptake rate by 66% where O_2 tension is at 15 μM.

Fig. 49 also illustrates a very large reduction in oxygen uptake rate between 11.5 and 11.8 μM KCN at 7 μM O_2. Such a reduction over a narrow range of KCN concentrations indicates a possible titration effect of KCN on the terminal oxidase. To test if KCN is titrating a specific group, the concentration of cells in the oxygraph was increased by one-half. This assay was carried out with batch grown A. vinelandii. With 1.0 ml of culture in the oxygraph well, 10 μM KCN was required to obtain a noticeable reduction in rate of oxygen uptake but with 1.5 ml approximately 25 μM KCN was required to obtain a noticeable reduction in rate.

Fig. 48. The rate of oxygen uptake at different dissolved oxygen concentrations for oxygen limited chemostat-grown A. vinelandii, in the presence of

No KCN ○———○

11.50 μM KCN □———□

11.75 μM KCN △———△

15.0 μM KCN ○-----○

20.0 μM KCN □-----□

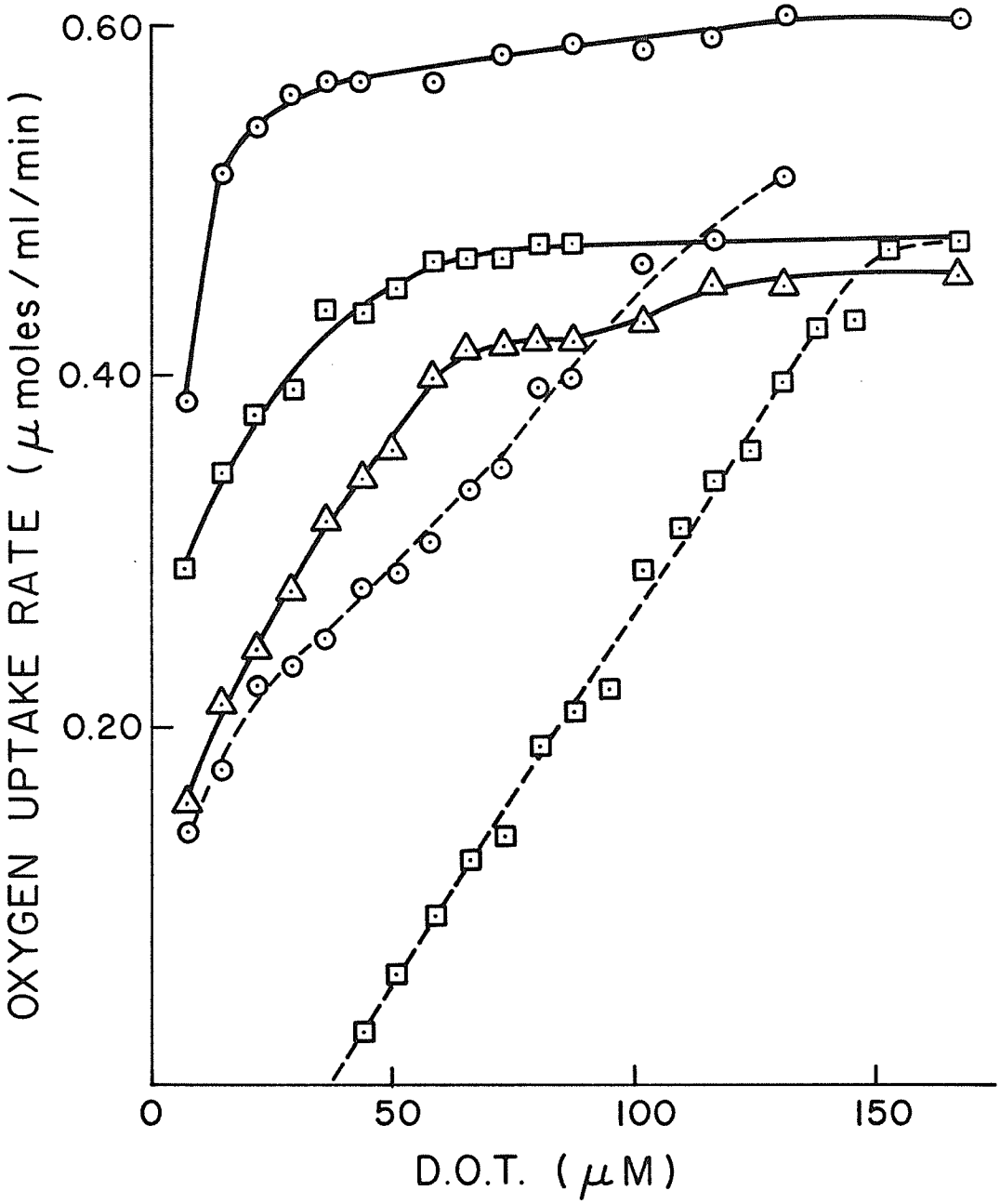
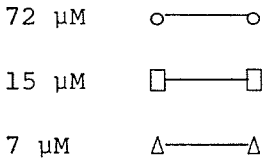
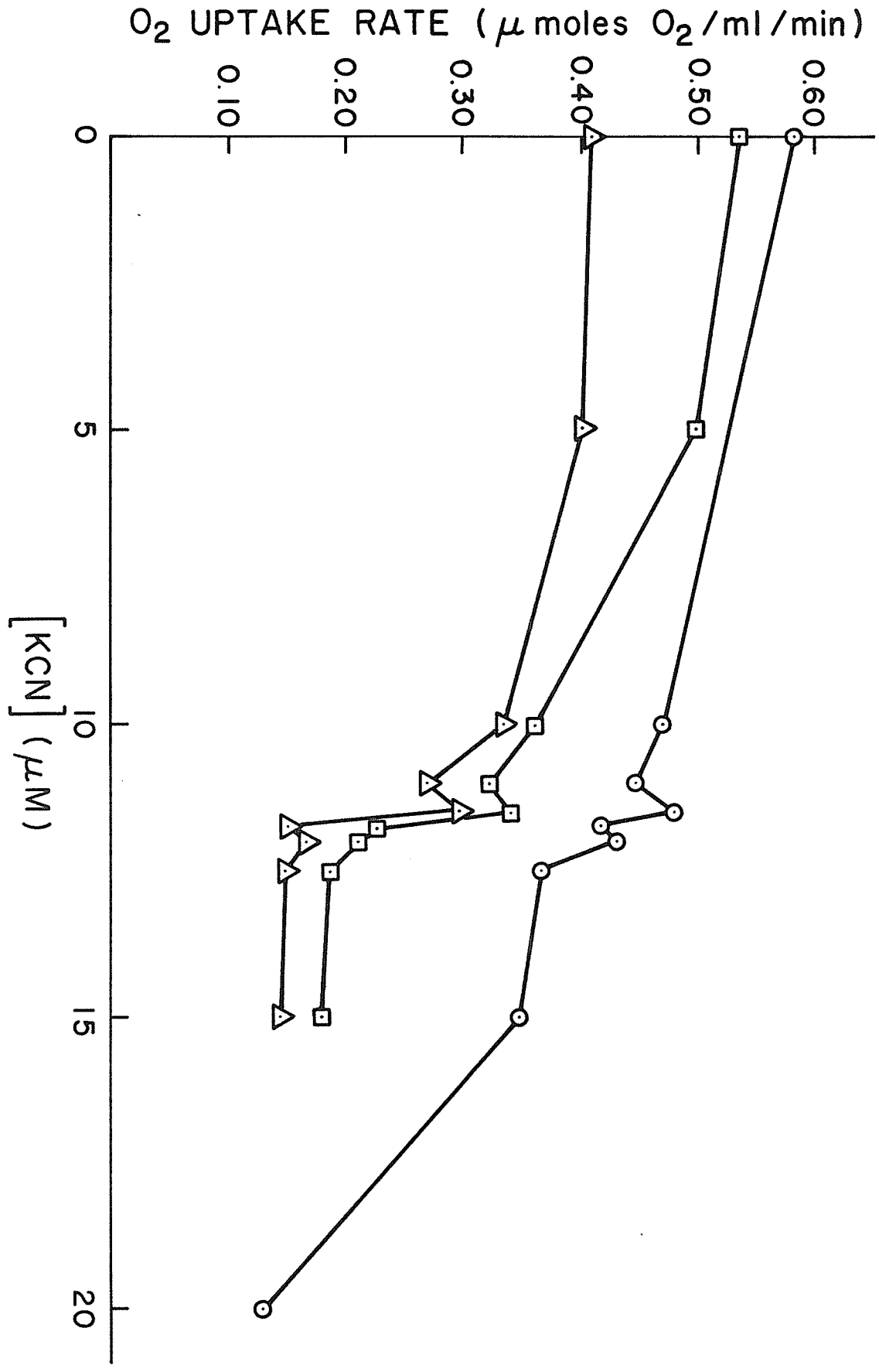

