THE ROLE OF OXYGEN PROTECTIVE AND SCAVENGING MECHANISMS IN THE RESPONSE OF NITROGEN FIXING *AZOTOBACTER CHROOCOCCUM* TO OXYGEN

BY

ALFRED G. BUCHANAN

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DEDICATION

For Monique Nicole - and the future she represents,
with the fervent hope that her
world will be better than mine.

ABSTRACT

The response and marked sensitivity of Azotobacter chroococcum, and of the nitrogenase complex to oxygen, is well documented. To date however, no explanation has been given which adequately considered the role of oxygen protective and scavenging enzymes in this organism. The present work examines the contribution of the two primary oxygen-protective enzymes - catalase and superoxide dismutase - to the physiological response of Azotobacter to oxygen. Other enzyme activities - cytochrome oxidase, nicotinamide adenine dinucleotide oxidases, and others, which may influence such a response - are also examined.

In the absence of what was felt to be a convenient and reliable assay for superoxide dismutases, particularly applicable to crude extracts, a method of assay was devised which made it possible to monitor accurately SOD (superoxide dismutase) levels in crude extracts prepared from the organism grown in continuous culture with intense vortex aeration.

The levels of SOD present in the organism did not vary significantly with the oxygen content of the medium in which the organism was grown. In contrast to SOD, the catalase activity of Azotobacter chroococcum whole cells doubled in organisms grown with 30% oxygen on comparison to those grown with 20% or 5% oxygen. Catalase thus responded to the oxygen supplied to the organism, a

necessary response in view of the assumption that hydrogen peroxide is probably the most stable and most relatively long-lived product of the dismutation processes involved in metabolic utilisation of oxygen. H_2O_2 has been implicated in several instances of damage done to cell membranes, and in physiological changes having profound effects on the energy relationships of organisms.

The possible effect of O_2^- on Azotobacter was indicated by a reproducible inhibition of whole cell nitrogenase activity by enzymically generated exogenous O_2^-. The degree of inhibition of whole cell nitrogenase produced by O_2^- was apparently related to the oxygen supply of the organism; nitrogenase activity in cells cultured with 30% O_2 was more sensitive to inhibition by exogenous O_2^- than was that in cells grown with 20% or 5% O_2. This inhibition of whole cell nitrogenase was specifically attributed to O_2^-, since the addition of bovine SOD prevented these inhibitions. Moreover, free radical scavengers - ethanol and sodium benzoate - were not effective in reversing the inhibition produced by the xanthine-xanthine oxidase O_2^- - generating system. Some indication was given (by varying the quantities of O_2^- produced in the presence of differing concentrations of cells) that exogenously produced O_2^- may act competitively in its inhibition of whole cell nitrogenase activity. Exogenously added peroxides (H_2O_2, and ethyl hydrogen peroxide) also inhibited whole cell nitrogenase activity; the

inhibition so produced was not prevented by catalase and/or horse radish peroxidase. Viability studies indicated that the inhibitions noted as being effected by these exogenous agents were not attributable to general cytotoxicity.

Some idea of the adaptive response of nitrogenase (under the growth conditions specified) was obtained from examination of the enzyme levels in cells which were suddenly switched from growth with 5% O_2 to that with 20% O_2. Nitrogenase levels in whole cells so treated initially fell, rose after several hours, and returned to former levels after several days. The repression of nitrogenase activity by ammonium showed a similar pattern of response.

The location of SOD (and catalase) was inferred from analysis of cell fractions. Most of the SOD activity detected was found in the "soluble fraction" of the cell. Smaller quantities were associated with the "large fragment" (membranes, vesicles), and minimal quantities were detected in the "small fraction". The enzyme was apparently released by osmotic shock, hence may occur as a soluble enzyme in Azotobacter. A structural-functional model for nitrogenase and membrane vesicles is proposed; the vesicles are regarded as being susceptible to attack by O_2^- (and similar species) to effect exposure of the previously protected nitrogenase to inactivation resulting from distortion and/or rupture of the membranes. O_2^- reacts with sulphydryl groups - which are thought to be an integral part of nitrogenase catalysis -

thus may produce conformational changes in the enzyme, exposing previously protected groups, and depriving the nitrogenase complex of conformational protection.

Exogenous O_2^- also inhibited O_2 uptake by Azotobacter whole cells, suggesting quite general effects on the organism's physiology, effects which conceivably could alter the energy producing mechanism of the cell, with attendant consequences on growth and other cellular processes.

The SOD was apparently not secreted by actively growing cultures of Azotobacter chroococcum under normal growth conditions. There were apparently two or three forms of SOD present in crude extracts, initially differentiated by varying mobilities on polyacrylamide gels. An SOD enzyme was partially purified and characterised according to its molecular weight, reactions with cyanide, and electrophoretic mobility.

Changes in macromolecular composition - protein, polyhydroxy butyrate, among others, were as found in previous reports. Relatively greater quantities of NADH oxidase were found on increasing the O_2 content of the growth medium. Cytochrome oxidase activity also varied as previously found. A functional peroxidase was not detected. It was therefore proposed that Azotobacter chroococcum was sensitive to oxygen intermediates produced during respiration, and that adverse effects on the organism indicated production of reactive intermediates at a rate (or in quantities) which override the normal protective capacity of the cell, as produced by growth of the organism in elevated oxygen supply.

The ability of the bacteria to process large quantities of oxygen is regarded as being a compromise effected between efficient protection of the sensitive nitrogenase and normal cellular oxygen requirements. This compromise is effectively maintained under normal growth conditions - when the oxygen protective mechanisms are adequate - but cannot adapt to the degree required to ensure survival and functioning of the nitrogenase complex under conditions where oxygen is abundant or excessive. A limit is thus imposed on the organism's protective capacity.

A model was proposed to explain the control over nitrogenase activity exercised by oxygen. The nitrogenase was seen as functional when in contact with a membrane, in the "on-site" position. Inactive nitrogenase was produced by loss of contact with the membrane, in the "off-site" position. This 'off-site' position was deemed attributable to electrostatic repulsion and/or allosteric changes in nitrogenase, which were presumably mediated by an abnormally rapid rate of production of O_2^- during oxygen stress. The 'off-site' form is viewed as being equivalent to the conformationally protected inactive nitrogenase.

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ABBREVIATIONS

Ac.c	-	acetylated cytochrome c.
ATP	-	adenosine triphosphate
ATPase	-	adenosine triphosphatase
DAB	-	diaminobenzidine
DEAE	-	diethylaminoethyl
DER	-	diglycidyl ether
DMAE	-	dimethylaminoethanol
DNA	-	deoxyribonucleic acid
DNA se	-	deoxyribonucleotidase
EDTA	-	ethylenedinitrilotetraacetic acid
EPR	-	electron paramagnetic resonance
ERL	-	vinyl cyclohexane
ESR	-	electron spin resonance
HRP	-	horse radish peroxidase
HTP	-	hydroxylapatite
NADH	-	nictoinamide adenine dinucleotide (reduced)
NADPH	-	nicotinamide adenine dinucleotide phosphate (reduced)
NBT	-	nitroblue tetrazolium
NMR	-	nuclear magnetic resonance
NSA	-	nonenyl succinic anhydride
O_2^-	-	superoxide anion
.OH	-	hydroxyl radical
pHB	-	polyhydroxybutyrate

ppm	-	part per million
SDS	-	sodium dodecyl sulphate
SOD	-	superoxide dismutase
TEMED	-	tetramethylethylenediamine
tRNA	-	transfer ribonucleic acid

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INTRODUCTION

INTRODUCTION

The Azotobacteriaceae are characterised by intake of large amounts of oxygen, and by a marked sensitivity of their nitrogenase enzyme complex to oxygen. Numerous reports have documented a large number of oxygen mediated effects on the general physiology, growth, and metabolism of Azotobacter chroococcum. The assumption had been made that the effects noted were attributable to molecular oxygen. However, evidence now available indicated that molecular oxygen itself was probably not as toxic a species as previously assumed. The toxic effects noted were therefore more properly assigned to the reactive intermediates produced by the metabolic utilisation of molecular oxygen. These reactive intermediates, namely, the superoxide anion (O_2^-), the hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), and singlet molecular oxygen ($^1O_2$), are reportedly produced by a large number of biological processes.

The toxic effects of the above-named oxygen by-products have been demonstrated in a large number of organisms, ranging from bacteria to vertebrates, including man. These reactive intermediates effect inactivation of deoxyribonucleic acid (DNA); they destroy cell membranes; they inhibit enzyme systems, and are lethal to numerous organisms. All aerobic organisms are capable of producing, and do produce, all or several of these reactive and

toxic by-products. It was therefore regarded as vital that organisms be equipped with the necessary defense mechanisms to confer protection from the markedly adverse and potentially lethal effects of these intermediates. These defense mechanisms consisted of the superoxide dismutases and the catalases, with peroxidases contributing in some organisms. These enzyme systems were responsible for transformation of the previously toxic reactants to relatively innocuous species.

The defense mechanisms provided by the superoxide dismutases, catalases, and peroxidases, were regarded as prerequisites for an aerobic existence. Their presence and distribution - particularly that of the superoxide dismutases - were correlated with the degree of adaptability to aerobic existence displayed by organisms.

In view of the toxicity hitherto attributed to molecular oxygen now being attributed to the above reactive by-products, it became desirable to reassess the responses of Azotobacter chroococcum to oxygen as mediated by these intermediates. This report therefore documents some of the responses of A. chroococcum which may, directly or otherwise, be mediated by the superoxide anion, the hydroxyl radical, or by hydrogen peroxide, and by singlet oxygen.

It became necessary therefore, to see which of the defense mechanisms A. chroococcum possessed. The organism had been shown to have an active catalase, so their superoxide dismutase activity was examined. There was then no reliable method which

enabled the facile examination of crude extracts of biological material as to SOD content. An assay procedure to satisfy these requirements became a prerequisite to examination of A. chroococcum's response to oxygen, as influenced by this enzyme. The assay method herein developed became an integral part of this study.

The role of the nicotinamide adenine nucleotides in the response of Azotobacter to oxygen has been regarded as pivotal. The effect of oxygen supply on related enzyme activities thus acquired some significance, and was examined.

A major section of the literature relative to the response of A. chroococcum to oxygen, had been based on methods of cultivation involving batch culture methodology. This method had imposed some limitation on the validity of the data so obtained, and the continuous culture of A. chroococcum now provides a more acceptable method of growth. Information relating to the adaptive responses of A. chroococcum in this study was obtained largely therefore from continuous cultures.

The nitrogenase of the Azotobacter has been shown to be extremely sensitive to oxygen. Information on the response of nitrogenase to the superoxide anion and other species herein mentioned has been sparse and largely coincidental. Nitrogenase control, as influenced by oxygen, has been regarded as being based on respiratory and conformational protection. This present work

attempts to provide an adequate explanation for the observed effects of oxygen on nitrogenase, as mediated by the reactive intermediates produced from utilisation of molecular oxygen. Evidence is presented which documents some direct interaction between the nitrogenase complex and these intermediates. The theory of conformational and respiratory protection of nitrogenase was re-evaluated to accommodate the role of these toxic intermediates.

The present work therefore extends a more modern view of oxygen utilisation to embrace the responses of a bacterial species which, on the one hand, is an efficicient user of moleccular oxygen,and on the other, is markedly sensitive to oxygen. The study contributes to the growing body of information on aerobic existence, and as such relates in some fashion, to the fate of all organisms - from bacteria to man - in an environment which is largely aerobic.

HISTORICAL

HISTORICAL REVIEW

THE PRIMITIVE EARTH

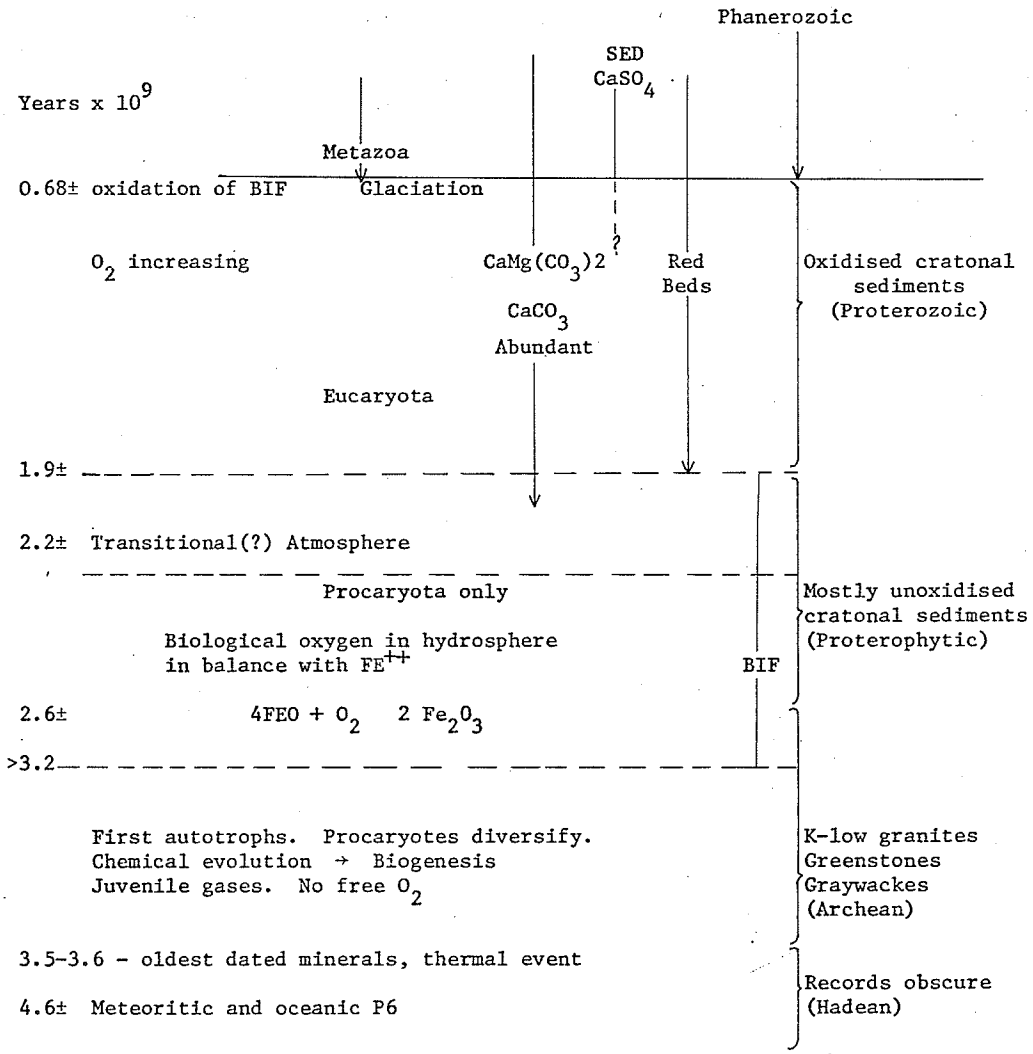
The earth's atmosphere at present contains approximately twenty percent oxygen. Early primitive atmospheres were anaerobic. Life on earth likely arose beneath an essentially anaerobic atmosphere some 3.4×10^9 years ago, as noted from the oldest known rock sediments. Oxygen utilising aerobic organisms could not have evolved until sufficient quantities of oxygen became available in the environment. Trace amounts of oxygen - thought to be locally present at that time - were presumably produced non-biologically, possibly by ultra-violet induced photodissociation of water (Moorbath, O'Nions and Pankhurst (1973). The small amounts of oxygen so produced were apparently consumed rapidly by reaction with previously unoxidised materials - volcanic gases, unoxidised iron compounds etc. - which were readily available in the earth's early environment. Quantitatively important alternate sources, producing large quantities of oxygen, must have existed to account for the onset of widespread oxygenic conditions. Organisms with a green plant type metabolism are such an alternate source. These organisms are efficient producers of oxygen; in the blue green algae, for example, the offspring from one gram of these organisms can ideally photosynthetically produce oxygen in amounts equal to that now present in the earth's atmosphere in less than forty days. One can reasonably assume that the onset of oxygenic conditions was a direct result of the appearance of relatively primitive algae. Paleological data now available indicates that

increasing quantities of oxygen may have been added to the environment, and that the transition from an oxygen deficient to an oxygenic environment started some 2000 million years ago, when communities of microscopic, oxygen producing blue green algal types possibly became widespread (Nagy 1974; Barghoorn and Tyler 1965). Such a transition was to have eternally profound impact on biological evolution, irrevocably altering the previously anoxygenic evolutionary pattern (see Figure 1).

An oxygen free - or virtually oxygen deficient environment, was probably mandatory for early geochemical and molecular developments. Free oxygen, in more than trace quantities, would have effectively precluded the origin of life as it is now postulated. The essential precursor molecules for living systems would not likely have been formed, since these precursors were readily oxidisable. The most probable high energy source then was ultra violet radiation, which would not have penetrated the ozone screen resulting from the presence of free oxygen in concentrations greater than one percent of the present atmospheric level (Cloud, 1974). Even if these precursor molecules had been formed, rapid oxidation in the presence of oxygen would have prohibited their accumulation in the quantities required to permit evolution, accumulation, and combination of such precursors to form the self-replicating systems described as living.