0.60
0.40
0.20
OXYGEN UPTAKE RATE (μ moles / ml / min)
0
50
100
150
D.O.T. (μM)

Fig. 49 The rate of oxygen uptake for oxygen-limited chemostat grown A. vinelandii in the presence of various concentrations of KCN, measured at the following dissolved oxygen concentrations:




O_2 UPTAKE RATE (μ moles O_2 / ml / min)
0.10
0.20
0.30
0.40
0.50
0.60
0
5
10
15
20
[KCN] (μM)

6.1 The effect of KCN on oxygenated cells

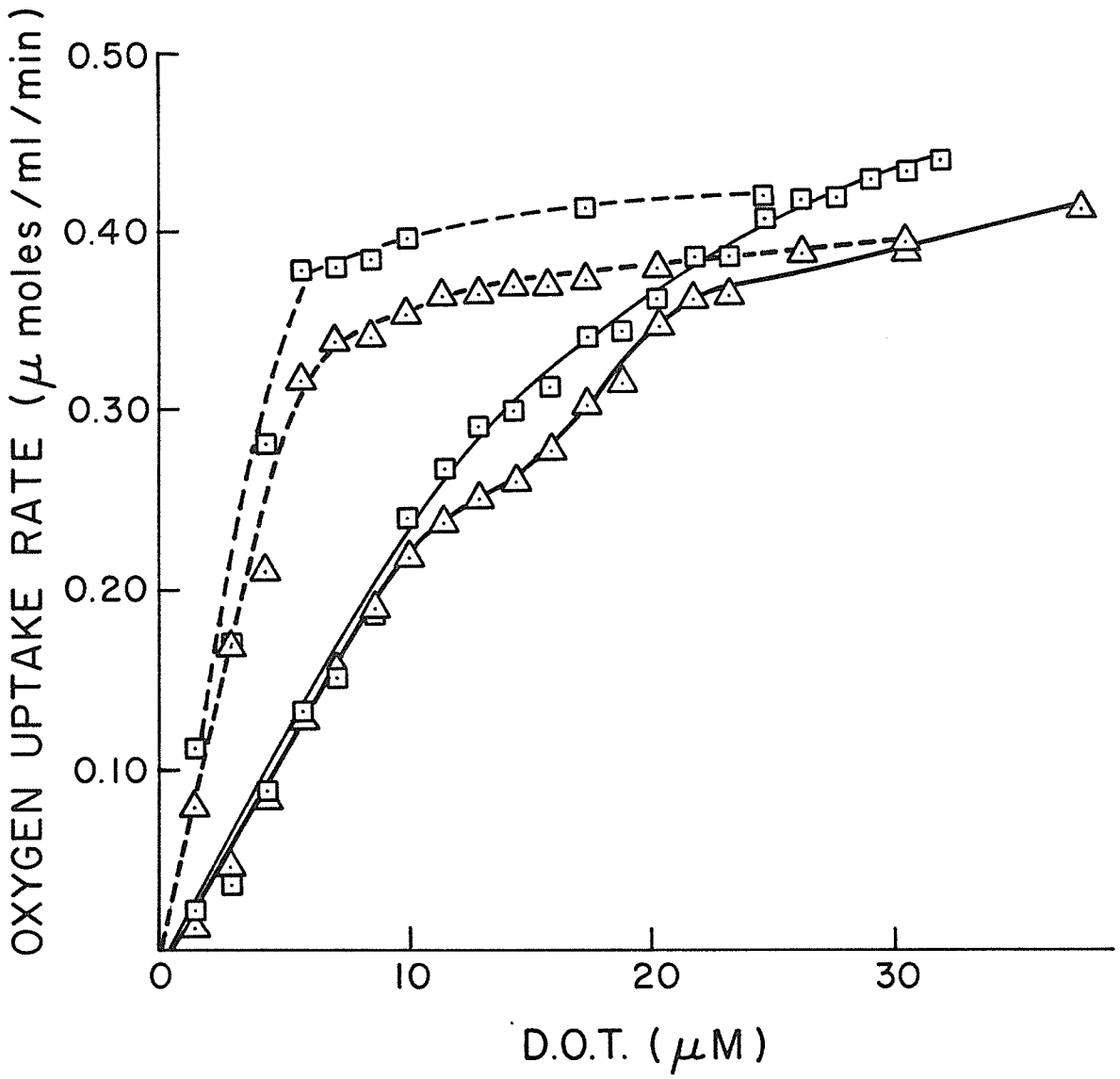
In order to determine if oxygenation inhibits only the minor terminal oxidase pathway, (i.e. terminating with cytochromes $\underline{a}_1$ and $\underline{o}$) the rate of oxygen uptake of cells in the presence of low concentrations of KCN was compared to the rate of oxygen uptake by cells which had been exposed to pure oxygen. The cells used were taken from an oxygen-limited chemostat (0.20 hr^{-1}) and the rate of oxygen uptake was immediately determined. Oxygenated cells had been prepared by sparging with pure oxygen for 10 mins immediately before use. The results are presented in Fig. 50.