Some primitive organism may have evolved in a localised reducing environment, but such an organism would not have survived transfer to

Figure 1 Evolution of the primitive earth



From: Cloud, P. 1972.

an oxidising atmosphere in the absence of suitable oxygen protective mechanisms; oxygen would have been lethal to this organism in the absence of such protection. Biochemical evolution has in fact been effected so that most biological oxidations occur by hydrogen removal rather than by addition of oxygen, (Wald, 1964; Cloud, 1972), and a large number of basic metabolic processes in all organisms are anaerobic.

Primeval evolutionary circumstances notwithstanding, oxygen, a by-product of green plant-type photosynthesis, is now essentially involved in the major energy yielding biochemical processes in extant living systems, which oxidise organic compounds to produce biochemical energy through aerobic respiration. The majority of modern microorganisms and virtually all higher life forms are now dependent on atmospheric oxygen (Schopf, 1975).

Oxygen utilisation provides biochemical energy to drive life processes, why then is it necessary that an oxygen utilising organism should require protection from the very same substance on which its functioning depends? The nature of oxygen - its reactivity and transformation during metabolic processing, its reactive by-products and their effects - has to be examined to provide an explanation.

Some properties of molecular oxygen

Molecular oxygen, in the normal or ground state, is much less of an active oxidant than is expected, primarily attributable to

the imposition of a spin restriction as determined by the electron configuration of oxygen. Ground state or normal molecular oxygen contains two unpaired electrons having parallel spins, which prohibits direct entry of paired electrons, and requires that addition of spin-paired electrons to ground state molecular oxygen be effected so that inversion of one electron spin occurs, avoiding placement of two parallel spins in the same orbital (Taube, 1965). Moreover, the time required for a change in spin state is far greater than the life-time of colliding oxygen molecules and electrons. Univalent pathways of reducing molecular oxygen, as influenced by the energetics of the process, are thus more likely to occur than are divalent pathways, and these univalent reduction steps provide the basis for the toxicity attributed to oxygen.

Many biological reductions occur through multivalent pathways, for example, cytochrome oxidase (which accounts for much of aerobic oxygen consumption) produces water as the first readily detectable product of oxygen reduction (Malkin and Malmstrom, 1970). There are, however, a large number of biological oxidations which directly or indirectly generate some univalently reduced state of oxygen, in the form of the superoxide anion (O_2^-), and the hydroperoxy radical (.OH) (Fridovich, 1974). The complete reduction of oxygen to water requires the addition of four electrons, thus hydrogen peroxide (H_2O_2) may also be produced. The superoxide anion, the hydroperoxy radical, and hydrogen peroxide are thus the intermediates in question. Singlet oxygen may also be implicated.

Radical production from molecular oxygen

The superoxide anion is produced by a one-electron reduction of ground state molecular oxygen. The O_2^- anion may be regarded as the ionised form of univalently reduced oxygen, while the hydroperoxy radical may be regarded as the protonated form. The O_2^- and .OH radicals have been examined and characterised by several workers. The .OH radical is a weak acid with a pKa of 4.8 (Behar, Czapski, Rabani, Dorfmann and Schwarz, 1970), and can also be produced by univalent oxidation of hydrogen peroxide. Haber and Weiss (1934) have attributed the reactivity of a mixture of iron salts and water as a hydroxylating agent (Fenton's reagent) to the formation of the hydroxyl radical during the following series of chain reactions:-

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot$$

$$OH\cdot + H_2O_2 \rightarrow H_2O + H^+ + O_2^-$$

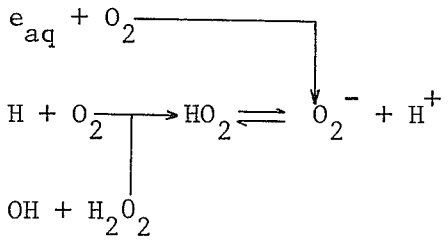
$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH\cdot$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + 2H^+ + O_2^-$$

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$$

The hydroperoxy radical (OH·) has been deemed the most potent oxidant known, and attacks virtually every type of organic molecule (Neta and Dorfman, 1968). This radical, which is highly energised, reacts at a rate approximating to that with which it collides with

other molecules; with ethanol, for example, reaction occurs with a bimolecular rate constant of $10^{9}M^{-1}$ sec^{-1} at $37^{o}C$ (Neta, 1967). Such an extreme reactivity results in existence of the species normally at very low concentrations, in the order of $10^{-4}M$ to $10^{-9}M$ (Pryor, 1976). The hydroperoxy radical can be readily generated by photochemical and radiation-induced reactions; in solution the radical can be produced by secondary reactions of solvated electrons, and hydrogen and hydroxide species;- (Czapski, 1971).



Some chemical reactions - the oxidation of hydrogen peroxide by cobalt and cerium salts; reaction of hydrogen peroxide with copper for example, produce the hydroperoxy radical (Czapski and Katakis, 1966).

The superoxide anion (O_2^-)

The superoxide anion (O_2^-) can be produced by the univalent oxidation of hydrogen peroxide, or by the univalent reduction of oxygen. O_2^- is also produced by a variety of physical and chemical methods, for example the reduction of oxygen by hydrated electrons, (Adams, Boog and Michael, 1965); the reduction of oxygen by reduced dyes on flavins, (Nishihimi, Rao and Yagi, 1972); and by the ferredoxins

(Misra and Fridovich, 1971). Several biological processes also produce O_2^-, as with phagocytosis (Patriarca, Dri, Kakinuma, Tedesko and Rossi, 1975); oxidative enzymes (xanthine oxidase, for example, Fridovich, 1970); tryptophan dioxygenase (Hirata and Hayaishi, 1971); aldehyde oxidase (Rajagopolan, Fridovich and Handler, 1964); reaction of reduced iron sulfur proteins with oxygen (Orme-Johnson and Beinert, 1969); and illumination of chloroplasts (Harbour and Bolton, 1975). Several other sources of O_2^- are given in a recent review by Fridovich (1976). O_2^- absorbs in the ultra-violet region, and has an extinction coefficient of $\Sigma_m O_2^- = 2000$ at 245nm (Behar et al., 1970).

Hydrogen peroxide

Hydrogen peroxide is the most stable of the intermediates produced by the reduction of molecular oxygen; this compound is also the least reactive and most easily detectable intermediate. Hydrogen peroxide may be generated directly by the divalent reduction of oxygen, or indirectly by the univalent reduction of molecular oxygen by dismutation of the superoxide so produced. Numerous oxidases also produce hydrogen peroxide during the reduction of oxygen, as in the case of uricase, and D-amino acid oxidase. The production of hydrogen peroxide has also been observed in liver slices (Portwich and Aebi, 1960); in mitochondria (Boveris, Oshino and Chance, 1972); tissue homogenates (Portwich and Aebi, 1960); illuminated chloroplasts (Halliwell, 1974); microsomal suspensions (Thurman, Ley

and Scholz, 1972); phagocytosing granulocytes (Paul and Sbarra, 1968); and in aerobic microorganisms (Zobelland, Little, 1967). The toxicity of hydrogen peroxide is well documented, as evidenced from the fact that the oxygen tolerances of aerobes and obligate anaerobes has been based on the toxicity of hydrogen peroxide; aerobes were thought to contain the enzyme catalase as a defence mechanism, whereas anaerobes presumably lacked catalase and were thus killed on exposure to molecular oxygen (Gordon, Holman and McLeod, 1953).

Examination of the interaction of the superoxide anion, the hydroperoxy radical, and hydrogen peroxide, with biological material, will demonstrate that the biological significance of oxygen metabolism rests basically on these reactive intermediates produced during utilisation of oxygen. The contribution of singlet oxygen to these phenonema will also be examined.

The Biological Effects of Oxygen Intermediates

The biological effects of the oxygen intermediates are attributable to their free radical characteristics. Many of the reactions reported for other free radicals have been documented to occur with one of more of the reactive intermediates from molecular oxygen. The incubation of tissue homogenates or suspensions of subcellular particles in the presence of air or oxygen produced peroxides as a result of the reactions initiated and/or propogated by free radical species (Barber and Wilbur, 1959).

Hydrogen peroxide has reportedly effected a lowering of adenosine triphosphate (ATP) levels in platelets (Holmes and Robkin, 1977), and in bacteria (Swedes, Sedo and Atkinson, 1975; and Chapman, Fall and Atkinson, 1971), and toxicity has been associated with the peroxidation of lipid in the endoplasmic reticulum (Slater and Sawyer, 1971). The peroxidation of membrane lipids has also been demonstrated to effect the destruction of many susceptible membranes and cytoplasmic constituents, such as oxidisable small molecules and enzymes (Bernheim, Wilbur and Kenaston, 1952); membraneous structures (Tappel, 1968); and cytoplasmic proteins (Tappel, 1966). Lysosomal membrane lipids have been observed to undergo peroxidation by chain reactions prompted by the perhydroxy radical (Fong, McKay, Poyer, Steele and Misra, 1973).

The rate of peroxidation of unsaturated fatty acids by free radical chain reaction is proportional to oxygen only at fairly low partial pressures, though hyperbaric oxygen toxicity has been partially equated to peroxide formation. The effect of oxygen may be indirect, resulting in increased radical formation through the increased activity of normal enzymatic processes (Gerschmann, Gilbert, Nye, Dwyer and Fenn, 1954). Certain atmospheric oxidants may initiate chain reactions leading to peroxide formation in membrane lipids. Nitrogen dioxide in relatively low concentrations has been found to induce lipid peroxidation in vitro, and in lung lipids of rats exposed to 1ppm of this gas (Thomas, Mueller and Lyman, 1968). Wills and Wilkinson (1966) have demonstrated that the disruption of lysosomal

membranes - with the release of lysosomal enzymes- could be effected by radiation and other conditions leading to peroxide formation. Hydrogen peroxide has been found to cause alteration in isolated deoxyribonucleic acid (DNA) to liberate all four bases. Hydrogen peroxide apparently disrupts the DNA sugar-phosphate backbone sufficiently to inactivate DNA (Freese, Gerson, Taeber, Rhaese and Freese, 1967; Rhaese and Freese, 1968; Uchida, Shigematsu and Yamafugi, 1965; Yamafugi and Uchida, 1966). Hydrogen peroxide and a photoproduct of tryptophan irradiation - in the presence of oxygen - acted synergistically with near u.v. radiation to kill cells and inactivate T_7 phage; and to enhance single strand breaks in DNA (Ananthaswamy and Eisenstark, 1976). Ananthaswamy and Einsenstark (1977) have attributed an increased sensitivity of Escherichia coli mutants to hydrogen peroxide to a reduced capacity in these mutants to repair single strand breaks induced by hydrogen peroxide.

The superoxide anion has been implicated as a mediator in drug induced oxidative hemolysis (Goldberg, Bruce and Stern, 1976); and in the toxicity of nitrofurantoin to produce pulmonary fibrosis, (Mason and Holtzmann, 1975) and in the lethality of streptonigrin to E. coli (White and Dearman, 1965). The exposure of DNA to a source of O_2^- resulted in single strand breaks in the DNA, brought about by a direct attack on the DNA or by generation of other radicals which subsequently attacked the DNA (White, Vaughn and Yeh, 1971). The superoxide anion may have caused depolymerisation of acid polysaccharides (McCord, 1974), and may also have caused oxidation of epinephrine, in addition to having acted as a chain-propagating radical

in its autoxidation (Misra and Fridovich, 1972). Thiol groups have been oxidised by the superoxide anion, in a reaction which also involved hydrogen peroxide (Misra, 1974). The peroxidation of lipids may also be initiated by the superoxide anion, which generated singlet oxygen to produce fatty acid hydroperoxides on reaction of the latter with lipids (Pederson and Aust, 1973). Nicotinamide adenine dinucleotide (reduced) (NADH) in free solution reacted very slowly with O_2^-, but this reaction proceeded rapidly on binding to lactate dehydrogenase; this reaction was initiated by O_2^- radicals, and propagated by oxygen (Bielski and Chan, 1973). Hydrogen peroxide, and the superoxide and perhydroxy radicals have been observed to cause hemolysis in erythrocytes of vitamin-E deficient rats (Fee and Teitelbaum, 1972). Goldstein and Weismann (1977) have suggested that O_2^- and related reactive molecules were capable of perturbing lipid bilayers to cause leakage of relatively impermeant anions, an observation which fitted the model for oxidative damage to membranes as proposed by Suwa, Kimura and Schaap (1977). The O_2^- anion has also been observed to have effected a decrease in the adenosine triphosphatase (ATPase) activity of bovine erythrocyte membranes, which were hemolysed on exposure to O_2^- (Bartosz, Fried, Grzelinska and Leyko, 1977).

It is thus apparent that all three of the oxygen intermediates - O_2^-, H_2O_2 and $\cdot OH$ - referred to above, are potentailly lethal, and can have a markedly adverse effect on several biological processes. In addition H_2O_2 and O_2^- can react to produce $\cdot OH$, an event which

occurred readily in buffered aqueous systems (Fridovich, 1976).

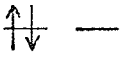
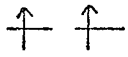
The above-mentioned intermediates are not the only reactive types with potentially harmful effects on biological processes. Excited or energised oxygen has two metastable singlet states, namely, $^1\Sigma$, and $^1\Delta$. The $^1\Delta$ singlet state is relatively long-lived and is likely that formed by biological systems (Kellog and Fridovich, 1975). The relationship between the different states assumed by oxygen is represented as shown in Table 1.

Singlet oxygen can be generated by several processes, among which are included (i) photosensitised oxidation (Foote, 1968), (ii) the reaction of hydrogen peroxide and O_2^- (Kellog and Fridovich, 1975), (iii) the disproportionation of hydrogen peroxide (Smith and Kulig, 1976), (iv) the electron generated radical ion transfer reaction (Mayeda and Bard, 1973), (v) the transfer of an electron from O_2^- to some suitable acceptor in illuminated chloroplasts (Takahama and Nishimura, 1975), (vi) the disintegration of lipid hydroperoxides produced by liver microsomes oxidising reduced nicotinamide adenine dinucleotide phosphate (NADPH).

Jefford et al., (1976), have presented evidence to show that monoxygenases which required α-ketoglutarate as cofactor produced singlet oxygen. The production of singlet oxygen from photochemical and biological systems is summarised in Fig. 2.

Singlet oxygen has been implicated in the oxidative destruction of histidine, which was shown to be the major cause of the inactivation of several enzymes (Spikes and Straight, 1967). Singlet oxygen may

TABLE 1. Oxygen states and their energy levels.

State of Oxygen Molecule	Symbol	Energy Above Ground State	Orbitals
2nd excited	1_{Σ}	37 K. cal	
1st excited	1_{Δ}	22 K. cal	
Ground	3_{Σ}		

(Table 1 according to Foote (1968))

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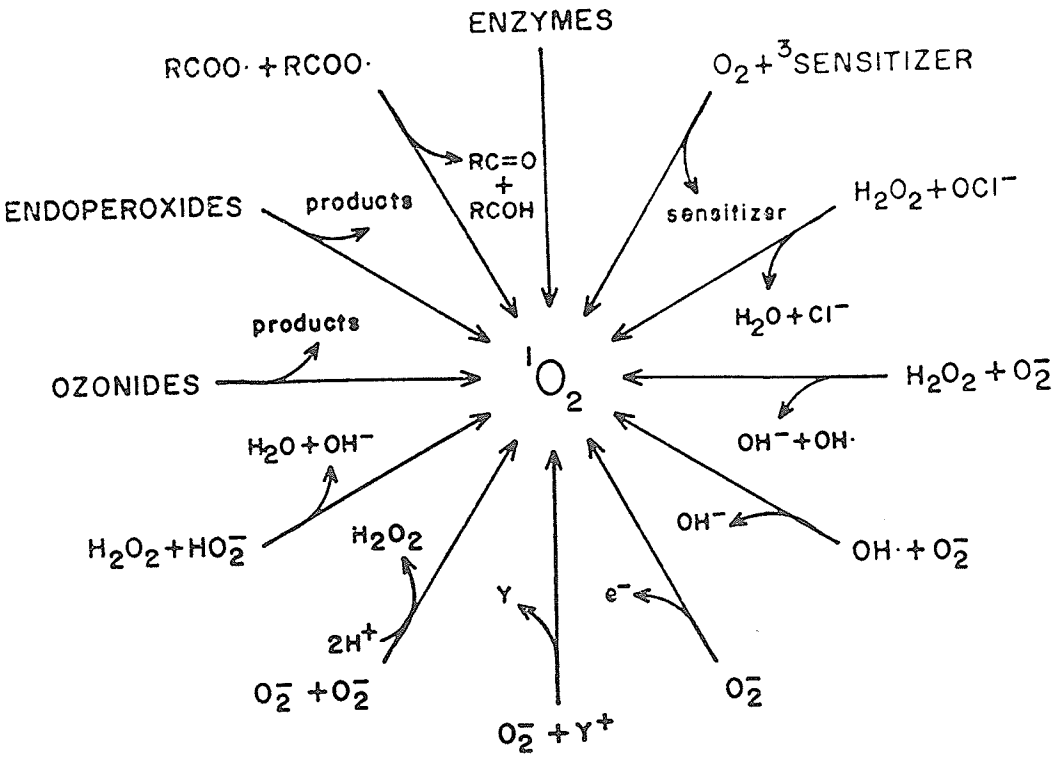
Figure 2 The production of singlet oxygen ($^1O_2$) by photochemical, chemical and biological systems.