The rate of oxygen uptake of untreated cells at oxygen tensions below 6 µM O_2 appears to be dependent upon the dissolved oxygen concentration, however the rate of oxygen uptake at dissolved oxygen tensions above 6 µM becomes independent of the oxygen concentration. Since the rate of O_2 uptake may depend on either electron flow or oxygen supply it is likely that the rate of oxygen uptake would become limited by the flow of reducing equivalents to the oxidase at high oxygen concentrations.

The reduction in the maximum rate of oxygen uptake, in the presence of 10 µM KCN represents a decrease of 10% of the electron flow to the terminal oxidase. Therefore low concentrations of KCN which are known to inhibit the minor path (Jones & Fedfearn 1967) also reduce the electron flux to the terminal oxidase. That is to say, electron flow to the terminal oxidase is dependent upon the amount of active oxidase present.

Prior exposure of the cells to pure oxygen induces an inhibition of respiration resulting in the loss of affinity to a much greater

Fig. 50. The oxygen uptake rates at different dissolved oxygen concentrations, for oxygen limited chemostat-grown A. vinelandii cells, which by pretreatment have (—) and have not (--) been sparged with oxygen, and in the presence (Δ) or absence (□) of 10 μM KCN.


OXYGEN UPTAKE RATE (μ moles / ml / min)
0.50
0.40
0.30
0.20
0.10
0
10
20
30
D.O.T. (μM)

extent than seen in cells treated with low concentrations of KCN. Although the maximum rate of oxygen uptake is slightly greater than for cells which have neither been exposed to excess oxygen or KCN, the rate of oxygen uptake at oxygen concentrations below 20 μM is lower for oxygenated cells compared to untreated cells or to untreated cells in 10 μM KCN (i.e. 128 vs 318 μM O_2 min^{-1} m^{-1} at 7.3 μM O_2).

The effect of KCN on the oxygen uptake rate by oxygenated cells is also shown by Fig. 50. At very low oxygen tensions there is no apparent effect of 10 μM KCN on oxygenated cells. Above 8 μM O_2 the rate of oxygen uptake appears to be affected to a greater extent and the curve relating oxygen uptake rate to d.o.t. demonstrates a definite biphasic response with regard to dissolved oxygen concentration.

6.2 Effects of KCN on acetate oxidation

The apparent relief of acetate inhibition of respiration by KCN led me to investigate the effect of this inhibitor on acetate oxidation. Cells grown in batch culture utilizing acetate as the sole carbon source were examined with respect to their oxygen uptake rates in the presence of various concentrations of KCN. The results of these assays are shown in Fig. 52. At high concentrations of KCN these cells also appear to develop a reduced affinity for oxygen, but unlike chemostat-grown mannitol oxidizing cells, cells oxidizing acetate appear more resistant to KCN being capable of reducing the d.o.t. to zero at 20 μM KCN. The concentration of KCN which produces the dramatic loss of affinity for oxygen is higher for acetate grown cells, i.e. between 20 to 30 μM KCN compared to approximately 12 μM KCN for mannitol oxidizing chemostat grown cells.

The oxygen uptake rate of acetate utilizing cells at 40 μM KCN is non-linear with respect to the d.o.t. in contrast to the linear response, observed at 20 μM KCN in mannitol oxidizing chemostat-grown cells. The electron flow to the acetate oxidizing cells also appears to be rapidly reduced by CN^-, as indicated by a decrease in the V_{max} for oxygen uptake by these cells.

6.3 Effect of oxygen on KCN inhibition

The apparent linear decrease in the oxygen uptake rate with decreasing dissolved oxygen observed in cells from oxygen-limited chemostat cultures in the presence of high concentrations of KCN (Figs. 47 and 48) was also observed in batch grown A. vinelandii cells from mid-log growth. This linear decrease in activity does not intercept the dissolved oxygen tension axis above zero as had been

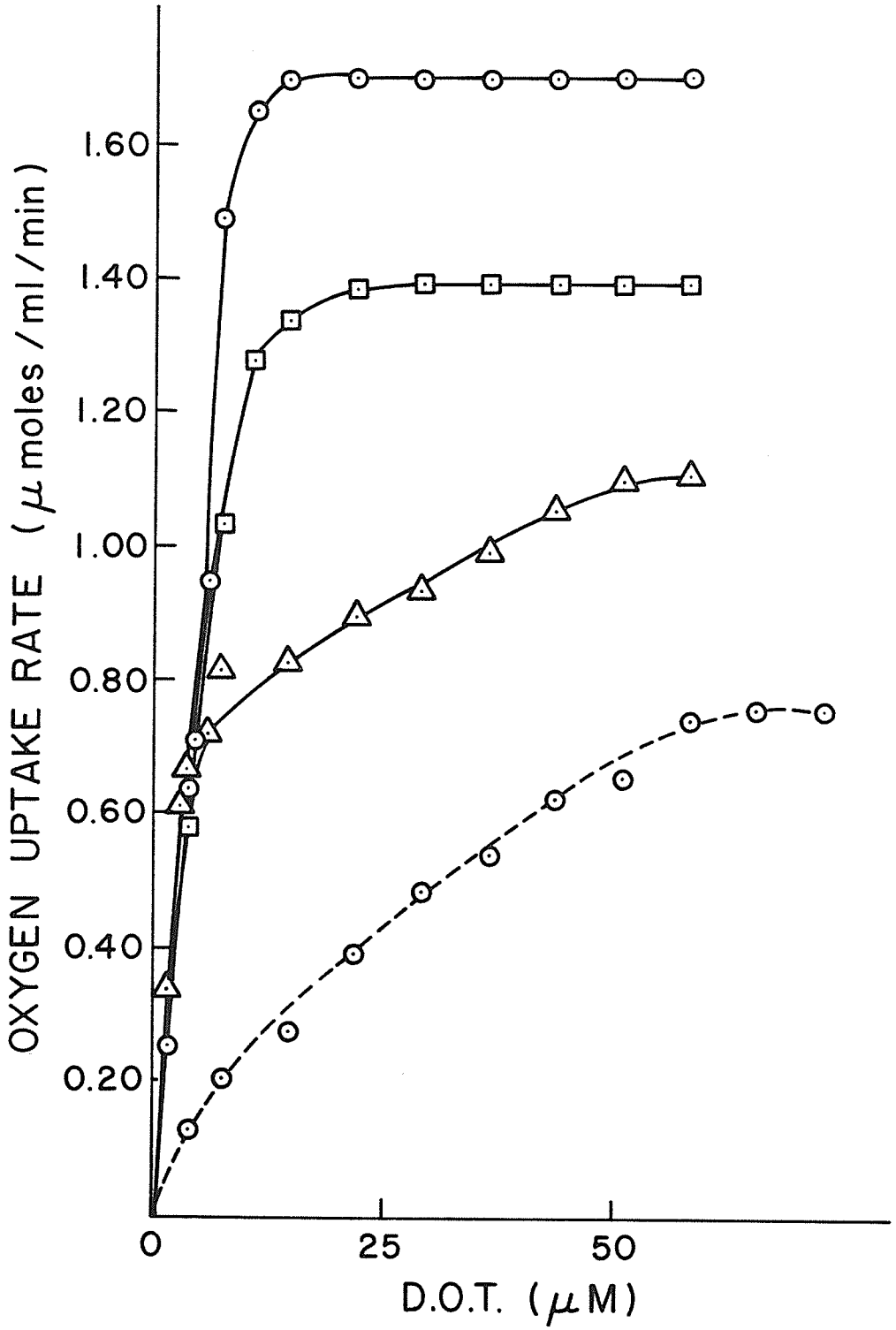
Fig. 51. The oxygen uptake rate for batch grown acetate oxidizing A. vinelandii at different dissolved oxygen concentrations, in the presence of:

No KCN o———o

20 µM KCN □———□

30 µM KCN Δ———Δ

40 µM KCN o-----o


OXYGEN UPTAKE RATE (μ moles / ml / min)
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0
25
50
D.O.T. (μM)

observed for chemostat grown-cells. Rather, the oxygen concentration decreased to zero at an extrapolated rate value of 25 mmole min^{-1} ml^{-1} (Fig. 52) These cells were allowed to remain in the anaerobic state for approximately two minutes, at which time they were lightly sparged with air. The oxygen uptake rate of these aerated cells returned to approximately what it had been prior to anaerobosis (Fig. 52) indicating the reduced cytochrome oxidase does not bind CN^-, confirming the spectrophotometric analysis of Kauffman (1973b). The rate of decline of activity with decreasing d.o.t., of these aerated cells was also slightly reduced (Fig. 53). Such a reduction in the rate of decline in activity indicates a higher oxygen-to-cyanide ratio occuring after a significant amount of CN^- has been bound, which would provide a competitive advantage to the oxygen over CN^- for binding to the remaining active sites.

To test whether the oxygen to CN^- ratio is a factor in the inhibition of the terminal oxidase, batch grown A. vinelandii cells were exposed to KCN. The oxygen tension was either 1) allowed to decline due to respiration, 2) maintained at a high d.o.t. and then allowed to decline, or 3) allowed to decline and then maintained at a low d.o.t. The oxygen uptake rate at different oxygen concentrations is shown in Fig. 54. At low d.o.t. the rate of decline was fastest for cells which were allowed to immediately reduce the d.o.t. by respiration. Cells which had the d.o.t. maintained artificially high had intermediate rates of decline and cells which had been maintained at low d.o.t. had the lowest rate of decline.

The rate of oxygen uptake is similar for CN^- treated cells which have been maintained at a high oxygen tension and cells maintained at low

Fig. 52 The oxygen uptake rate for batch-grown mannitol-oxidizing cells at different dissolved oxygen concentrations in the presence of 25 μM KCN (o———o) and after the d.o.t. is manually increased to approximately 120 μM (□———□).

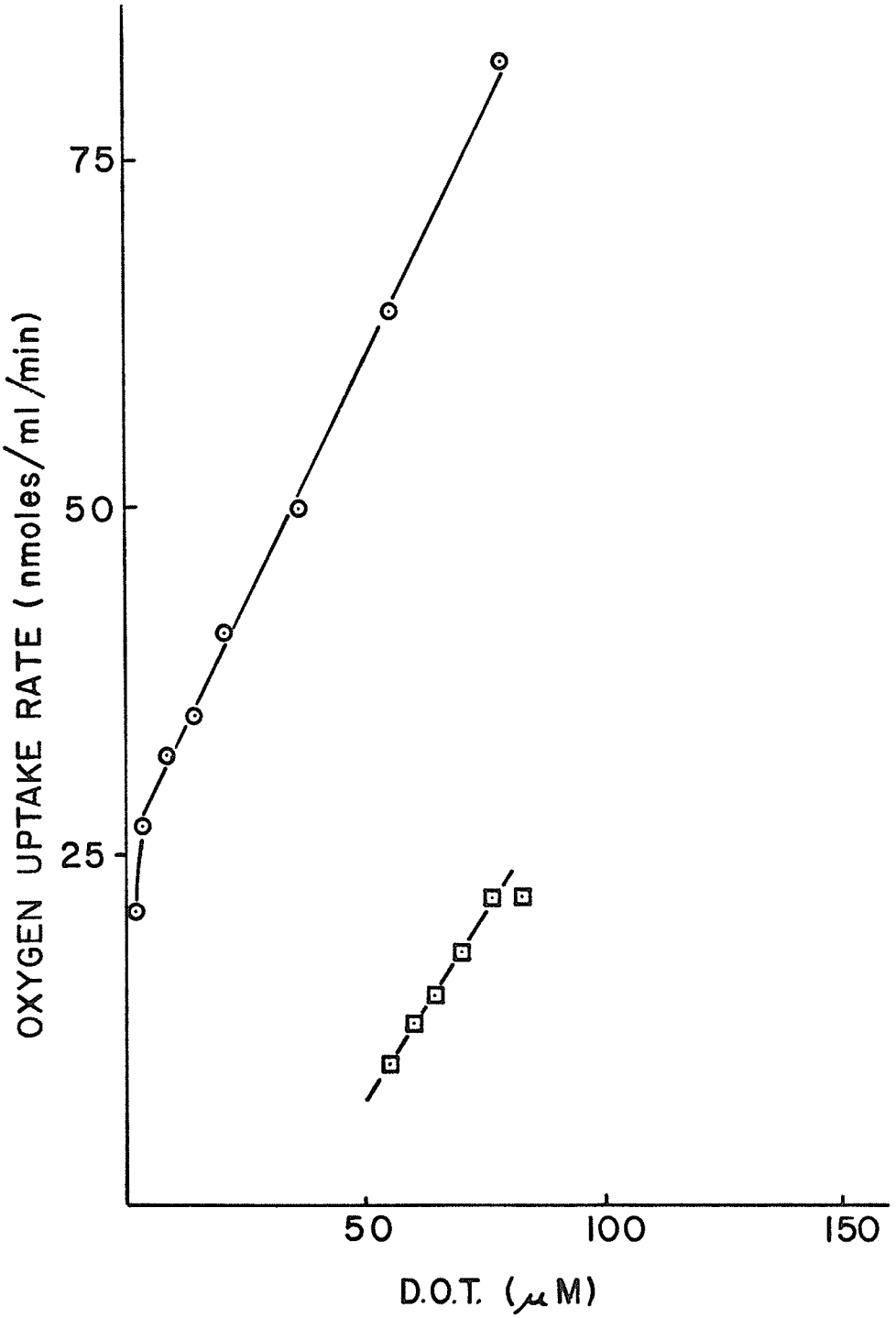

OXYGEN UPTAKE RATE (nmoles/ml /min)
75
50
25
50
100
150
D.O.T. (μM)

Fig. 53. Results from Fig. 52 as a function of time.