(Taken from Krinsky, 1977).


ENZYMES
RCOO· + RCOO·
RC=O + RCOH
O_2 + ^{3}SENSITIZER
sensitizer
ENDOPEROXIDES
products
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_2^-
H_2O_2
OH^-
OH· + O_2^-
Y
e^-
$2H^+$
O_2^- + O_2^-
O_2^- + Y^+
O_2^-

also exert some influence on the reactions of tryptophan, methionine and cysteine (Weil, 1975). Guanine and purine derivatives were reportedly photoxidised in events which involved singlet oxygen (Simon and van Vunakis, 1965).

The production of reactive intermediates by biological systems

Free radicals are produced during operation of the terminal portion of the electron transfer chain involving the reduction of oxygen. Numerous enzymes and biological reactions have also been shown to produce free radicals. Fong, McKay, Poyer, Steele and Misra (1973), have shown that several oxidative enzymes produce the superoxide anion as a normal product of the one-electron reduction of oxygen. Fridovich (1970) has extensively analysed the production of superoxide by milk xanthine oxidase, and Khan (1970) has suggested that the generation of singlet oxygen may also occur in enzymatic systems. Sugioka and Nakano (1976), and King, Lai, and McKay, (1975), have presented evidence to support such a conclusion, and showed that the microsomal oxidation of NADPH resulted in singlet oxygen production, possibly from the breakdown of lipid hydroperoxides formed during microsomal oxidation. Superoxide production by rat liver microsomal fractions has also been reported (Mishin, Pokrovsky and Lyakhovich, 1976). Harbour and Bolton (1975), have documented the light induced generation of superoxide by chloroplasts in the presence of oxygen. Superoxide production was observed in the reaction of reduced iron-sulfur proteins with oxygen (Orme-Johnson and Beinert, 1969), and Misra and Fridovich, (1971) demonstrated this during the autoxidation of

ferredoxins. Ohtaki, Mashimo and Yamazaki (1973) have described a H_2O_2 generating system in hog thyroid microsomes, and Kakinuma, Boveris and Change (1977), have shown that subcellular leucocyte fractions generate hydrogen peroxide.

It now therefore has to be accepted that the generation of reactive free radical intermediates from oxygen during its metabolic utilisation does occur in several biological processes; this being so, it is appropriate to consider the protective mechanisms which exist to negate the demonstrably toxic effects of these reactive intermediates.

Oxygen scavenging and protective mechanisms

The toxicity of molecular oxygen is likely due to the highly reactive by-products of cellular oxygen consumption; namely, singlet oxygen, and the hydroxyl and superoxide radicals - all of which have been shown to have devastating effects on many biological processes. Obviously, the organisms best equipped to utilise molecular oxygen will be those organisms containing the necessary enzyme mechanisms, which essentially are:-

i) superoxide dismutases - to destroy the superoxide anion without the production of singlet oxygen

ii) catalase and/or peroxidase

iii) an agent which scavenges singlet oxygen, or confers some protection from this radical.

The superoxide dismutases generate ground state oxygen from the superoxide anion, preventing the spontaneous dismutation of this anion to singlet oxygen (Paschen and Weser, 1973). Superoxide dismutase also decreases the intracellular concentration of O_2^- to levels

which may prevent the generation of singlet oxygen by the Haber-Weiss reaction (Krinsky, 1977). It is the tandem role of superoxide dismutase and catalase to prevent the occurrence of the Haber-Weiss reaction and thus permit aerobic organisms to survive. Khan (1970) pointed out that even small quantities of singlet oxygen could have markedly adverse effects.

Hydrogen peroxide was initially thought to be responsible for oxygen toxicity (McLeod and Gordon, 1923; Callow, 1923), and though catalase may have conferred some protection (Callow, 1923), its activity alone does not allow aerobic growth. The relative importance of catalase, compared to superoxide dismutase, can be inferred from the observation that many organisms capable of aerobic growth do not contain catalase (Gledhill and Casida, 1969; Jones, Watkins and Meyer, 1970), and some strict anaerobes show catalase activity, yet are aerointolerant (Prevot and Thouvenot, 1952). McCord, Keele and Fridovich, (1971), have surveyed various microorganisms as to their superoxide dismutase and catalase contents, and have found that:-

(i) strict anaerobes did not exhibit superoxide dismutase activity

(ii) all aerobic organisms containing cytochrome systems displayed both superoxide dismutase and catalase activities

(iii) aerotolerant anaerobes - lacking a cytochrome system, yet capable of metabolising oxygen in a limited fashion - had superoxide dismutase activity, but lacked a catalase.

Such a distribution suggested that the prime physiological function of superoxide dismutase was the protection of oxygen metabolising organisms from the potential damage posed directly or indirectly

by the superoxide anion. McCord et al., (1971) have suggested that superoxide dismutase may be the single most important enzymic activity enabling organisms to survive in the presence of molecular oxygen. The occurrence of superoxide dismutase among all aerobic organisms, and its distribution among various mammalian tissues, within which superoxide is presumably generated, support this proposed physiological role. Myriad observations lend adequate credibility to this view. Among these are the following:-

(i) Superoxide dismutase was induced by oxygen in Streptococcus faecalis (Gregory and Fridovich, 1973a); in Escherichia coli (Gregory and Fridovich, 1973b); and in Saccharomyces cerevisiae (Gregory, Goscin and Fridovich, 1974). Cells with induced high levels of superoxide dismutase were rendered resistant to hyperbaric oxygen.

(ii) In Bacillus subtilis, an organism in which oxygen induced catalase but not superoxide dismutase, resistance to hyperbaric oxygen was not conferred (Gregory et al., 1973).

(iii) Catalase and peroxidase, but not superoxide dismutase, were induced by oxygen in E. coli K12, but these organisms did not become resistant to hyperbaric oxygen. Mutant organisms, with temperature-sensitive defects in superoxide dismutase activity, exhibited parallel defects in oxygen tolerance (McCord, Beauchamp, Goscin, Misra and Fridovich, 1973).

(iv) Superoxide dismutase has been said to confer some protection (McCord et al., 1973b) from the antibiotic streptonigrin,

which is thought to produce the superoxide anion during its action.

The protection provided by superoxide dismutase from the events mediated by the superoxide anion is also displayed by eucaryotic organisms. Superoxide dismutase has been shown to protect fetal calf myoblasts against photochemically generated superoxide anion (Michelson and Buckingham, 1974). The superoxide dismutase activity in the lungs of rats - which had adapted to tolerate 100% oxygen - was shown to increase to coincide with such an adaptive response (Crapo and Tierney, 1974). Superoxide dismutase has also been documented to prevent the swelling (induced by glutathione) of rat liver mitochondria (Fridovich, 1975). Superoxide dismutase reportedly conferred protection from radiation damage, during which reactive intermediates of reduced oxygen were produced, as in the erythrocytes of X-ray irradiated mice (Petkau, Kelly, Chelack and Barefoot, 1976); in bone marrow stem cells (Petkau, Kelly, Chelack, Pleskach, Barefoot and Meeker, 1975); in post-irradiated mice (Petkau, Chelack and Pleskach, 1976; Petkau, Chelack, Pleskach, Meeker and Brodsky, 1975); in Acholeplasma laidlawi (Petkau and Chelack, 1974); and phospholipid model membranes (Petkau and Chelack, 1976). Lavalle, Michelson and Dimitrejevic (1973), have shown that SOD confers protection in various biological systems, including bacteria, bacteriophage, yeast ribonuclease and transfer ribonucleic acid (tRNA) ligase.

It is thus evident that the superoxide dismutases are of primary importance, enabling organisms to overcome the dangerous and potentially

lethal effects of the reactive intermediates produced during the utilisation of molecular oxygen. The nature, occurrence, and characteristics of the superoxide dismutases will now be examined.

The superoxide dismutases

The superoxide dismutases can be grouped according to their metal constituents and occurrence as follows:-

1. copper and zinc-containing SOD
2. manganese-containing SOD of procaryotes
3. manganese-containing SOD of eucaryotic mitochondria
4. iron-containing SOD

1. The copper and zinc SODs

These have been found in the cytosols of eucaryotic cells. They have been obtained from a variety of organisms; namely, mammals (Beem, Rich and Rajagopolan, 1974); birds (Weiseger and Fridovich, 1973); plants (Asada, Urano and Takehashi, 1973; Sawada, Okayama and Yamazaki, 1972); and fungi (Misra and Fridovich, 1972). These enzymes are very similar except for relatively minor differences in amino acid composition and in details of electron spin resonance (ESR) spectra. The enzyme has been found to have a molecular weight of 32,000, and consists of 2 identical subunits, each subunit containing copper and zinc. The copper present was apparently essential for activity, which was partially restored by replacement of the copper, with full activity being produced by the replacement of both copper and zinc. The zinc may be replaced by cobalt, mercury, or cadmium without

apparent loss of activity (Forman and Fridovich, 1973; Beem, Rich and Rajagopalan, 1974; Fee, 1973; Rotillo, Calabreese and Coleman, 1973).

The structure of the bovine copper-zinc enzyme has been shown to consist of a cylinder with its walls made up of eight strands of the sequence in an antiparallel β structure. The metals were close together, and joined by the imidazole ring of histidine as a common ligand. The copper present was relatively exposed to solvent, whereas the zinc was buried inside the cylinder (Richardson, Thomas, Rubin and Richardson, 1974). The copper present was associated with a ligand field of histidines, and the copper at the active site was apparently surrounded by three or four imidazole rings (Fridovich, 1974; Fee and Gaber, 1972; Rotillo, Morpurgo, Giovagnoli, Calabreese and Mondovi, 1972; Stokes, Hill, Bannister and Bannister, 1973). The modification of tyrosine, lysine, and histidine residues has been correlated with a loss of activity (Barra, Bossa, Rotillo, Roberts and Fielden, 1975). The reaction of the bovine SOD apparently consisted of a mechanism in which the reaction of one of the copper atoms present, in either the uni-or divalent form, rendered the other transiently non-reactive to other superoxide species. A reaction scheme has been proposed (Fielden, Roberts, Bray, Lowe, Mantner, Rotillo and Calabreese, 1974), which postulated two singly reduced enzyme species, one of which had the divalent copper atom reactive while the monovalent copper was not reactive; this was apparently reversed in the other enzyme species. Such a scheme

has been thought to involve an allosteric type mechanism. The anaerobic reduction of the bovine copper-zinc enzyme with dithionite effected a bleaching of the divalent copper (EPR) electron paramagnetic resonance spectra, which indicated that the enzyme was capable of accommodating one electron per divalent copper atom (Weser, Bunnenberg, Commack, Djerassi, Flohe, Thomas and Voelter, 1971).

Direct electron transfer between the superoxide anion and divalent copper required that the metal be accessible to water; nuclear magnetic resonance (NMR) indicated that the divalent copper was exposed to solvent (Gaber, Brown, Koenig, Fee, 1972; Bowden, Holmes and Knowles, 1974). Cyanide was reportedly bound to the divalent copper, with the carbon on the cyanide liganded to the metal (Hoffner, and Coleman, 1973), to reversibly inhibit the enzyme. Hydrogen peroxide has been shown to reduce the divalent copper present, and irreversibly inactivated the enzyme at concentrations in excess of 10 μM (Simonyan, and Nalbandyan, 1972; Rotillo, Calabreese, Morpurgo and Mondovi, 1973; Fee and Dicorleto, 1973; Bray, Cockle, Fielden, Roberts, Rotillo and Calabreese, 1974).

The N-terminus of the bovine enzyme can be blocked by acetylation (Fridovich, 1974). The ultra violet absorption spectrum of the bovine copper-zinc enzyme was reportedly atypical, and exhibited a maximum at 258 nm, thus resembled the absorption spectrum of phenylalanine (Keele, McCord and Fridovich, 1971). The metal prosthetic groups may have contributed to the u.v. absorption noted, since the apoenzyme

absorbed only half as much as the holoenzyme, both copper and zinc being required for full restoration of the spectrum (Bannister, Bannister and Wood, 1971). The blue colour of the copper-zinc enzyme was attributed to a weak absorption at 680 nm (McCord and Fridovich, 1969). This enzyme was found to be remarkably stable for several hours at room temperature, and was capable of tolerating treatment with 95% ethanol for several hours (McCord and Fridovich, 1969). Assays have been conducted in the presence of 9.5M urea; the enzyme was found to be stable up to pH 11.5, but was irreversibly inactivated at pH 12.0 (Rotillo, Finazzi, Calabreese, Bossa, Guerreri and Mondovi, 1971); brief boiling also resulted in irreversible inactivation. The enzyme was also found to be stable in 0.1N hydrochloric acid, and in 6.0M guanidium chloride, but was irreversibly inactivated by 0.1M hydroxylamine.

Manganese superoxide dismutase

This enzyme has been isolated from several microorganisms, including E. coli β (Keele, McCord and Fridovich, 1970), Streptococcus mutans (Vance, Keele and Rajagopalan, 1972), and Bacillus stearothermophilus (Brock, Harris, and Sato, 1976). The bacterial enzyme (which apparently is totally unrealted to the copper and zinc enzymes) is a dimer made up of two subunits of equal size, each subunit containing one atom of manganese; and has a molecular weight of 40,000. The manganese present is trivalent (Villafranca, Yost and Fridovich, 1974), and was deemed essential for enzymic activity (Brock et al., 1976). However, the site to which manganese normally

bound could accommodate divalent cobalt, divalent nickel, or divalent zinc (Ose and Fridovich, 1976). These same metals, in 100 fold molar excess over manganese, competed effectively with the manganese (Ose and Fridovich, 1976). The manganese enzyme, in contrast to that containing copper and zinc, was progressively less active as the pH was raised (Forman and Fridovich, 1973). The enzyme obtained from E. coli was closely related to the mitochondrial manganese enzyme, and to the iron-containing enzyme (with which an eighty percent sequence homology was shown) but was totally unrelated to the bovine copper-zinc enzyme (Steinman and Hill, 1973). The manganese enzyme was also insensitive to cyanide, differing in this respect from the bovine copper-zinc enzyme. The manganese superoxide dismutase from E. coli was purple-red, had an absorption maximum at 473 nm with an extinction coefficient of 400. This superoxide dismutase exhibited the usual protein u.v. absorption maximum at 283 nm, and contained three methionine residues and twelve of tyrosine (Keele et al., 1970). A manganese-containing SOD isolated from the blue green alga Plectonema boryanum (Asada, Yoshikawa, Takahashi, Mayeda and Enmanji, 1975), was found to be resistant to hydrogen peroxide and retained its activity after incubation for 24 hrs in 5 mM hydrogen peroxide. The enzyme from P. boryanum was also insensitive to p-chloromercuribenzoate, which indicated that the sulphydryl groups did not participate in the enzymatic reaction (Asada et al., 1975).

Mitochondrial superoxide dismutases

The mitochondrial superoxide dismutase was strikingly similar to the procaryotic manganese-containing SOD, but contained four subunits, compared to the two present in the latter SOD, and had a molecular weight of 80,000 (Weisiger and Fridovich, 1973a, Weisiger and Fridovich, 1973b). The similarity between the two enzyme forms was also reflected in their partial amino acid sequences (Steinman and Hill, 1973), an observation which has been cited as evidence in support of the view that mitochondria developed from a procaryotic organism that entered into an endocellular symbiosis with a protoeucaryote (Fridovich, 1975).

Iron superoxide dismutases

Iron superoxide dismutases have been shown to be present in, among others, E. coli (Gregory and Fridovich, 1973); Plectonema boryanum (Asada et al., 1975), and in Pseudomonas ovalis (Yakamkura, Suzuki and Mitsui, 1976). The enzyme from P. boryanum had an estimated molecular weight of 40,700, and consisted of two subunits of equal size without a disulphide bridge between the subunits (Asada et al., 1975). Acid-labile sulphur was apparently absent; one sulphydryl group per molecule of enzyme, and 2 gram atoms of iron, were indicated. The u.v. spectra of the enzyme displayed peak absorption at 280 nm, with shoulders present at 260 and 290 nm. An absorption coefficient of 66,900 was estimated at 280 nm (Asada et al., 1975).

The enzyme obtained from P. boryanum was found to be similar to that obtained from E. coli, although the iron was present in the trivalent form in E. coli (Yost and Fridovich, 1973). Levels of this enzyme in E. coli could be nutritionally modified (Gregory and Fridovich, 1973).

Assay procedures, and catalytic properties, of superoxide dismutase

Superoxide dismutase catalyses the reaction

$$O_2^- + O_2^- + 2H+ \rightarrow H_2O_2 + O_2.$$

The relative instability of the superoxide anion (O_2^-) requires that routine assays be indirect. Assay procedures consists basically of the coupling of an O_2^- generating system with an O_2^- scavenger, and of allowing the SOD to compete with the scavenger for the flux of O_2^- generated, thus inhibiting the rate of the reaction between O_2^- and the scavenger. Assay conditions should be carefully specified since SOD competes with O_2^- and the radical scavenger.