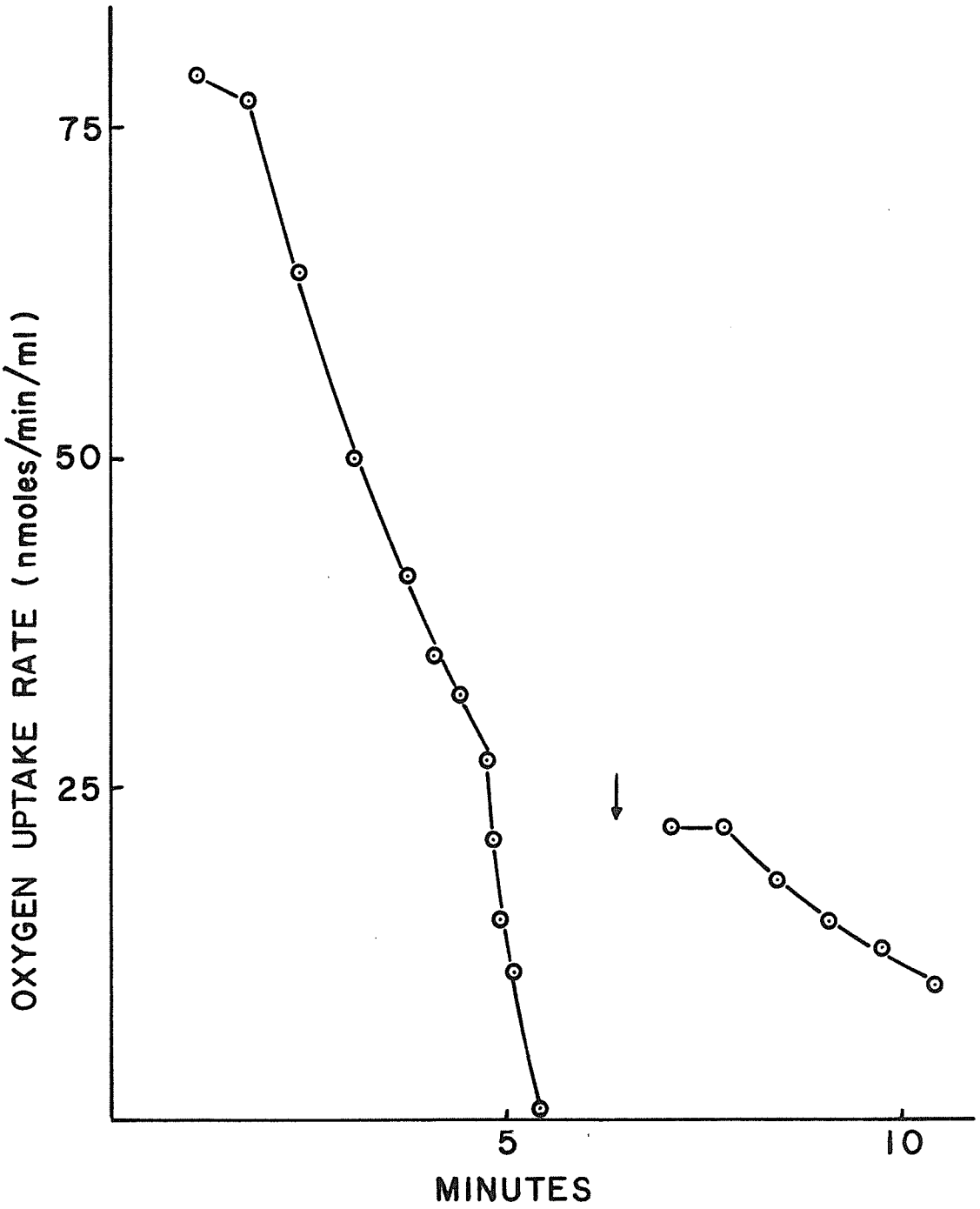

75
50
25
OXYGEN UPTAKE RATE (nmoles/min/ml)
5
10
MINUTES

Fig. 54. The oxygen uptake rates for batch-grown mannitol oxidizing A. vinelandii at different dissolved oxygen tensions in the presence of 25 μM KCN after either

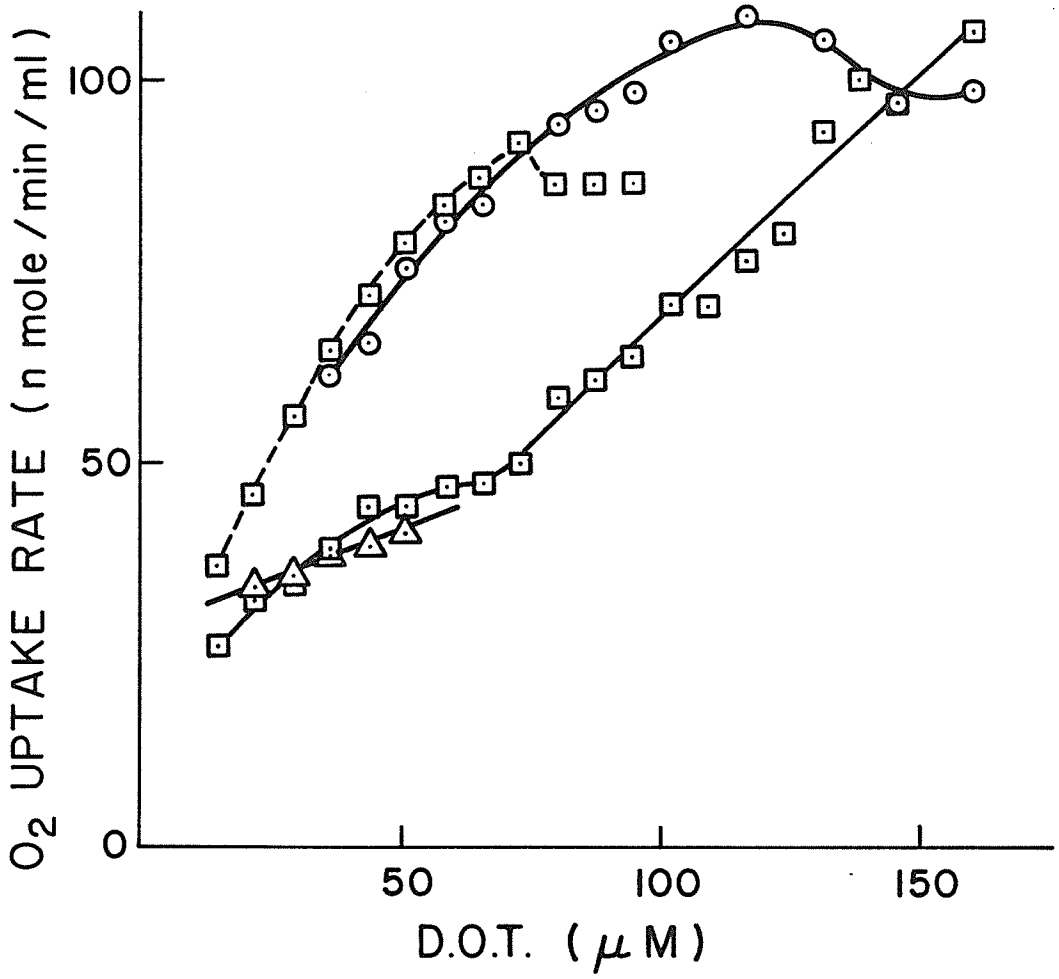
allowing oxygen tension to decrease immediately (□-----□, ○——○)

or

oxygen tension was maintained at approximately 180 μM for 2 minutes. (□——□)

or

oxygen tension was allowed to decrease to approximately 60 μM and maintain at this concentration for 2 minutes (Δ——Δ)