A turnover number of at least 3×10^6 has been estimated from stop-flow measurements, and a second order rate constant of 6×10^9 M^{-1} sec^{-1} for the reaction of O_2^- and SOD has been calculated (Fee and Gaber, 1971; McCord and Fridovich, 1969). Rigo, Viglino, and Rotillo (1975), have presented evidence which suggested that the catalytic sites of SOD could be saturated by O_2^-.

Xanthine oxidase, acting aerobically on xanthine, generates O_2^-, which reduces cytochrome c. The inhibition produced by SOD of the rate of reduction of cytochrome c, monitored at 550 nm, under specified conditions, served to quantitate the SOD present. A unit

of SOD was defined as that amount which caused a 50% inhibition of the rate of cytochrome c reduction under the conditions specified (McCord and Fridovich, 1969). The sensitivity of the assay procedure was increased by raising the pH, or by lowering the concentration of cytochrome c used.

More recently (Buchanan and Lees, 1976), assay procedures were expanded by the utilisation of the SOD inhibited reduction of acetylated cytochrome c by O_2^-; one unit of SOD activity was defined as that which inhibited the O_2^- mediated reduction of acetylated cytochrome c by 25%, under the conditions specified. The reaction between O_2^- and acetylated cytochrome c is specific (Azzi, Montecucco and Richter, 1975), thus lends itself readily to assays of crude extracts for example, and eliminates the potential problems of effecting inhibition of other enzyme systems, cytochrome oxidases and others, which may also act on the O_2^- scavenger in the assay to question the reliability of such an assay procedure.

Assay procedures have also utilised SOD inhibiton of free radical chain oxidations in which O_2^- served as an initiator or chain-propogating radical. The following are some examples of such reactions which have been utilised to assay SOD activity:-

i) oxidation of epinephrine (adrenaline) to adrenochrome (Misra and Fridovich, 1972)

ii) autoxidation of sulfite (McCord and Fridovich, 1969)

iii) autoxidation of pyrogallol (McCord and Fridovich, 1969).

The photochemical reducton of tetrazolium dyes to insoluble blue

formazans (Miller, 1970; Rajagopalan and Handler, 1964), has also been used as a method of assay, and formed the basis of an assay procedure which was applicable to free solutions of the enzyme, or to solutions of the enzyme applied to polyacrylamide gels (Beauchamp and Fridovich, 1971). The illumination of solutions containing riboflavin and tetramethylethylenediamine (TEMED) led to photoxidation of the TEMED and photo-reduction of riboflavin, which reacted (in the reduced form) with oxygen to generate O_2^-. Nitro-blue tetrazolium (NBT), added to the reaction mix in polyacrylamide gels, was reduced to the blue formazan except for areas in which SOD was present, such areas being indicated by achromatic zones in the now deep-blue coloured acrylamide gels. As little as 0.016 µg SOD was detected by this polyacrylamide gel electrophoresis procedure (Beauchamp and Fridovich, 1971).

Direct assays of SOD are also possible; such procedures usually consist of a means of introducing O_2^- into a solution, linked to a system with the sensitivity required to detect the O_2^- so produced. O_2^- has been generated in the reoxidation of reduced flavins in a stopped flow system; quantitation was achieved by EPR, SOD activity was measured or indicated by an accelerated rate of disappearance of the O_2^- EPR signal (Ballou, Palmer and Massey, 1969). The reduction of NBT by potassium superoxide dissolved in dimethyl sulfoxide has also been utilised as an assay for SOD (Henry, Halliwell and Hall, 1976). High concentrations of O_2^- have been generated in aqueous solutions by pulse radiolysis, and superoxide dismutase activity has been directly assayed spectrophotometrically (Klug, Rabani and Fridovich, 1972).

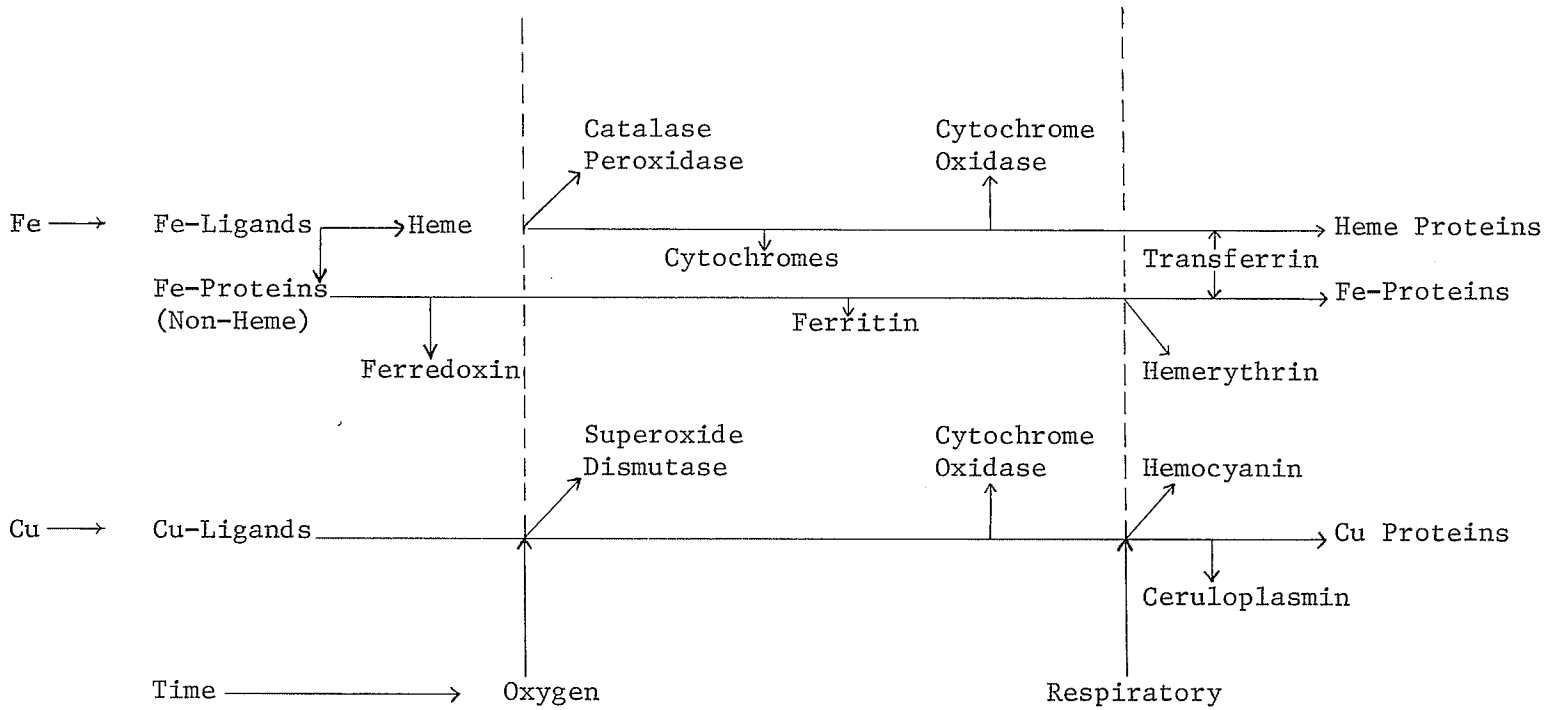
The superoxide dismutases are indubitably of paramount importance in providing biological processes and organisms with some protection against the potentially lethal effects of the energised states and free radicals produced from oxygen, but the contribution of the other members of the oxygen protecting triad - the catalases and peroxidases - also has to be considered. The relationship of these latter two enzyme activities to the superoxide dismutases, and their more salient features, will be examined.

The relationship of the catalases and peroxidases to the superoxide dismutases

An intimate biochemical association between the iron and copper proteins, the catalases, peroxidases and the superoxide dismutases, among others has emerged during evolutionary development. This association is reflected in their participation in providing defenses against the reactive by-products of oxygen utilisation, such by-products (O_2^-, H_2O_2) forming the substrates of these enzyme activities. The relationship between the primary oxygen-protective enzymes, and the other iron and copper proteins, is illustrated in Fig. 3.

Hydrogen peroxide (whether generated directly by the divalent reduction of oxygen, or indirectly by the dismutation of superoxide radicals) is scavenged by two classes of related enzymes, namely, the catalases and the peroxidases.

Figure 3 The evolutionary sequence and development of Fe and Cu metalloproteins.



Reference: Frieden (1976)

Catalase

The enzyme catalase (hydrogen peroxide: hydrogen peroxide oxidoreductase E.C.1.11.1.6.) has a molecular weight of 240,000, and contains four ferriprotoporphyrin groups per molecule, corresponding to a protohaem content of 1.1%, and an iron content of 0.09% (Aebi, 1974). Catalase absorbs maximally at 405 nm, and has a millimolar extinction coefficient of 380-400 per mole of enzyme, or of 100 per haem group. The enzyme is widely distributed in nature (Sumner and Dounce, 1937; Bonnischen, 1947; Herbert and Pinsent, 1948; Laskowski and Sumner, 1941; Jones, Diebel and Niven, 1964; Rao, Larson and Cox, 1964; Sulebelle and Rege, 1955), and can act either as a catalase or as a peroxidase, as indicated by the reactions -

catalase I - $2H_2O_2 \rightarrow 2H_2O + O_2$ - Decomposition of hydrogen peroxide

Peroxidase II - $ROOH + AH_2 \rightarrow H_2O + ROH + A$ - Oxidation of hydrogen donors

The reaction effected is determined by the concentration of the hydrogen donor, and the steady state concentration or rate of production of hydrogen peroxide in the system. The enzyme acts as a peroxidase when the concentration of hydrogen peroxide is low, and in the presence of electron donors. Initially, a catalase-hydrogen-peroxide complex I is formed, and in catalactic reactions a second molecule of hydrogen peroxide serves as a hydrogen donor for complex I (Aebi, 1974). The decomposition of hydrogen peroxide is very

rapid, and has, reportedly, a rate constant of approximately 10^7 sec^{-1} $mole^{-1}$, whereas peroxidactic reactions have been documented to have rate constants of the order of 10^2- 10^3 sec^{-1} $mole^{-1}$ (Aebi, 1974). The catalase content of biological material can be determined by monitoring the decomposition of hydrogen peroxide, or the production of oxygen, the latter of which can be effected manometrically (Beers and Sizer, 1953; Greenfield and Price, 1954). Hydrogen peroxide concentrations may be determined by titration, or by u.v. spectrophotometry at 230-250 nm (Beers and Sizer, 1952; Chance, 1949; Bergmeyer, 1955), or electrochemically with a platinum electrode (Damaschke and Todti, 1956). The kinetics of the catalase reaction are anomalous, in that saturation by the substrate does not occur at concentrations of up to 5M H_2O_2, but H_2O_2 in quantities greater than 0.1M rapidly inactivated the enzyme (Aebi, 1974). Adler (1963) has reportedly found no correlation between the activity of catalase and the sensitivity of E. coli to H_2O_2, and has suggested that the inability of catalase to protect E. coli from exogenous H_2O_2 may be attributed to the inactivation of catalase produced by high H_2O_2 concentrations. E. coli SASX76 however has been shown to be partially protected from inactivation by the presence of exogenous catalase (van Hemmen and Meuling, 1977).

Peroxidase

Peroxidases catalyse the oxidation of many organic compounds by hydrogen peroxide. The enzyme is highly specific, and combines only

with hydrogen peroxide, and methyl and ethyl hydrogen peroxides. The reaction effected can be indicated thus:-

$$AH_2 + H_2O_2 \rightarrow 2\ H_2O + A$$

where "AH_2" is the hydrogen donor, and "A" is the oxidised form of the hydrogen donor. Assay procedures for measuring peroxidase activity usually have involved the monitoring of the oxidation product or of the unchanged substrate (Luck, 1965). The substrates, p-phenylenediamine, and guaiacol (Boch and Zubkawa, 1921; George, 1953), have been used in assay procedures, and diaminobenzidine formed the basis of a relatively sensitive colorimetric procedure (Fahimi and Herzog, 1973), a procedure which has been adapted to demonstrate of both catalase and peroxidase on acrylamide gels (Gregory and Fridovich, 1974).

The protective role of the catalases and peroxidases against the potentially harmful effects of oxygen metabolites has been acknowledged (Fridovich, 1975; Lavalle, Michelson and Dimitrijevic, 1973; Gregory and Fridovich, 1973; McCord, Keele and Fridovich, 1971). The synthesis of catalase in E. coli was shown to be regulated by repression-induction and catabolite repression (Yoshpe-purer, Henis and Yashpe, 1977). Catalase was shown to be induced in Rhodopseudomonas and Saccharomyces (Clayton, 1960; Sulebele and Reye, 1968; Bhuvaneswarm, Sreenivasan and Rege, 1961). The organism Leptospira pomona, grown in high concentrations of oxygen,had five times as much catalase compared to that present in growth with low concentrations of oxygen (Rao, Larson and Cox, 1964). Increased

oxygen tension also effected an increase in the catalase content of Streptococcus faecalis (Jones, Diebel and Nieven, 1964).

The Biological Functions of catalase and peroxidase

Respiring cells produce H_2O_2, which, due to its inherent toxicity, cannot be allowed to accumulate in significant quantities in biological material. The catalases and the peroxidases serve the indispensable role of preventing the accumulation of H_2O_2. In view of the relative stability of hydrogen peroxide, and the number of defenses against its effects, the importance of those reactions which scavenge H_2O_2 is not immediately apparent. Accordingly, there are some microorganisms which lack catalase or peroxidase enzymes, and thus secrete H_2O_2, but such microorganisms normally live in mixed cultures with other cell types which do contain catalases and peroxidases, or in soil containing inorganic catalysts - iron for example - which facilitate the decomposition of H_2O_2 (Fridovich, 1976). The cells of acatalactic organisms were reportedly damaged by dilute solutions of H_2O_2 (Aebi and Suter, 1970). The erythrocytes of ducks reportedly lack catalase (Aebi and Suter, 1970), but compensation for this lack is effected by a marked increase in the levels of glutathione and glutathione peroxidase. In Homo sapiens, high levels of catalase were found in the liver, in the kidney, and in blood, but very little was found in the brain, in the thyroid, and in the testis. Other tissues also had relatively low levels of catalase, and it was thought that in such tissues, the H_2O_2 produced was removed by the

circulating blood (Hartz, Funakosi and Deutsch, 1973). In some tissues, for example the thyroid, normal functioning precludes the existence of a catalase, since H_2O_2 reputedly serves (in the thyroid) as an essential intermediate in iodine metabolism (Stanbury, 1972). The production of H_2O_2 by leucocytes is apparently an essential part of phagocytosis by leucocytes, which contain a peroxidase facilitating the bactericidal effect observed through the peroxidation of halide ions (Babior, Kipnes and Carnutte, 1973).

Catalase is remarkably well designed to function as a scavenger of H_2O_2 irrespective of the levels of its substrate, since the mode of action, as determined partially by substrate concentration, may be either that of a catalase or a peroxidase. Catalatic action does not require a cosubstrate other than H_2O_2, whereas peroxidatic action does require a cosubstrate. Methanol, ethanol, nitrite, or formate, may serve experimentally as cosubstrates for the peroxidatic reaction, but their natural counterparts are reportedly unknown (Aebi and Suter, 1972). The enzyme acts as a catalase in liver peroxisomes and in mitochondria, both of which are associated with relatively high levels of H_2O_2.

Peroxidases - which act preferentially at low levels of H_2O_2 - are widely distributed in mammalian and other cell types, and its importance in the scavenging of H_2O_2 has been widely recognised (Mills, 1959; Cohen and Hochstein, 1963). Glutathione peroxidase can act upon lipid hydroperoxides, as well as on H_2O_2, and in this capacity provide protection from a large number of potentially toxic peroxides (Little and O'Brien, 1968; Christopharson, 1969; Little, 1972; Chow, Reddy and Teppel, 1973). Humans with genetically defective

erythrocyte glutathione peroxidase are reportedly prone to the development of hemolytic anaemia (Necheles, Boles and Allen, 1968), apparently attributable to the lack of an adequate defence against the oxidative effect of H_2O_2 (Jacob and Jandl, 1966). Glanzmann's thrombocytopenia is produced by a similar defect in platelets (Karpatkin and Weiss, 1972). Several types of peroxidases - differing in respect to their substrate specificity - occur among several organisms. Yeast contains a cytochrome c peroxidase (localised in the mitochondrial intermembrane space) which serves to scavenge H_2O_2 generated inside the mitochondria, consequently, the mitochondria of yeast produce relatively smaller quantities of H_2O_2 when compared to their mammalian counterpart (Erecinski, Oshina, Lob and Brocklehurst, 1973). A pseudomonad has also been shown to contain a cytochrome c peroxidase (Ellfolk, Ronnberg and Soininen, 1973). Among plants, peroxidases acting on several phenols and arylamines, have been found (Saunders, Holmes-Siedle and Stark, 1964). The relative abundance of the peroxidases in some plants suggests that relatively large quantities of H_2O_2 are generated, and that the scavenging of H_2O_2 in plants is apparently as necessary as it is in animals.

The enzyme triad - of the superoxide dismutases, of the catalases, and of the peroxidases - is thus demonstrably vital to the defences and protective mechanisms which allow aerobic life, these enzymes effecting a method for reducing the effects of the potentially lethal or toxic by-products of the metabolic utilisation of oxygen.

A partial discussion of the reported effects of oxygen on microorganisms and on Azotobacter in particular now follows.

The effects of oxygen on microorganisms with particular reference to Azotobacter.

The effects of oxygen on microorganisms and biological systems are many and varied. In aerobic organisms, molecular oxygen normally serves as the final electron acceptor during the oxidative processes which ultimately generate the requisite energy for effecting vital activities. Anaerobes usually are incapable of tolerating significant quantities of oxygen, whereas facultative organisms can effect some adaptation to allow the utilisation of molecular oxygen. This present discourse will center mainly on aerobic microorganisms. The general methodology of investigating the effects of oxygen on microorganisms varies; a frequent method of study involves growth of the organism in batch or continuous cultures, with appropriate equipment or procedures (oxygen electrodes for example) to monitor the concentration of dissolved oxygen available to the organism. The inherent limitations imposed by the use of batch cultures (Dalton and Postgate, 1969; Harrison, 1972) often restricts examination of such cultures to the more qualitative aspects of the organisms' response. Far greater use is now being made of continuous cultures to provide more meaningful data. The theory and applications of continuous culture to the study of the effects of oxygen on microorganisms will be reviewed in the appropriate section.

The effects of oxygen on microorganisms may be conveniently examined as they relate to:-

1. physiology and metabolism

2. redox potentials

3. growth yields.

1. General effects on physiology and metabolism.

a) Respiratory rate.

Harrison and Pirt (1967), working with Klebsiella aerogenes grown in continuous culture, showed that the organism demonstrated a higher respiration rate at low oxygen tensions than under conditions of excess oxygen. These authors defined a 'critical oxygen tension' as that above which the organism's respiratory rate was independent of the dissolved oxygen tension. They also concluded that a switch to a higher respiratory rate, with a lower growth efficiency, occurred at oxygen concentrations below this critical oxygen tension. Similar results were obtained by McLennan and Pirt (1970), and by Harrison, McLennan and Pirt (1969), who found that a Pseudomonas displayed an increased affinity for dissolved oxygen at low oxygen tensions. That the above was also applicable to E. coli grown in continuous culture was demonstrated by Harrison and Loveless (1971), who suggested that the relatively high QO_2 values obtained with a low oxygen tension and an associated high growth rate, represented an uncoupling of either the ATP generation or the ATP utilisation processes. Anaerobically grown cells were adjudged to display poor respiratory control. McLennan, Ousbey, Vasey and Cotton (1971) found that at oxygen tensions varying from 28 to 110 mm Hg, alteration of the oxygen tension had no perceptible effect on the QO_2. The observed QO_2 rose as the oxygen tension fell below 28 mm Hg, but at 7 mm Hg and less the cultures became oxygen limited and cell dry weight concentration

fell. The production of carbon dioxide (CO_2) increased on raising the dissolved oxygen tension from 100 to 560 mm Hg. Oxygen tensions above 560 mm Hg, up to 675 mm Hg, resulted in a decrease in CO_2 production. At these relatively high dissolved oxygen tensions (560 mm Hg. for example) alteration of the oxygen supplied resulted in 'steady-state conditions' after several days, which may have indicated that some selection of a different bacterial strain was occurring.

b) Respiratory enzymes

Numerous reports have been published on the changes in cytochrome levels of microorganisms in response to dissolved oxygen tension. These reports generally showed that highest cytochrome concentrations were obtained under low oxygen tensions. Some of these findings are summarised in Table 2. In both E. coli and Klebsiella aerogenes, Moss (1956) found larger quantities of cytochrome a, and a_2 at low oxygen tensions, whereas the content of cytochrome o did not change as greatly as that of cytochrome a_2 (Harrison 1972). Maximum cytochrome content in cells was generally obtained under oxygen-limited conditions, a finding applicable to cytochromes a, a_2, a_3, b, c, and o in both obligate aerobes and facultative organisms. Groot and Poyton (1975) investigated the effect of oxygen on cytochrome c oxidase synthesis in isolated mitochondria of Saccharomyces cerevisiae, and found that oxygen acted directly on the mitochondrion, concluding that at least one step in mitochondrial transcription-translation required oxygen. Oxygen most likely regulated the synthesis of subunits I and II of cytochrome oxidase directly at the level of mitochondrial translation.

Table 2. Reported effects of oxygen status on cytochrome content.

Organism	Culture Method	O_2 Monitor	Cytochrome	Anaerobic	O_2 status O_2 limited	O_2 Excess	Reference
Escherichia coli	Continuous	Yes	a_1	+	+++	++	Moss (1952)
			a_2	+	+++	++	
			b_1	+	++	++	
Escherichia coli	Continuous	Yes	C552	++	-	-	Wimpenny,Cole(1967)
Klebsiella aerogenes	Continuous	Yes	a_1	++	+++	-	Harrison *et al.*(1970)
			a_2	++	+++	+	
			b_1	++	++	++	
			o	++	++	++	
Hemophillus parainfluenzae	Batch	No	a_2 }		+++	++	Sinclair,White(1970)
			a		+++	++	
			o		+++	++	
			b		+++	++	
			c		+++	++	
Spirillum iternosi	Batch	No	b		+++	++	Clark-Walker *et al.*(1967)
			c		+++	++	
Saccharomyces cerevisiae	Batch	No	aa_3	-	++	+++	Biggs,Linnane(1963)
			b_1	-	++	+++	
			$c \ddagger c_1$	-	++	+++	
Staphylococcus epedermis	Batch	No	b_1	++		+++	Jacobs *et al.* (1967)
			o_1	++		+++	

Continued.....

Table 2 continued.....

Organism	Culture Method	O_2 Monitor	Cytochrome	Anaerobic	O_2 status O_2 limited	O_2 Excess	Reference
Azotobacter vinelandii	Batch	No	b		+++	++	Lisenkova,Hkmel(1967)
			c		+++	++	
Bacillus liceniformis	Batch	No	a_1	-	++	+++	Schulp,Southamer(1970)
			b_1	-	++	+++	Moss et al., (1969)
Candida utilis	Continuous	Yes	aa_3	++	+++	++	
			b	++	+++	++	
			c	++	+++	++	
Saccharomyces carlsbergensis	Continuous	Yes	aa_3	+	++	++	Moss et al., (1971)
			b	++	++	+++	
			c	++	++	+++	
Micrococcus dinitrificans	Batch	No	aa_3		++	+++	Sapshead,Wimpenny(1970)
			o		+++	++	
			c	+++	++	+	
			b	+++	++	+	
Achromobacter	Batch	No	a_1		+++	++	Arima, Oka (1965)
			a_2		+++	++	
			o		+++	++	
			b_1		++	+++	

KEY: Blank, not measured; -,absent; +, low levels present; ++, present; +++, at a maximum.
REFERENCE: Harrison, D.E.F.

Rogers and Stewart (1973), working on S. cerevisiae, showed that the synthesis of cytochrome c peroxidase and cytochrome -succinate reductase enzymes was particularly sensitive to oxygen. They presented evidence which suggested that the developmental pattern of mitochondrial components in this organism may be a differential rather than a coordinate response to environmental oxygen, with respect to the particulate lipids and cytochromes on the one hand, and to the mobile electron carriers and accessory enzymes on the other. Cytochrome b and c syntheses were more sensitive to oxygen than those ofcytochrome a, and a_3, and may have reflected the sequence of assembly during development.

Farago and Gibbins (1974) found that in chemostat cultures of the facultatively anaerobic Erwinia amylovora, a marked increase in acid production occurred as the dissolved oxygen tension decreased. An increased ability to oxidise succinate was noted at low dissolved oxygen tensions. Similar results were also obtained by Wimpenny and Necklen (1971), who found that there was an initial increase in the levels of isocitrate dehydrogenase, and fumarase. Tempest and Herbert (1965) reported similar behaviour for the enzymes NADH oxidase, succinate dehydrogenase and the cytochromes.

Several enzymes, respiratory and other, have been shown to be inhibited by oxygen. Included among these are the following:-

i) glyceraldehyde-3-phosphate dehydrogenase (Balazs, 1959) from rat brain mitochondria.

ii) succinic dehydrogenase (Dickens, 1946).

iii) xanthine oxidase (Mann and Quastel, 1946).

iv) d-amino acid oxidase (Stadie and Haugaard, 1945).

v) pyruvate and lactate oxidases (Mann and Quastel, 1946).

vi) citrate metabolising enzymes in pea tissues (Turner and Quartley, 1956).

vii) purified cytochrome c reductase (Dixon, Maynard and Morrow, 1960).

viii) yeast cytochrome b_2 (Armstrong, Coates and Morton, 1960).

c) Adenine nucleotides - NAD, NADP, NADH, NADPH

NAD occupies a key position in biological oxidation-reduction reactions, thus its role in the physiological response of bacteria must be considered. Wimpenny and Firth (1971) showed that the levels of NAD, NADH, and total nucleotides varied in different organisms; twice the amount present in facultative anaerobes was found in the anaerobe, Clostridium. Facultative anaerobes, grown aerobically or anaerobically, had remarkably constant levels of NADH. The concentration of NAD may have been proportional to the degree of aerobiosis of cells from these organisms. Wimpenny and Firth (1971) suggested that regulation in bacteria was geared towards maintaining a constant NADH pool size; the synthesis of NAD was influenced, directly or indirectly, by oxygen. NAD was thought to act as an inducer of aerobic respiratory enzymes and carriers, while NADH synthesis may be prompted by aerobiosis. The ratio of NADH to NAD as found in the bacterial cell has been used to indicate the redox potential of such

cells, hence the effect of oxygen on the redox potentials of bacteria will now be considered.

2. Redox potentials

Redox potential can be defined by the expression

$$Eh = Eo + \frac{RT}{nF} \text{Ln} \frac{(\text{OXIDANT})}{(\text{REDUCTANT})} \qquad \text{(Tang, 1933)}$$

where Eh is the potential in reference to the hydrogen electrode,
Eo is the standard potential of the system at 30°C with the reactants assigned a unit value.
R is the gas constant
T, the absolute temperature
F, the Faraday
n, the number of electrons transferred in the reaction.

Wimpenny, (1969) implied (under conditions of severe oxygen limitation) that it was redox potential rather than dissolved oxygen tension to which the cells responded; however, it is difficult to assign an overall redox potential to so complex an assemblage as a bacterial cell. Wimpenny and Necklen (1971) pointed out that over the range of Eh which had most effect on cell constituents, i.e., Eh + 150 to -250 mV, a change of a few mV would in fact represent a large change in the dissolved oxygen tension. Harrison (1973) on the basis of Wimpenny's work, suggested that redox probes may be used to obtain empirical relationships between oxygen and metabolic changes at levels of dissolved oxygen below the sensitivity of membrane electrodes. At such low oxygen tensions however, the biochemistry of

a bacterial culture would tend to undergo such changes as to alter the relationship between dissolved oxygen tension and redox potential, thus the concept of an overall redox potential was of little apparent value in studying the responses of growing microbial cultures to oxygen.

3. Growth yields

A yield coefficient, "Y", can be defined as the weight of dry cell material produced from a unit weight of substrate, as in YATP, which indicates the mg. bacteria synthesized/mmole ATP consumed (Nagai, Nishizawa and Aiba, 1969). The yield can also be expressed in terms of the number of ATP molecules generated from the substrate (Bauchop and Elsden, 1960), or by the use of P/O ratios (Chen, 1964; Hadjipetrou, Gerrits, Teulings and Stouthamer, 1964). Stouthamer, (1970) based yield coefficients on oxygen consumed (Yo_2). Gunsalus and Schuster (1961) using YATP values, concluded that aerobic growth was highly inefficient, which has been supported by Nagai et al., (1969), who showed that high levels of oxygen may have inhibited cell synthesis and energy metabolism. Low dissolved oxygen tension (Harrison and Pirt, 1967) and high dissolved oxygen tension (Dalton and Postgate, 1968), have both been shown to contribute to low growth yields. Hempfling (1970), showed that the level of ATP reached a constant value for several minutes after exposure to oxygen, and did not fall again on exhaustion of oxygen. Harrison and Loveless (1971), working with K. aerogenes and E. coli, showed that the

efficiency of aerobic growth was low during anaerobic to aerobic transitions. Oxygen shock was thought to effect uncoupling of the cells' energy production, or that some appreciable time elapsed before complete coupling was regained when anaerobic cells were aerated.

In considering the effects of oxygen on microbial organisms, the concept of Y_{ATP} will not be examined in the work here presented.

Oxygen effects on Azotobacter and on Nitrogenase

The pioneering studies of Burk (1930), and of Parker and Scutt (1960) showed that the respiration rate in Azotobacter reached a clearly defined maximum at 0.15 to 0.30 atm-oxygen. Dilworth and Parker (1961) presented evidence to show that respiration in Azotobacter vinelandii was inhibited by oxygen, an effect claimed peculiar to Azotobacter. The addition of sulfhydryl (-SH) group stabilising reagents failed to protect the organism from the toxic effect of oxygen. Dilworth (1962), showed that the oxygen inhibition of respiration was reversible and increased with time. A decrease in oxygen uptake was found to correlate with the accumulation of keto acids, namely, pyruvic and ∝-ketoglutaric acids. Cell-free extracts were also sensitive to oxygen when pyruvate was substrate, but not when succinate was used. Dilworth concluded that pyruvic oxidase was sensitive to oxygen.

Burk (1930) suggested that oxygen toxicity was attributable to a general inhibition of growth in Azotobacter, an idea supported by the work of Khmel, Gabinskaya and Ierusalimsky (1965), who reported that Azotobacter vinelandii yields were highest at low aeration rates.

Tschapek and Gaimbiggi (1955) proposed that nitrogen fixation was influenced by oxygen through intimate linkage with respiration. The conclusions obtained above related to batch cultures, which inherently imposes restrictions on the interpretation of the resultant data. A more apt methodology is offered by continuous cultures, which have been extensively used by Postgate and others (1969,1972)to offer what must now be accepted as a more plausible explanation of the effects of oxygen on Azotobacter.

Dalton and Postgate (1969a), (citing the work of Bulen and LeComte (1966); Kelly, Klucas and Burris (1967), and Kelly (1966)), pointed out that in aerobic bacteria, the components of nitrogenase were intrinsically sensitive to oxygen, and that the aerobic organism must have some mechanism for protecting nitrogenase from oxygen. This protective mechansim was thought to be two-fold, namely conformational protection, and respiratory protection. Conformational protection was inferred from the oxygen tolerance of particulate preparations, which suggested that some steric arrangement of nitrogenase components conferred a tolerance to oxygen, either by the inaccessibility of the oxygen sensitive sites to oxygen, or by stabilisaton such that the necessary conformational features of nitrogenase existed in a conditon which was not damaged by oxygen. Respiratory protection essentially implied that respiration was used to scavenge oxygen from the site of nitrogenase action. Conformational protection was thought to function as a supportive or 'back-up' device under conditions of oxygen stress when respiratory protection was

inadequate (Postgate, 1974). The nitrogenase complex was inactive in the 'conformational protected' state (Dalton and Postgate, 1969). Phillips and Johnson (1961) had earlier suggested that respiration served as an oxygen-wasting system in such a fashion that a low E_h value was maintained intracellularly, providing favorable circumstances for nitrogen fixation. Dalton and Postgate (1969a) based their assumption that respiration was a protective device for the nitrogenase complex on the following observations:-

1. In nitrogen fixing cultures which are nitrogen-limited, respiration is so effected that a balance in oxygen supply results, with the process of nitrogen fixation not being significantly affected. Nitrogen fixation was very inefficient - in respect to the quantities of carbon utilised - when cultures were highly aerated. Conversely, the process was much more efficient at very low oxygen tensions.
2. Carbon-limited nitrogen fixing cultures were extremely sensitive to oxygen, since such cultures could not increase their respiratory rate in the presence of high oxygen tensions.
3. Indications were obtained to suggest that a balance between oxygen solution and oxygen utilisation was actively maintained, based on the observation that the maximum tolerated concentration of oxygen in the culture medium was relatively the same, even though the inhibitory oxygen tension for the cultures was dependent on the population density.

4. Cultures which were not fixing nitrogen, i.e., grown with ammonia, showed no abnormal sensitivity to oxygen. The Azotobacter species are characterised by unusually high Q_{O_2} values of 4000 to 5000 µl O_2/mg. dry wt/hr (Williams and Wilson, 1954), which, as pointed out by Dalton and Postgate (1969), may represent a mechanism for excluding oxygen from parts of the cell. Oxygen sensitivity was thus regarded as an expression of the respiratory protection of nitrogenase (Dalton and Postgate, 1969b). This respiratory protection was deemed to be operative through the cytochrome pathway, as suggested by the sensitivity of phosphate-limited cultures to oxygen (Dalton and Postgate, 1969a).