O_2 UPTAKE RATE (n mole / min / ml)
100
50
0
50
100
150
D.O.T. (μM)

oxygen tensions. Yet the deceleration of rates was greater for cells maintained at high oxygen tensions. This indicates that the amount of CN^- present after inactivating a given amount of oxidase is greater if the cells have been maintained in a fairly high concentration of oxygen. Therefore there may be a detoxification process occurring in cells at lower oxygen concentrations which removes a large amount of CN^-. The most likely enzyme responsible for this process is nitrogenase. This enzyme is capable of reducing cyanide to ammonia and methane. The enzyme activity, as was previously shown, is also reversibly sensitive to O_2, i.e. no N_2 was fixed when the dissolved oxygen rose above 60 μM.

A word of caution should be included with regard to comparisons between the batch grown and chemostat grown cultures. The large differences in cytochromes along with other differences (i.e. polysaccharide levels and cytochrome levels) make it difficult to relate conclusions from the results of one type of cultural condition to the second type.

DISCUSSION

DISCUSSION

1. Control of respiration in A. vinelandii

The very high rates of respiration, as well as the ability of Azotobacter cells to adapt their respiration to the rate of oxygen supply raises the question as to what specific enzymes are controlling the rate of oxygen uptake. Haaker and Veeger (1976) have suggested control of respiration may occur at the level of the uptake of carbon in carbon-limited cells. They found the rate of sucrose oxidation which is low under oxygen-limited conditions equaled the rate of pyruvate oxidation when the dissolved oxygen tension was high. These results suggest an inducible translocase enzyme, which may meter the carbon flow into the cell. Also, since the oxygen uptake rates of these two substrates are similar for cells grown in the presence of excess oxygen, the activity of the pyruvate dehydrogenase complex may limit the oxidation rate of both sucrose and pyruvate. The results presented in this dissertation indicate the control of respiration in Azotobacter may exist at three sites: the carbon substrate translocase, the pyruvate dehydrogenase complex and the NADH dehydrogenase.

Control over the first site, mannitol uptake, occurs under conditions of oxygen-limited growth. Cells taken from a chemostat culture grown under conditions of mannitol limitation demonstrate a much greater rate of ^{14}C-mannitol incorporation than oxygen-limited cells. The absence of significant acid production by cells in an oxygen limited state in the presence of excess mannitol also indicates that "in situ", the rate of mannitol incorporation into the cell is tightly controlled. As opposed to this, the rate of glucose uptake is apparently not under such strict control since an O_2 limited-culture oxidizing glucose produced a significantly greater amount of

acid than mannitol oxidizing cultures.

The increased activity of the translocase in mannitol-limited cells places the cell in a very competitive situation: however, a sudden increase of mannitol concentration turns the advantageously high translocase activity into a detrimental process. Under these conditions the cell absorbs a large amount of mannitol which is not completely oxidized but rather excreted as pyruvic acid. The reduction in pH of the medium to 5.2 then results in complete cessation of respiration. This situation is analogous to the excretion of metabolic intermediates by K. aerogenes, termed overflow metabolism (Neijssel and Tempest 1975). One important feature of overflow metabolism is that the products formed are always of a higher oxidation level than the substrate from which they are derived indicating acid production is not due to oxygen limitation. In K. aerogenes the two most important physiological considerations described by Neijssel and Tempest relate to (a) the affinity for the carbon substrate and (b) the pyridine nucleotide dehydrogenase. Both factors are apparently important in Azotobacter. The increase in translocase was observed during extreme mannitol limitation and since glucose, which is not immediately oxidized, allows the production of acid, the level of NADH in the cell may not play an important role in determining the carbon uptake rate.

The high rate of pyruvate production after mannitol addition and its continued production after the d.o.t. has been reduced to zero indicates that the increased activity of the translocase is not mediated by the NADH/NAD ratio, nor by the levels of some intermediate nor by the presence of excess oxygen in the culture. The

control of the translocase activity by adenine nucleotide levels also seems unlikely since a culture which has been completely without an easily oxidizable carbon source for several minutes, and so would be expected to have very low ATP levels, is still capable of producing pyruvate when given mannitol. Reduction in the amount of acid produced in the presence of dinitrophenol suggests that either ATP hydrolysis or an energized cytoplasmic membrane is required for the uptake of carbon. However since acid is still produced under these conditions it would appear that production of acid is the result of the high levels of translocase rather than simply high ATP levels.

The large amount of pyruvate produced and the apparent absence of other acids being formed indicate the second controlled site of respiration is at the pyruvate dehydrogenase enzyme. Dilworth (1961) described the activity of this enzyme to be inhibited by oxygen, on the basis that very high oxygen concentrations induced pyruvate production in A. vinelandii. However my results show pyruvate is produced when a pulse of mannitol is supplied to mannitol-limited cultures, under air, which had only sufficient mannitol to maintain the d.o.t. at zero. Therefore it appears unlikely that "in vivo" the pyruvate dehydrogenase would be inhibited by oxygen. Bresters (1975) has shown the activity of the enzyme to be competitively inhibited by NADH with respect to NAD^+ (Ki = 5 μM) or by acetyl-CoA with respect to pyruvate (Ki = 40 μM). Under conditions of a sudden high rate of carbon incorporation, the ratio of $NADH/NAD^+$ may be expected to increase thus inhibiting the enzyme. Therefore the high levels of pyruvate so produced may be a direct result of two factors, the increased translocase activity and the inhibition of pyruvate

dehydrogenase by NADH.

The role of pyruvate dehydrogenase as a regulating enzyme is also demonstrated by the nature of substrates which can induce the complete inhibition of oxygen uptake under conditions used to measure this activity. All substrates tested, with the exception of lactate, which enter the catabolic pathway prior to pyruvate dehydrogenase do not induce inhibition of respiration; however, Krebs cycle intermediates, glycollic acid and lactate all will inhibit respiration completely. This inhibition appears to be mediated by high NADH levels, which become inhibitory to NADH oxidation. This reduction of activity is similar to that reported by Hatefi and Stempel (1969) in which high NADH concentrations reduced the activity of mammalian mitochrondrial membrane-bound NADH dehydrogenase.

The pyridine nucleotide of anaerobic cells might be thought to be completely reduced, however this is not the case. Haaker and Veeger (1976) demonstrated that 5 to 30% of the pyridine nucleotides are present in the oxidized form when measured in anaerobic A. vinelandii cells. The results presented in Fig. 46 also indicate that the levels of reduced pyridine nucleotide increased upon the addition of substrate to anaerobic cells. However the greater rate of NADH formation indicate the cells are unable to control the oxidation of citrate and lactate as well as mannitol and also that the accumulation of reducing power beyond that which occurs in anaerobic conditions is possible.