The above conclusions were ably supported by a subsequent report by Lees and Postgate (1973), who noted that oxygen limited cultures, on being subjected to excess O_2, were able to increase their respiration so as to keep the level of dissolved oxygen low, but that phosphate-limited cultures were incapable of so doing. Phosphate limited cultures ceased to grow when the oxygen tension in the growth medium was increased, suggesting that the terminal-respiration system was malfunctioning, possibly through an effect of excess oxygen on the ADP/ATP ratio. In this same report, it was noted that Azotobacter chroococcum, grown in phosphate limited cultures and subjected to excess O_2, remained viable if the organisms were tested on a medium containing ammonium. This observation thereby ably supported the assumption that 'oxygen stress specifically affected nitrogenase or something closely associated with it,' and thus gives even greater

credibility to the postulated respiratory protecton of nitrogenase. Postgate (1974) considering evolutionary trends displayed by nitrogen-fixing systems, hypothesised that the internal membranous network of Azotobacter represented a highly evolved oxygen-exclusion apparatus not found in the less evolved nitrogen fixers. A physiological evolution of respiratory protection, starting with Clostridium (which lacked respiratory protection), and ending with the Azotobacteriaceae, was postulated.

The conclusions which were made in respect to the general effects of oxygen on microorganisms, in particular, changes in cytochrome profiles, growth yields and respiratory rates, are also applicable to Azotobacter. Khmel and Ierusalimskii (1967) showed that the economy factor, i.e., the ratio of cell mass formed to substrate consumed, was highest under oxygen-deficient conditions, and that excessive aeration led to a decrease in the economy factor and the nitrogen fixation rate.

In respect to cytochrome levels, Lisenkova and Khmel, (1967) working with batch cultures of Azotobacter vinelandii, concluded that cytochrome synthesis had an optimal aeration rate of 0.6 g oxygen per litre per hour; that the cytochrome content decreased in iron-deficient medium; and that nitrogen-fixing cells contained more cytochromes than cells grown in a medium containing ammonium. Haaker and Veeger (1976) using continuous cultures of this same organism, showed that the composition of respiratory membranes with respect to cytochromes $c_4 + c_5$, b, and d, differed in cells grown in oxygen-limited

conditions. The maximum activity of nitrogenase was dependent on the type of substrate oxidised. Ackrell and Jones (1971) presented data to show that there was a general increase in cytochrome concentration resulting from oxygen limitation, which may have been an attempt by the organism to maintain maximal rates of electron flow under these conditions. A cyanide-sensitive minor terminal oxidation pathway was thought to carry a higher fraction of the total terminal electron flow in cells growing under oxygen limited conditions compared to those grown in excess oxygen. The respiration rate of the organism was deemed to be controlled by the (ATP)/(ADP)(Pi) ratio; a low (ATP)/(ADP)(Pi) ratio in whole cells would produce high respiratory activity, and a high (ATP)/(ADP)(Pi) ratio would lead to a low respiratory activity. Membranes prepared from cells grown with excess oxygen were regarded as having lost respiratory control associated with NADH oxidation, thus had greatly lowered (ATP)/(ADP)(Pi) ratios, or stated differently, displayed high respiratory activity. The lowered (ATP)/(ADP)(Pi) ratios observed in the presence of excess oxygen were deemed to lead to an increase in the rates of mannitol breakdown and terminal respiration to produce the very high $^{Q}O_2$ values for which the Azotobacteriaceae are famous. The increased rate of mannitol catabolism was attributable to the fact that several key enzymes were inhibited by ATP, or stimulated by high ADP or AMP concentrations (Yates, 1970; Atkinson and Walton, 1967; Weitzmann, 1970). Ackrell and Jones (1971) explained the low $^{Q}O_2$ values obtained from oxygen-limited cells by stating that an increased (ATP)/(ADP)(Pi) ratio was present in such cells, which displayed high phosphorylation

efficiencies and respiratory control through NADH.

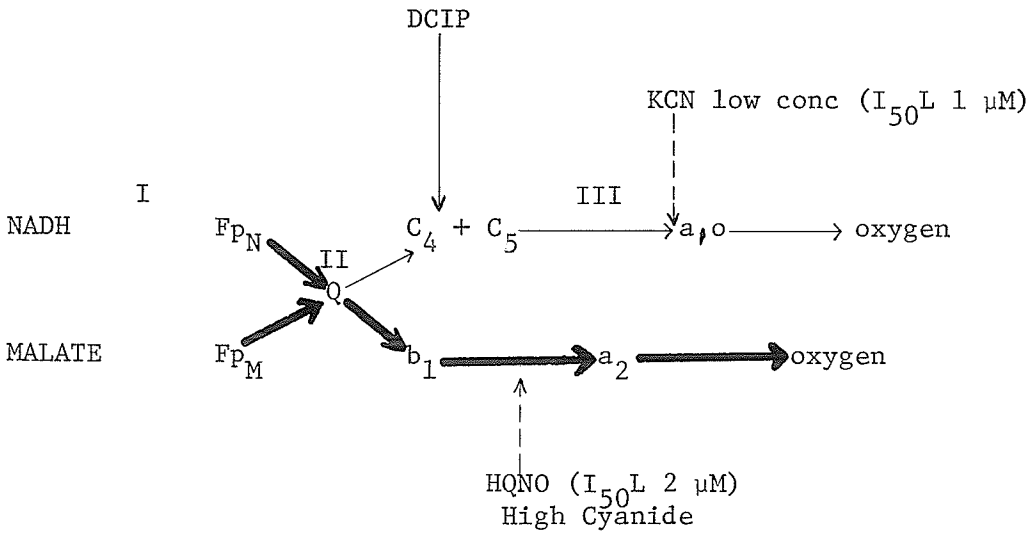
The branched electron transport system of Azotobacter vinelandii is shown in Fig. 4(i).

Oxygen limitation and polyβ-hydroxybutyrate (PHB) metabolism in Azotobacter.

It is now generally accepted, as has been reported by several groups (Lees and Postgate, 1973; Jackson and Dawes, 1976; Stockdale, Ribbons and Dawes, 1968; Senior, Beech, Ritchie and Dawes, 1972), that oxygen limitation in Azotobacter results in the production of increased quantities of poly β-hydroxybutyrate. Lees and Postgate (1973), examined the behavior of Azotobacter chroococcum in oxygen and phosphate-limited continuous cultures, and showed that there was an inverse relation between biomass and dilution rate, accounted for largely by increased polysaccharide and polyhydroxybutyrate content. The chemostat experiments conducted by Senior et al., (1972) revealed that oxygen limitation, rather than nitrogen limitation, initiated synthesis of poly β-hydroxybutyrate and confirmed the observation of Lees and Postgate (1973) to the relationship of growth rate andPHB content.

In Azotobacter beijerinckii, PHB metabolism is reportedly regulated by a sharing of acetyl CoA between citrate synthase and β ketothiolase, representing the initiating enzymes of the (TCA) tricarboxylic acid cycle and polymer formation respectively (Jackson and Dawes, 1976). The sharing is effected by the intracellular levels of free CoA and NADH (Senior and Dawes, 1973). Under oxygen limitation, the sharing system operates so that reducing equivalents which would have utilised

Figure 4. Branched Electron Transport System of A. vinelandii.



From: Ackrell and Jones (1971), and Jones and Redfearn (1967)

NADH-reduced nicotinamide adenine dinucleotide; HQNO, 2n-alkyl-4-hydroxyquinoline.

Fp-Flavoprotein; DCIP, 2-6 dichlorophenol idophenol; KCN-potassium cyanide. Q-ubiquinone. I, II, III - phosphorylation sites.

oxygen as the terminal electron acceptor are now diverted to PHB synthesis, PHB thus serving as an electron sink (Jackson and Dawes, 1976). Intracellular NADH/NAD ratios rose initially on imposing oxygen limitation, but readjustment occurred at the start of PHB synthesis. The levels of β-ketothiolase and acetoacetyl CoA reductase are influenced directly by environmental oxygen concentration, though they may still be present under highly aerobic conditions (Jackson and Dawes, 1976).

Senior et al. (1972), concluded that in the absence of exogenous substrate and intracellular reserve material, the process of respiratory protection envisaged by Dalton and Postgate (1969) would be impossible, and viability threatened. Large quantities of readily oxidisable poly-β-hydroxybutyrate would confer a distinct advantage to cells which had to increase their oxidative capacity in the absence of exogenous substrate. PHB thus apparently serves in the Azotobacteriaceae as a method of regulating the oxygen environment of their environment so as to remain effectively viable.

Pate, Shah and Brill (1974), using batch cultures of Azotobacter vinelandii, presented evidence which seemed to indicate that the internal network of membranes might be produced in response to oxygen availability rather than to nitrogen source. The cells apparently responded to dissolved oxygen concentration by synthesizing more membrane material when oxygen was limiting, in an attempt to effect an increase in surface area to sequester enough oxygen to allow the bacteria to remain in the exponential growth phase. These conclusions are indeed highly questionable, and will be assessed as they relate to

other findings in the appropriate section of the results presented subsequently. The following section, relating to the possible location of nitrogenase in the bacterial cell, shows that the question is far from being unequivocably settled.

The possible intracellular location of nitrogenase and its relation to oxygen damage

The exclusion of oxygen from nitrogenase could possibly be effected by comparmentation, i.e., restriction of the nitrogenase to a site inaccessible to oxygen. Postgate (1974) has even viewed conformational protection as intracellular comparmentation of nitrogenase, in association with a membrane. The internal membranous network of Azotobacter then presumably represented a highly evolved oxygen-exclusion apparatus. The location of nitrogenase within Azotobacter may therefore be an important factor in protection against oxygen damage.

The literature to date contains conflicting reports as the possible location of nitrogenase in Azotobacter and other bacteria. Bulen, Burns and LeComte (1965), presented evidence to show that nitrogenase in A. vinelandii was particulate; a similar result has also been obtained for A. chroococcum (Yates and Daniel, 1970; Kelly, 1968). Oxygen tolerant particulate nitrogenase has been obtained from Mycobacterium flavuum (Biggins and Postgate, 1971), whereas those obtained from Azotobacter, as noted above, were oxygen-sensitive. Oppenheim, Fisher, Wilson and Marcus, (1970), showed that nitrogenase

obtained from A. vinelandii by osmotic shock was soluble and more oxygen-sensitive than similar preparations obtained by disruption of cells in a French press. Oppenheim and Marcus (1970) showed that nitrogen-fixing cells had an extensive internal membrane network which was absent from ammonia grown organisms; they therefore interpreted these findings as indications of a definite structural modification in the cell to protect nitrogenase from O_2 inactivation. A structural functional model was proposed which had the internal membranes as sites of respiratory activity surrounding the soluble nitrogen-fixing enzyme system to create a barrier between the cytoplasm and the relatively oxygen-rich environment of the rest of the cell.

In contrast to the findings of Oppenheim et al., (1970), Reed, Toia, and Raveed (1974) found that so-called 'azotophore' membranes containing nitrogenase could be obtained in high yield from A. vinelandii ruptured mechanically or osmotically. These azotophore membranes were readily seperable from the intracytoplasmic membranes containing the cytochromes by density gradients or differential sedimentation.

More recently, Haaker and Veeger (1977), subjected A. vinelandii cells to rupture by lysozyme treatment, osmotic shock, or by use of a French press. They concluded that no evidence was found for a so-called particulate nitrogenase, or for an interaction between nitrogenase and cytoplasmic membrane vesicles. The nitrogenase was seen to sediment as a complex comparable to that of the pyruvate dehydrogenase complex. Sedimentation behavior was viewed as being independent of the method of cell rupture, and the association which may occur between nitrogenase and membrane fragments was seen as only a weak interaction. The oxygen

stability of nitrogenase so obtained was attributed to a complexation of the nitrogenase components with an Fe-S (iron sulfur) protein, and it was proposed that the "switched off" state of nitrogenase in Azotobacter cells was caused by oxidation of flavin hydroquinone rather than by a reversible inactivation of the nitrogenase.

The work of Postgate and colleagues (cited previously), of Haaker and Veeger (1977), and of Scherings, Haaker and Veeger (1977), can be applied to an interpretation of the control and functioning of nitrogenase activity.

Control and functioning of nitrogenase

Dalton (1974), reviewing the fixation of dinitrogen by free-living microorganisms, postulated that nitrogenase function in Azotobacter could be controlled by controlling synthesis of nitrogenase or by control of activity. The synthesis of nitrogenase was viewed as being repressed by ammonium, which when present in high concentration in the cells' intracellular pool, produced a halt in the synthesis of nitrogenase. Synthesis of nitrogenase would be partially repressed in normal cells fixing nitrogen, and maximum synthesis would be effected in ammonium limited chemostat cultures. Gross control over nitrogenase activity was achieved by the enzyme assuming a 'switched-off' state in the presence of excess oxygen, with 'switching-on' occurring when the oxygen tension was reduced. 'Fine control' was seen as being due to alterations in the (ATP)/(ADP)(Pi) ratios as previously discussed (Yates and Jones, 1974).

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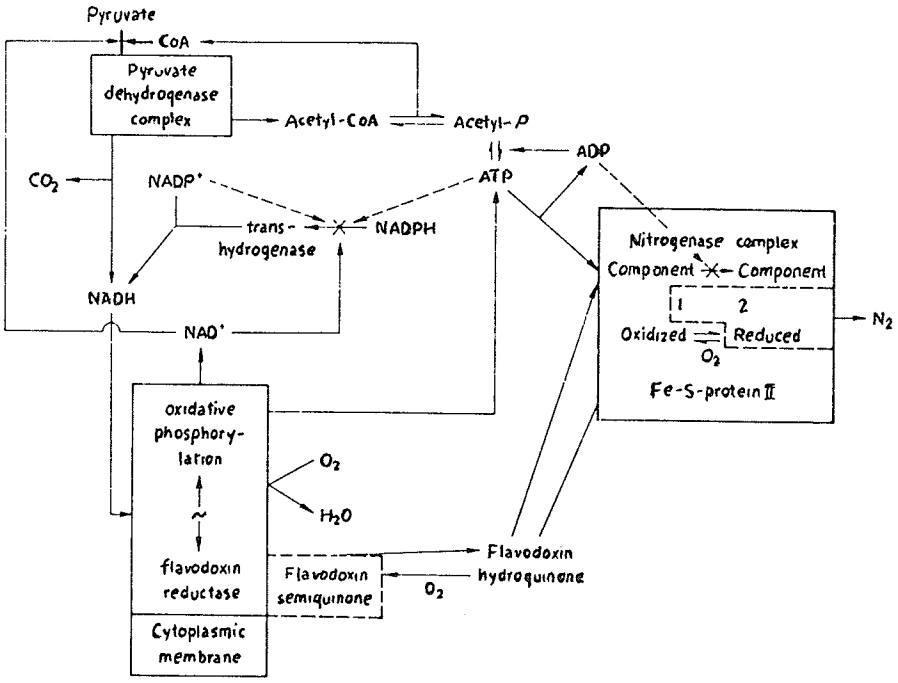
Figure 4(ii) Proposed scheme for electron transport from NADH to nitrogenase.

The nitrogenase complex consists of components 1 and 2 and Fe-S (iron sulphur) protein II. It is assumed that only the reduced form of Fe-S protein II modifies the properties of components 1 and 2.

From: Scherings, Haaker and Veeger (1977).


Pyruvate
CoA
Pyruvate dehydrogenase complex
Acetyl-CoA
Acetyl-P
ADP
ATP
CO_2
NADP'
trans-hydrogenase
NADPH
NADH
NAD'
Nitrogenase complex
Component
Component
1
2
Oxidized
Reduced
O_2
N_2
Fe-S-protein II
oxidative phosphorylation
O_2
H_2O
flavodoxin reductase
Flavodoxin semiquinone
O_2
Flavodoxin hydroquinone
Cytoplasmic membrane

Yates and Jones (1974) suggested that a very small increase in $^{Q}O_2$ values would inhibit electron supply to nitrogenase to cause the "switching-off" of the enzyme. A second possible method of control was related to the reactivity of flavodoxin hydroquinone towards oxygen, in that excess oxygen in the cell interior would cause or enable the hydroquinone to transfer electrons preferentially to oxygen rather than to nitrogenase to effect an instant "switch off" of functioning. Similar results were obtained by Scherings et al., (1977), who found that the flavodoxin hydroquinone (Fe-S protein II) regulated nitrogenase activity (see Fig.4(ii))and that the reaction rate of nitrogenase was strongly dependent on the redox potential of the flavodoxin semi-quinone/hydroquinone couple. This Fe-S protein II protected nitrogenase from oxygen inactivation, in that extracts containing Fe-S II retained 60% of its initial activity after 4 days storage without dithionite, whereas complete inactivation resulted in the absence of Fe-S II in extracts under similar conditions.

The above interpretations of the results in question point to acceptance of an alternative mechanism to conformational protection to account for the almost instantaneous "switch-on" and "switch-off" of nitrogenase activity. Acceptance of the alternative explanation does not preclude the existence of a conformational mode; as pointed out by Yates and Jones (1974), both processes could occur simultaneously. In vitro conformational changes in nitrogenase in the presence of ATP have been documented, and such changes were associated with an increased sensitivity of nitrogenase components to oxygen (Yates, 1972). Irrespective of the issues herein raised, interpretation of the work of

Lees and Postgate (1973) among others, implied that the regulation of conformational protection was in response to similar signals as those that initiated respiratory protection (Yates and Jones, 1974).