The increased level of NADH in cells provided with lactate is apparently contrary to the results of Jones and Redfearn (1966), who showed lactate to be oxidized independent of pyridine nucleotides and

suggested a membrane bound lactate oxidase exists. However, unlike the other Krebs cycle intermediates the translocating system of lactate is constitutive, (Postma and Van Dam 1971) it may therefore easily enter the cell and overwhelm the membrane-bound dehydrogenase with reducing power. NADH, produced by the Krebs cycle intermediates present in the cell, would not be oxidized and would tend to accumulate.

The oscillatory respiration of mannitol-grown, carbon-limited cells when exposed to acetate may be a result of the reversible inhibition of NADH oxidation. When the dissolved oxygen tension of the medium is high the rate of NADH formation is balanced by the high rate of oxygen penetration into the cell. As the d.o.t. is reduced, the oxygen penetration rate into the cell would be lower than the high rate of NADH production from acetate. This would likely result in partial inhibition of respiration which in turn would permit an increased d.o.t. The higher d.o.t. would allow more oxidation of NADH, which by removal of the inhibitory effect of NADH would reduce the d.o.t. thus producing regular fluctuations in respiration noted in Fig. 41.

The oxygen concentration at which this oscillation occurs is similar to the 32 μM O_2 described by Achrell and Jones (1971) as the concentration at which log growth became limited by oxygen in batch cultures of A. vinelandii, and so may represent the oxygen tension which just provides sufficient oxygen to the terminal oxidase to balance the supply of reducing power.

The inhibition of A. vinelandii respiration, in situ, by citrate reflects the high production of reducing power by this intermediate.

The apparent lack of inhibition by malate *in situ*, however, may reflect the rate of oxygen penetration in highly agitated cells being greater than the uptake of malate and/or the formation of reducing power. Under conditions used to measure oxygen uptake rate which permit inhibition of respiration by malate, the agitation of the cells is mild compared to the chemostat culture where no inhibition was observed. In the former condition the rate of oxygen transfer to the cell oxidase may be much lower than the rate of reducing power production, thus leading to the inhibition of respiration by the high levels of NADH.

In order to prevent the inactivation by NADH the cell must have mechanisms to control the level of reducing power. This may be one of the major roles assigned to the formation of poly-β-hydroxybutyrate. Ritchie *et al*. (1969) and Senior *et al*. (1972) demonstrated that under anaerobic conditions, reoxidation of NAD(P)H occurs principally via PHB formation. It was suggested this polymer acted as an electron sink into which excess reducing power could be channelled. The consumption of excess reducing power would then release the inhibition of glucose oxidation and citrate metabolism. High levels of PHB were found in *A. vinelandii* cells growing at a very low dilution rate, Table 1, and PHB granules could be observed in cells which have been grown with sufficient mannitol to lower the d.o.t. to zero. Under both conditions the cells are oxygen-limited. In the first condition (low dilution rate) the rate of oxygen transfer per cell may be lower than some minimum threshold value resulting in the accumulation of reducing power which then must enter PHB formation.

Competition between Respiration and Nitrogen Fixation

The competition between respiration and nitrogen fixation for reducing power was first suggested by Phillips and Johnson (1961). In the presence of excess oxygen the two systems compete for reducing power, but the greater affinity of respiration (or the autooxidation of flavodoxin hydroquinone) results in the inactivation of nitrogenase and a greater proportion of reducing power is transferred to the reduction of oxygen.

The results of increasing the oxygen tension from 10 to 30% above a culture of A. chroococcum showed that the nitrogenase enzyme was not inactivated by such treatment. On the contrary, the acetylene reduction capability of such cells was immediately increased. But, the lack of actual nitrogen fixed in situ indicated the nitrogenase was inhibited by the presence of excess oxygen. The critical concentration of oxygen in solution below which nitrogenase is permitted to function appears to be between 30 and 60 μM O_2 which is again similar to the d.o.t. at which logrithmic growth changes to oxygen-limited growth in batch cultures of A. vinelandii.

The explanation of the inhibition of nitrogenase function by oxidation of hydroquinone in excess oxygen may be an oversimplification. The demonstration of nitrogen fixation in A. vinelandii cells, in the presence of excess oxygen, and the ability of these cells to grow for long periods in a steady state, indicate the reduction of oxygen is not given complete priority over nitrogen fixation. Also for carbon-limited cultures, and SO_4-limited cultures, the R.I. did not increase much more than twice the value under oxygen-limited conditions. This may be interpreted as indicative of two sites of

phosphorylation occuring in Azotobacter whereby one site is uncoupled by excess oxygen. Therefore at a d.o.t. of approximately 35 μM the amount of energy produced is half that of oxygen-limited cultures. And since these cells are still capable of fixing nitrogen and so evidently are capable of transferring sufficient reducing power for this process at d.o.t. between 0 and 35 μM O_2, the nitrogenase function may be limited by the energy produced from respiration. Therefore when the respiration becomes uncoupled from energy production due to the presence of excess oxygen (Achrell and Jones 1971b), the activity of the nitrogenase would also be limited to the same extent, resulting in a reduction of biomass production.

Toxic Effects of oxygen on Azotobacter

The presence of excess dissolved oxygen in a vortex stirred culture of A. vinelandii is apparently required in order to observe definite effects of oxygen on the metabolism of these organisms. By use of carbon limitation which, out of necessity introduces a second variable, the d.o.t. of a culture of A. vinelandii can be maintained at a high concentration. There are many effects accompanying an increase in d.o.t. brought about by mannitol limitation. The increase in R.I. is the most surprising in view of the carbon-limited nature of growth. These cells are apparently maintaining a process which wastes carbon at the expense of cell production, in an environment which selects for actively growing cells.

The increased levels of SOD production under conditions of excess oxygen is apparently contrary to that reported by Buchanan (1978) for A. chroococum cells. However this worker maintained the d.o.t. at zero and measured the effect of increased rate of oxygen

transferred to these cells rather than measuring the direct effect of excess oxygen.

The increased level of catalase reflects higher production of hydrogen peroxide in A. vinelandii under conditions of excess d.o.t. The high rate of respiration of these cells makes it likely that the hydrogen peroxide may originate from the incomplete oxidation of oxygen at the cytochrome oxidase level. Both of the main oxidases which are apparently operating are capable of the formation of an oxygenated species (Kauffman 1973, Yang and Jurtshuk 1978). The cytochrome o from Vitreoscilla has been shown to produce hydrogen peroxide as a normal product in the reduction of oxygen (Webster and Orii 1977). In the mechanism proposed for Vitroscilla, two molecules of cytochrome o bind one molecule of oxygen to form a compound "D"; this compound undergoes further oxygenation to produce the oxygenated form i.e. $(b^{+2}b^{+2})\text{-}O_2$ which must interact with a second reduced unoxygenated cytochrome o molecule for oxidation. In Azotobacter the general low d.o.t. as well as the low level of catalase under conditions of zero d.o.t. suggest the cytochrome o may completely reduce oxygen at the level of compound D. However in the presence of excess oxygen, which results in an apparent loss of affinity by both oxidases the compound D may be oxygenated. Since total reduction of oxygen to H_2O depends upon the presence of two reduced cytochrome molecules bound to one oxygen molecule, the cytochromes if present as an excess in the oxygenated form, may be unable to obtain reducing power to reduce $H_2O_2 \rightarrow H_2O$ and so release H_2O_2 into solution after a simple two electron transfer.