Having examined the control of nitrogenase functioning, it is only fitting that some of the basic mechanics of nitrogenase functioning, in particular the electron transfer to nitrogenase, be stated.

Mechanism of nitrogenase action, and electron transfer to nitrogenase

The two components of the nitrogenase enzyme, the molybdenum iron protein (Mo-Fe protein), and the iron (Fe) protein, together with a reducing agent, ATP, and magnesium (Mg^{2+}), are all essential for activity (Newton, Corbin and McDonald, 1976). The reducing agent can be reduced ferredoxin; flavodoxin; a reduced viologen; or sodium dithionite (Eady, Smith, Thorneley, Yates and Postgate, 1975). Neither protein alone, even in the presence of MgATP and when fully reduced, has been shown to catalyse any substrate reduction. Electron paramagnetic resonance (EPR) studies have shown that MgATP binds to the Fe protein, which also accepts 1 electron, and that electrons flow from the Fe protein to the Mo-Fe protein during catalysis (Orme-Johnson, Hamilton, Ljones, Tso, Burris, Shah and Brill, 1972). EPR (Zumft, Palmer and Mortenson, 1973) and gel equilbration experiments (Tso and Burris 1973; and Bui and Mortenson, 1968), have indicated that 2 mols of MgATP are bound to each Fe protein dimer. This binding has been shown to effect a lowering of the midpoint potential of the reduced Fe protein from -290 mV to -400 mV (Zumft, Mortenson and Palmer, 1974). Approximately 4 mols MgATP appear to be hydrolysed per reducing

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Figure 5 The interrelationship of nitrogenase components during enzyme catalysis, and their interactions with substrate, MgATP and reducing agent.

MoFep - molybdenum iron protein

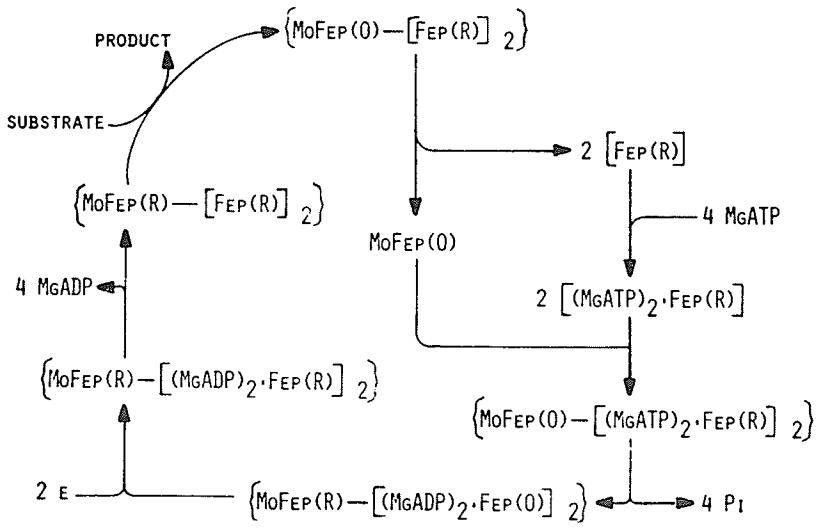
Fep - iron protein

MoFep (o) - oxidised MoFep (physiologically oxidised EPR active state)

MoFep (R) - reduced MoFEP

MgATP - magnesium adenosine triphosphate

From: Newton, Corbin, and McDonald (1976).


PRODUCT
SUBSTRATE
{MoFeP(O)—[FeP(R)] 2}
2 [FeP(R)]
{MoFeP(R)—[FeP(R)] 2}
4 MgATP
MoFeP(O)
4 MgADP
2 [(MgATP)2·FeP(R)]
{MoFeP(R)—[(MgADP)2·FeP(R)] 2}
{MoFeP(O)—[(MgATP)2·FeP(R)] 2}
2 E
{MoFeP(R)—[(MgADP)2·FeP(O)] 2}
4 PI

equivalent (2 electrons) used, which may indicate an active complex stoichiometry of two Fe proteins per Mo-Fe protein (Hadfield and Bulen, 1969). The data here summarised re nitrogenase mechanism can be represented as in Fig. 5.

To initiate catalysis, one electron is donated from the reducing agent to each of the two Fe proteins, which then bind 2 mols of MgATP each. Both FeP(R) - $(Mg\ ATP)_2$ species then combine with a single oxidised Mo-Fe protein. MgATP is visualized as binding by its adenine moiety to an adenine-specific site on the Fe protein, the terminus of the triphosphate chain is seen as binding to the Mo-Fe protein. The ADP portion of ATP is apparently required for binding to the Fe protein, while interaction of the terminal phosphate directly with molybdenum of the Mo-Fe protein is necessary for catalysis. MgATP may also serve to complex the Fe protein with the Mo-Fe protein to effect the requisite orientation such that interaction and electron transfer occurs between the exposed Fe_4S_4 chromophore of Fe protein and the electron-accepting site on the Mo-Fe protein (Newton et al., 1976; Thorneley and Eady, 1973; Walker and Mortenson, 1973). A site for substrate coordination on molybdenum is simultaneously cleared (Newton et al., 1976).

After ATP hydrolysis and phosphate departure, electrons flow to the Mo-Fe protein. The electrons are initially accepted by iron-sulphur clusters, which direct the electrons to the molybdenum-containing substrate binding and reducing site. The interaction between electron-transfer sites, as influenced by bound MgADP, holds the complex together, and reduction of the complexed Fe protein occurs. After MgADP dissociates, substrate is reduced on the Mo-Fe protein,

then protonated, and product released, leaving the complex of reduced Fe proteins with the oxidized Mo-Fe protein. This complex dissociates so that the Fe proteins may combine with more MgATP and reactivate the nitrogenase complex. The scheme here described (Fig. 5), as summarised by Newton et al., (1976), represents a two-electron ($C_2H_2 \rightarrow C_2H_4$) reduction cycle; a six electron reduction ($N_2 \rightarrow 2NH_3$) would involve three such cycles with the substrate bound to the MoFe protein throughout.

Rivera-Ortiz and Burris (1975), have presented evidence which suggested that nitrogenase serves as an "electron sink", and which indicated that substrates and inhibitors bonded at multiple modified sites on reduced nitrogenase. Acetylene was thought to be reduced by a less completely reduced "sink" than was required for the six electron transfer reaction to effect reduction of nitrogen to ammonia. Earlier work by Hwang, Chen, and Burris, (1973) has provided evidence of a similar nature, and these authors proposed the existence of five sites (or modified sites) for the nitrogenase complex; even though there may have been some doubt as to whether five specific sites (modified or otherwise) existed on nitrogenase, Hwang et al., (1973) stated that "it is clear that the multiple reactions of nitrogenase are not catalysed at a single, unmodified active site."

Electron transport to Nitrogenase in Azotobacter

A recently proposed scheme (Scherings, Haaker and Veeger, 1977) for the transport of electrons from NADH to nitrogenase, is shown in Fig. 6. Electron transfer is visualized as occurring through the cell membrane towards nitrogenase. On the basis of earlier work (Haaker and Veeger, 1977), it has been suggested that NADH can act as a donor of electrons to nitrogenase in Azotobacter if the cytoplasmic membrane was deemed capable of creating a difference in pH at the two sites of NADH-flavodoxin oxidoreductase (see Fig. 4). Flavodoxin hydroquinone was thought to be produced by membrane energisation. ATP, synthesized by a phosphotransacetylase in strong interaction with pyruvate dehydrogenase complex, was viewed as being coupled to nitrogenase (Haaker, Bresters and Veeger, 1972). NADPH formation was allegedly linked to nitrogenase by NADPH-NAD transhydrogenase, which enzyme was reportedly inhibited by $NADP^+$ and ATP, NADH formation occurring only at a high $NADPH/NADP^+$ ratio (Van den Broek and Veeger, 1971).

The ideas embodied in the schemes proposed for the mechanism of nitrogenase reaction, and for the transfer of electrons to nitrogenase in Azotobacter, will acquire profound significance in assessing the effect of the superoxide-mediated phenonema forming a significant part of the discourse subsequently presented, on the physiological responses of Azotobacter to dissolved oxygen, with specific emphasis on the role of oxygen-protecting and oxygen-scavenging mechanisms.

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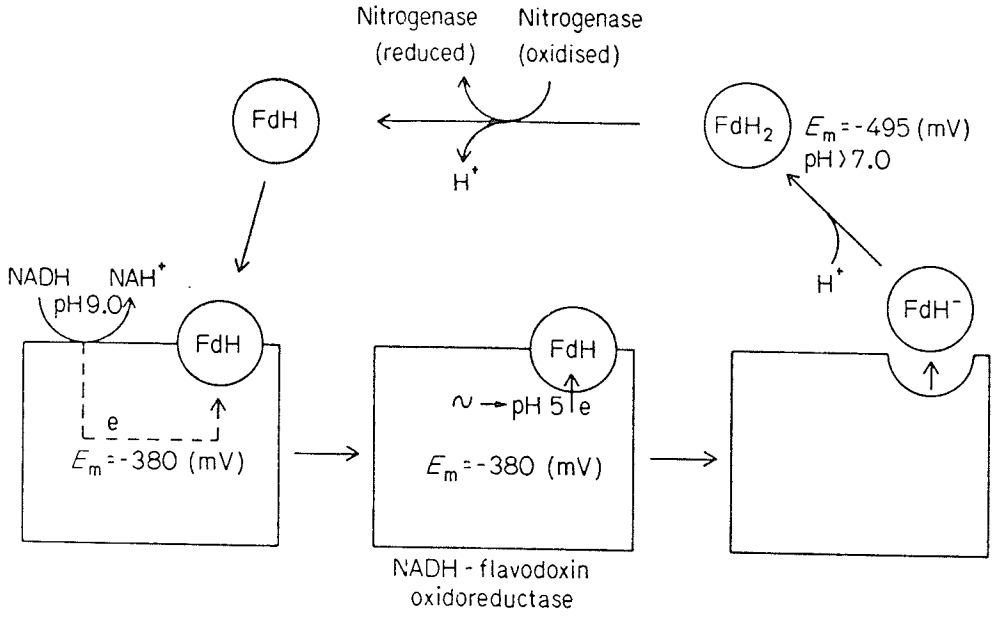
Figure 6 Proposal for electron transport to nitrogenase in Azotobacter vinelandii. Fd-flavodoxin.

From: Haaker and Veeger 1977.


Nitrogenase
(reduced)
Nitrogenase
(oxidised)
FdH
H+
FdH2
Em = -495 (mV)
pH > 7.0
NADH
NAH+
pH 9.0
FdH
e
Em = -380 (mV)
H+
FdH-
FdH
~ → pH 5 e
Em = -380 (mV)
NADH - flavodoxin
oxidoreductase

Continuous culture methodology and theory

The following is largely a summary of the ideas expressed by Malek, (1966) Pirt, (1975), and Pirt, (1973). Reference will be made to other sources as deemed appropriate. The basis for the utilisation of microorganisms in production and experimental research is their multiplication. The dynamics of multiplication as well as of other functions related to it are the most valuable properties of the microorganisms, but batch culture methods do not fully utilize these multiplication dynamics. To fully utilise culture multiplication dynamics, the volumes of batch culture would be prohibitively large, and an amount of nutrient would have to be supplied initially corresponding to the maximum growth possible in a given substrate volume. The constituent microorganisms of such batch cultures multiply from the start in a surplus of nutrients, which gradually decreases, with single components often being unevenly utilized, with simultaneous accumulation of growth products in the medium. The organisms grown in the first period of cultivation are different from those grown at later stages, producing a heterogenous culture of microorganisms which changes and develops with time. A typical growth curve thus results, consisting of an initial lag phase preceding a phase of logarithmic growth, and terminating with a stationary phase, which may turn downwards as death of the organisms in culture becomes more marked than growth.

In contrast to the classical batch culture, the chemostat consists of a culture into which fresh medium is continuously introduced at

a constant rate, and the culture volume is kept constant by the continuous removal of culture. Ideally, the mixing should be perfect, a drop of medium entering the vessel should instantly be distributed uniformly throughout the culture. Under the conditions of continuous culture the organisms have optimum conditions for muliplication as nutrients are continuously supplied in exact correspondence to immediate requirements. The volume of the culture is chosen in such a way as to allow optimum coordination of growth rate and nutrient inflow; the outflow of medium containing the grown organism is equal to the inflowing volume of nutrient fluid. These conditions are relatively constant, and there are no periods (under normal circumstances) of nutrient surplus or of partial and continuing insufficiency of nutrients. Consequently the physiological state of the microorganisms can be continuously maintained at a low level or in a form corresponding to the predetermined constant nutrient inflow.

The chemostat culture is characterised by a steady-state which is largely self-regulating, provided the system is operated at growth rates below the maximum, as determined by the principal limiting factor in the medium. The population size within such a culture is subjected to two opposing factors, namely; 1) a constant increase in population due to growth and 2) a constant decrease in the population caused by culture outflow from the culture vessel since the volume of the latter is kept constant. The rate of washout of cells can be defined by the expression

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

where x is the concentration of organisms (mg/ml);

D is the dilution rate, defined by F/V, (where "F" is the influx rate in ml/hr, and "V" is total volume of the culture in mls.)

The net rate of change of the population size within the culture vessel is given by the following:-

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

where μ is the growth rate constant, and the instaneous growth rate of the population is given by

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

The actual growth rate of the culture is given by the expression

$$\mu = \mu_{max} \frac{S}{Ks + S}$$ (Herbert, Elsworth and Telling, 1956)

where "μ_{max}" is the maximum value of μ, achieved when S is not growth limiting;

"S" is the concentration of the growth limiting substrate

"Ks" is the saturation constant (analagous to the K_m in Michaelis-Menten enzyme kinetics) equal to a substrate concentration at ½ the theoretical maximum growth rate.

A steady state is achieved when the increase in population size of the culture ceases at the point where some component of the inflowing medium becomes limiting, which limitation acts to produce a decline in the growth rate to the point where it becomes equal to the dilution rate. At this steady state level

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

and $$D = \mu_{max} \frac{(S)}{Ks + S}$$

The basic advantages of a chemostat over other methods of culture can be summarised as follows:-

1. A controlled growth rate achieved not by changing the nature of the substrate or physical conditions of the culture but by changing the concentration of growth-limiting substrate in the medium.
2. Physical and nutritional conditions can be changed whilst holding the growth rate constant.
3. Chemostat cultures afford a means of achieving "substrate-limited growth" with constant concentration of the limiting substrate, which has been utilised in a large number of investigations to examine and dissect the myriad effects of various factors on microbial and other organisms.
4. The unique steady state feature of a chemostat culture permits the biomass to adjust itself in any given environment, with such an environment being maintained indefinitely. The existence of a steady-state allows seperation of the effects of a given environment from those attributable to previous history of the organism.
5. A chemostat permits the most rapid conversion of substrate into biomass plus such growth limited products as carbon dioxide, features which lend themselves readily to large-scale biomass production, as evidenced from the markedly more significant yields obtainable from chemostats or

comparison to batch cultures.

Continuous cultures of the chemostat type, as referred to, thus provide a reproducibility not obtainable (usually or reliably) with the use of batch cultures, which are characterised by a continually changing environment, a feature which has formed the basis of elucidation for several factors affecting organisms' growth patterns and responses.