The apparent reduction in activity of the cytochrome oxidases at low d.o.t., after exposure to excess oxygen, is not observed

in cells which have been grown in batch culture. These cells differ at the level of the cytochrome oxidase by the presence of cytochrome $\underline{a}_1$, which is undetectable in chemostat-grown cells. It is tempting to speculate that the role of cytochrome $\underline{a}_1$ in these cells may be to interact with the oxygenated cytochrome $\underline{a}_1$ or $\underline{o}$ allowing complete reduction and thus maintaining high activity in the cytochrome oxidases.

Effect of KCN on the oxygen uptake rate of A. vinelandii

The respiratory response to cyanide by A. vinelandii has been previously studied (Kauffman and Van Gelder 1973, Jones, 1973) and CN^- has been used as a tool to differentiate the activity of the terminal oxidases of this organism. Artificial electron donor oxidations which are mediated by the minor respiratory pathway, are very susceptible to CN^- inhibition (Ki = 0.5 μM). The dissociation constant of the enzyme-inhibitor complex of the major oxidase (cytochrome $\underline{d}$) is believed to be much higher (15-115 μM KCN). Recently the specificity of CN^- for the minor pathway oxidases has been questioned (Hoffman et al. 1979), since this author found the V_{max} for cytochrome $\underline{d}$ to be decreased to almost one fifth by 12.5 μM cyanide.

The cytochrome $\underline{d}$ is believed to exist in at least three forms; one reduced and two oxidized forms. The reduced form is most prevalent when the cell has become anaerobic and it absorbs light at 631 nm. The first oxidized form occurs when cytochrome $\underline{d}$ is exposed to excess oxygen and can be identified by its absorption at 648 nm. The second oxidized form appears to be an intermediate species occuring upon reduction of the first oxidized form. This last form does not absorb light in the red end of the spectrum (Kauffman 1973a). The

formation of this oxidized intermediate species is dependent upon the turnover of the enzyme. CN^- does not appear to bind either the reduced or the first oxidized form, but does inactivate the oxidase when the cytochrome is in the oxidized intermediate form. Therefore, analysis of the kinetics of the CN^- inactivation of cytochrome d must take into account the fact that a large pool of cytochrome d exists, yet from this pool only a small portion may be available for inactivation at a specific instant. The size of the pool would depend upon the turnover of the enzyme demonstrable by the amount of oxygen which has been removed from solution.

Another parameter which may affect the results of CN^- inhibitor studies is the nucleophilic character of CN^-. This inhibitor has been shown to affect other enzymes, i.e. SOD, xanthine oxidase, diamine oxidase (Malmstrome et al. 1975, Bray 1975) and it also serves as an analogue substrate for the enzyme nitrogenase. This enzyme is capable of reducing cyanide to methane and ammonia and possibly other products. The Km for cyanide is believed to be of the order of 0.4 to 1.0 mM, approximately 10 fold greater than the Km for N_2 (0.03-0.12 mM) (Burns and Hardy 1975). Normally the relatively low affinity of nitrogenase for cyanide would not provide a significant detoxification process, but circumstances which may allow greater reduction rates of N_2 thus lowering the concentration of this gas in the cell may induce considerable reduction of CN^- as an alternate substrate. Such a case may occur if nitrogenase is suddenly activated by decreasing d.o.t. The activity of nitrogenase under these conditions would not be allosterically controlled by cellular pools of nitrogenous intermediates and the high rate of N_2 uptake may rapidly

reduce the N_2 within the cell, allowing greater detoxification of cyanide to occur. Reduction of cyanide levels within the cell would be accompanied by a plateau in the reduction in oxygen uptake rate with decreasing oxygen concentration. Such a plateau was observed in several assays, the most notable being oxygenated cells in the presence of 10 μM KCN (Fig. 50). Another plateau is plotted in Fig. 48 and occurs at a higher O_2 concentration, for cells treated with 11.75 μM KCN.

Both detoxification of CN^- and specificity of CN^- binding to the intermediate form of cytochrome d may be used to explain the observed pattern of reduction in oxygen uptake activity in A. vinelandii. The apparent linear decrease of activity with decreasing d.o.t. would be expected if cyanide binding depends upon the turnover of the enzyme. The relationship between the amount of CN^- bound and O_2 reduced appears to be constant at high d.o.t. (i.e. between 50-150 μM). This constant relationship indicates that oxygen may not interfere with CN^- binding. However, contrary to this, decreasing rates of oxygen uptake at very low oxygen concentration (i.e. below 20 μM) (Fig. 48, 15 μM KCN) indicate CN^- and oxygen may indeed compete for the active site.

Since the slope of the oxygen uptake rate versus d.o.t (Fig. 50) is less for 15 μM than for 20 μM KCN, the linear inactivation of cytochrome d would have a slope dependent upon inhibitor concentration. Initially because of an excess of oxidase and since electron flow limits O_2 uptake the inactivation of the enzyme would not likely be observable. However when the rate of O_2 uptake falls below the rate of maximum electron flow the decrease in observed rate appears

to be dependent on the rate of oxygen consumption. At lower concentrations of CN^- the observable inactivation of oxygen uptake would occur at a lower d.o.t. as seen in Fig. 50.

During the period when the cells have been maintained at high d.o.t. the nitrogenase activity would be expected to be reduced. When the concentration of oxygen is lowered to between 50 to 100 μM activity of the nitrogenase would be re-initiated. The high activity of the nitrogenase during this period may provide a certain degree of CN^- detoxification resulting in departure from linearity with a resultant plateau (observed in Figures).

The large amount of enzyme which has been inactivated by 20 μM KCN places the previous estimates of the Ki value of CN^- on cytochrome d in question. Using the maximum observed activity (an underestimate) and the minimum observed activity to describe enzyme levels the value of the Ki can be roughly calculated to be $\frac{(0.03)(20\ \mu M)}{(0.44)} =$ 1.36 μM. This value is a definite overestimate but illustrates that the binding of CN^- to cytochrome d occurs much more readily than has previously been shown. The Ki value appears to approach the Ki value of the minor path oxidase (0.5 μM). Therefore discrimination of the two pathways on the basis of CN^- inhibition would not appear very specific. The main difference between the cytochrome oxidase o and d with respect to CN binding may be the mechanism of binding rather than the dissociation rate of the enzyme inhibitor complex.

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