MATERIALS AND METHODS

MATERIALS AND METHODS

GROWTH CONDITIONS

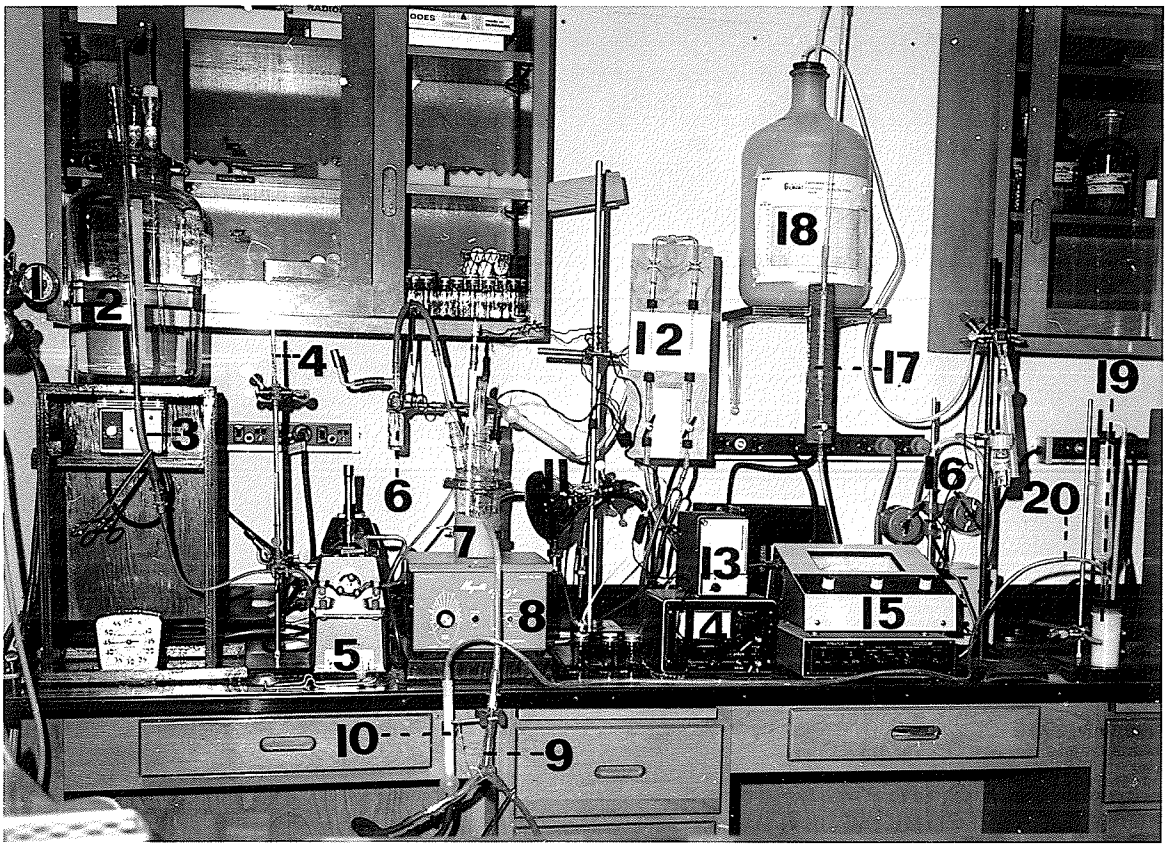
1. Continuous Cultures

Azotobacter chroococcum (ATCC 7493) was grown in continuous cultures with Burk's nitrogen-free medium essentially as described previously (Newton, Wilson and Burris, 1953), with mannitol in place of sucrose. The medium had the following composition (g. litre-1) Mannitol-10g KH_2PO_4-0.16; K_2HPO_4-0.64; NaCl-0.20; $MgSO_4 17H_2O$-0.20; $CaCl_2$-1.10. The trace element solution contained (g/litre) $FeSO_4.7H_2O$-2.5g; $H_3\beta O_3$-2.9; $CoSO_4.7H_2O$-1.2; $CuSO_4.5H_2O$-0.1; $MuCl_2.4H_2O$-0.09; H_2SO_4- 5ml. commerical stock; $Na_2MoO_4.2H_2O$-2.5; $ZnSO_4.7H_2O$-2.1; Nitrilotri-acetate-0.144. A quantity of 1 ml. of the above trace elements solution was added to each litre of medium. Stock cultures were used as inocula for 50 ml. medium contained in 125 ml. flasks, which were shaken initially at approximately 50 rpm; the rate of shaking was increased gradually as growth progressed. The resultant cultures were kept at $4^{\circ}C$ and used subsequently as inocula for the continuous cultures. Stock cultures were routinely subcultured.

The continuous cultures were started as batch cultures by the aseptic inoculation of 150 ml. medium, contained in culture vessel, with 50 ml. of stock culture. The culture vessel had a working volume of 200 ml. (see Fig. 7). The freshly inoculated culture was stirred overnight at approximately 500 rpm, with air being fed to the culture at a rate of about 150 c.c./min. The dissolved oxygen content of such cultures - as indicated by the oxygen electrode - was zero at the stir rate so specified.

Figure 7 The continuous culture assembly.

1. Gas supply
2. Medium reservoir with delivery tube, (3)
3. Delivery tube
4. Flow rate monitor (10 ml graduate pipette inserted into medium delivery tube by a glass "T" joint
5. Watson-Marlow H.R. Flow inducer pump, with variable setting to control rate of medium flow
6. Sample collection vial (20 ml. capacity, linked to glass tubing inserted into culture vessel)
7. Culture vessel; working volume of 200 ml. Dorsally sited inlets for medium and gas supply; pH control; thermostat; and O_2 electrode
8. Magnetic stirrer (variable speed; 60 cycles, 1 amp.)
9. Culture vessel outlet leading to overflow and bulk collection flasks
10. Air filter with cotton plug, and tubing, linked to CO_2 apparatus
11. Infra-red heat lamp-thermostatic; linked to $30^{o}C$ thermometer inserted into culture medium
12. Flow meters controlling air and/or gas supply to culture
13. Recording oxygraph
14. Oxygen meter; linked to O_2 electrode immersed in culture medium
15. pH meter; linked to pH electrode inserted into culture medium
16. Valves controlling addition of KOH from reservoir to culture vessel
17. Flow meter for air intake re CO_2 determination
18. Air reservoir (4 litre Nalgene bottle) for CO_2 determination
19. Percolation apparatus for CO_2 determination
20. Tubing linking percolation apparatus and culture overflow receiver.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The rate of stir was then increased to "vortex", i.e., about 1750 rpm, at which point the oxygen content of the medium rose rapidly. The concentration of oxygen in the gas mix supplied to the culture was then reduced by increasing the nitrogen content; the latter was gradually reduced by increasing the nitrogen content; the latter was gradually reduced until the actively growing cultures had attained the requisite cell density and attendant activity to maintain the medium's dissolved oxygen content at zero when supplied with air. Fresh medium was then fed into the culture vessel at progressively higher dilution rates until the desired rate was attained. The desired concentration of oxygen supplied to the continuous cultures was obtained by varying the relative quantities of nitrogen and/or oxygen in the incoming gas mix. Such cultures, grown as described, achieved a "steady-state" status in approximately three days as indicated by carbon dioxide production and dry weights. All continuous cultures used in this study were maintained at a dilution rate of 0.2 hr^{-1}, with a working culture volume of 200 ml.

Ammonium Supplemented Cultures

Ammonium succinate or ammonium sulfate (reagent grade) was added to the continuous culture vessel and to the medium reservoir, to give the required concentrations specified. Changes in culture pH which tended to be more marked at higher concentrations of ammonium salts, were controlled automatically be use of a Radiometer GK2303 C type combination pH electrode coupled to a Radiometer TT II Titrator (Radiometer, Copenhagen, NV, Denmark).

The pH of the ammonium-supplemented cultures was maintained at pH 6.6 by dropwise addition of requisite quantities of 1M NaOh through a Type MNV. magnetic valve (Radiometer).

2. Batch Cultures

There were grown in 1 litre flasks at 30°C by inoculation of approximately 250 ml. Burks nitrogen free medium as previous. The flasks were shaken at 120 rpm, and harvested after 3 days growth.

Batch and continuous cultures were routinely checked for purity by plating our culture samples onto trypticase soy agar plates which were incubated at 30°C for 2-3 days.

Sample Collection

Duplicate samples of 15 ml. volume were routinely collected - aseptically - from the continuous cultures into 20 ml sample bottles. Approximately 0.05 ml. concentrated sulphuric acid was added, and the samples were then stored at 4°C until analysed.

Larger volumes of sample - for the determination of enzyme contents for example, - were collected as overflow from the continuous culture vessel into 1 litre Erlenmeyer flasks; the latter was contained in an ice-filled insulated box. The cells so harvested were centrifuged and stored for subsequent use in the assay procedures stated herein for the respective enzymes.

Viabilities

The viabilities of Azotobacter chroococcum were estimated as described by Postgate, Crumpton and Hunter (1961), modified by the use of depression slides to accommodate filtered, agar supplemented mannitol B_6 medium. Inoculated slides were incubated at 30°C and examined after 18-24 hours.

Measurement of dissolved oxygen concentration in continuous culture medium

The dissolved oxygen content of the continuous cultures was measured using a steam-sterilisable galvanic (lead/silver) type oxygen electrode Type 50225, 225 mm long, which was supplied by the

L.H. Engineering Company Ltd., Bells Hill, Stoke Poges, Bucks, England. The oxygen electrode was hooked up to an oxygen meter, with the latter connected to a Rustrak recorder supplied by Rustrak Instrument Division, Gulton Industries Inc., Manchester, N.H., U.S.A. The sterilised O_2 electrode was then calibrated by passing nitrogen (to obtain a zero reading) and air (supplemented with oxygen as required) into the electrode container to obtain the maximum reading. The polypropylene membrane on the oxygen electrode was replaced periodically as required.

Oxygen uptake by whole cells

This was estimated by rapid transfer of cell samples from the respective continuous cultures to the reaction vessel of a Gilson oxygraph (Gilson Medical Electronics, 3000 W. Beltline Hwy., Middleton WI. 53562), equipped with a Clarke type oxygen electrode. The cells were added to the vessel with mannitol B_6 medium as used for growth.

Measurement of carbon dioxide produced by continuous cultures

The production of carbon dioxide by the continuous cultures was measured as described by Lees and Postgate (1973), with minor modification to the 4 litre receiver vessel as follows: The cotton plug at point A (Fig. 8) was replaced by a short length of glass tubing, which was covered by a metal cap; both the tubing and the metal cap were enclosed in a ground-glass joint, and the latter was fitted to the collection flask, allowing the release of air. The glass tubing was

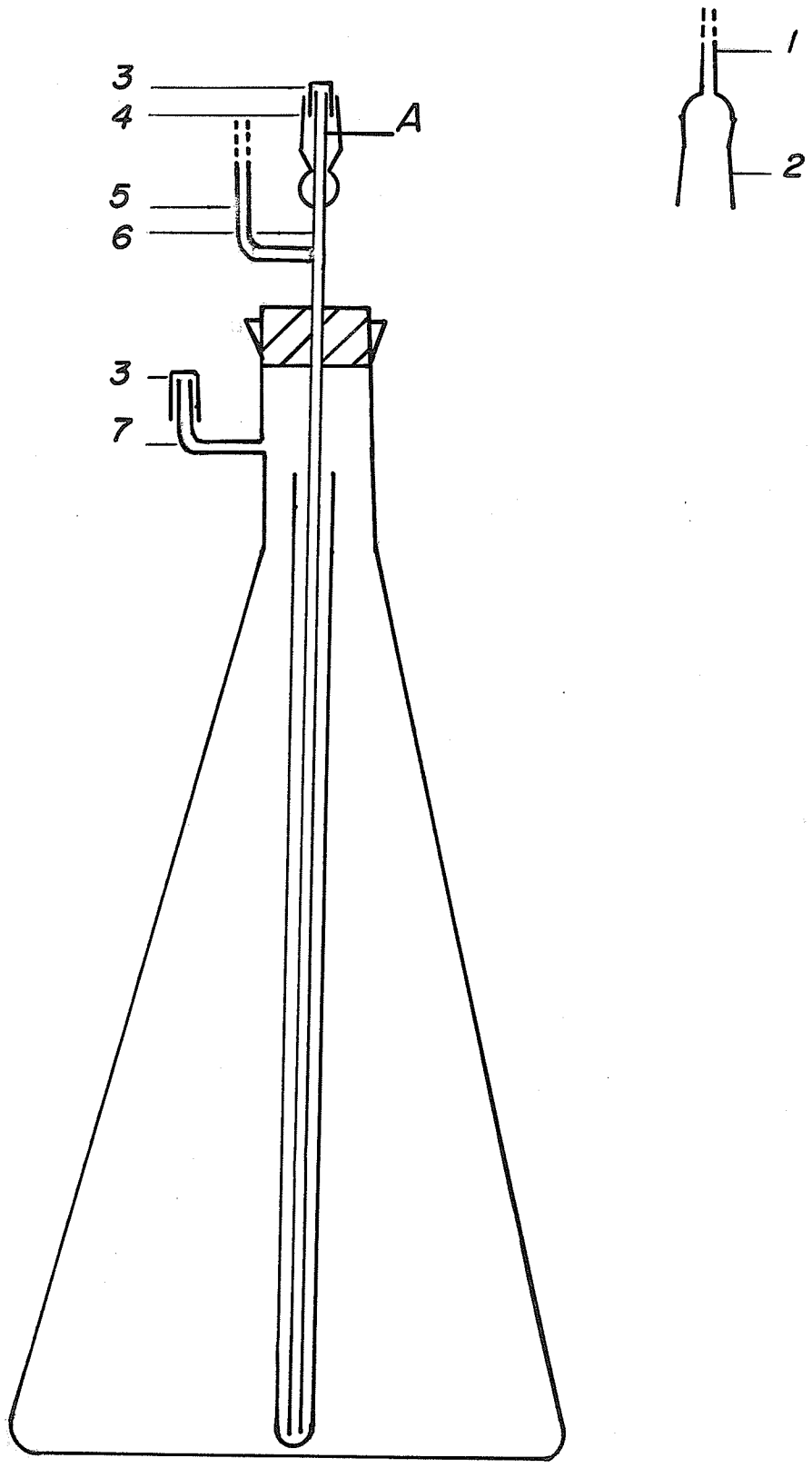
1
2
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Figure 8. The modified four-litre receiver vessel.

1. To CO_2 percolator
2. Female ground glass joint (inserted over 4)
3. Metal closures
4. Male ground glass joint
5. Culture inlet (overflow from culture vessel)
6. Grease application to prevent backflow
7. Air release.

lightly greased on the inside above the culture inlet to prevent backflow of the culture effluent.

MACROMOLECULAR COMPOSITION

1. Protein

Protein was determined according to Lowry, Rosebrough, Farr and Randall (1951), with bovine serum albumen as standard.

2. Polyhydroxybutyrate

This was determined as described by Law and Slepecky (1961), with commercially prepared β-hydroxybutyrate (Sigma) as standard.

3. Polysaccharide

The "anthrone" method - as described by Dalton and Postgate (1969) was used to determine polysaccharide, using glucose as standard.

4. Ribonucleic acid (RNA)

This was determined using the procedure reported by Morse and Carter (1949), with ribose as standard.

5. Dry weight

Duplicate samples of 10 ml volume, taken from the continuous cultures, were centrifuged at 5000 x g for 10 min, then washed 3 x with distilled water. Dry weights were determined by evaporation in pre-weighed aluminum dishes at 105°C until constant weights were obtained.

6. Mannitol

Residual mannitol in the medium of the continuous cultures was determined according to Neish, (1950).

ENZYME ASSAYS

1. Superoxide dismutase (SOD)

The superoxide dismutase activity of crude extracts of Azotobacter chroococcum, and partially purified enzyme from such sources, was determined by the method of Buchanan and Lees (1976). Superoxide dismutase activity was indicated by the inhibition produced by superoxide dismutase in the rate of reduction of acetylated cytochrome c (Ac c) by the superoxide anion; the latter was generaged by the reaction of 1×10^{-4} M xanthine and 40 µg. xanthine oxidase. Acetylated cytochrome c (2.5×10^{-5}, M, according to Azzi, Montecucco, and Richter 1975) was added to 1 ml quartz curvettes containing varying quantities of borate/ethylenediaminetetraacetic acid (EDTA) buffer, of 10^{-3}M EDTA and 10^{-2}M borate concentrations. The reduction of Ac. c was started by adding the requisite quantity of xanthine oxidase, which was the last addition to the reaction mix. Crude extracts, or partially purified enzyme, was added to the reaction mixtures before the addition of xanthine oxidase. The effects of various chemicals on the activity of superoxide dismutase was estimated by addition of the respective chemicals to the reaction mix, appropriate corrections being made for their effects (if any) on the rate of Ac. c reduction by the xanthine-xanthine oxidase system. A unit of SOD activity was defined as that which produced a 25% inhibition in the rate of reduction of 25 µM Ac. c by O_2^- at pH 9, and at 29°C with O_2^- being generated by the xanthine-xanthine oxidase system. Relevant details not here mentioned are given in the appropriate results. Cytochrome c (Type III) was obtained from Sigma, St. Louis, Mo; xanthine from Nutritional Biochemical Corp., Cleveland, Ohio.

O_2^- Production

O_2^- was produced from the aerobic action of xanthine oxidase on xanthine in the concentrations given for the respective experiments.

2. Nitrogenase

The nitrogenase activity of whole cells of Azotobacter chroococcum grown in continuous cultures was determined essentially as described by Dalton and Postgate (1969 b), and by Drozd and Postgate (1970). A ml.of cells was taken directly from the continuous cultures and rapidly transferred to 25 ml. flasks, which were then stoppered with Suba seals. The flask and contents were then flushed for 1 min with a gas mixture of 22% oxygen and 78% Argon (commercially prepared, Lynde speciality gases, Union Carbide Ltd., Toronto, Canada). 1 ml. of commercial acetylene was injected into the flasks, which were then incubated in a shaking water bath, at 30°C and approximately 120 rpm, for 1 hour. The reaction was terminated by addition of 0.2 ml conc. H_2SO_4. Ethylene production was estimated by injecting 0.2 ml. of the flask's gas phase into the injection port of a Pye Series 104 chromatograph (W.G. Pye & Co. Ltd., Cambridge, England), fitted with a 6' x 1/8" column packed with Porasil-C (phenyl isocyanate) 80 - 100 polar material (Durapak Type 27246). Column temperature was maintained at 45°C. Ethylene produced was estimated by comparison of peak heights produced by known quantities of ethylene under similar conditions. Flow rates of N_2 and H_2 were 50 cc/min, and that of compressed air approximately 630 cc/min.

The effect of O_2^- was examined by adding 2×10^{-5} M xanthine and 200 µg xanthine oxidase enzyme just before adding the acetylene. Appropriate controls were similarly treated.

3. Cytochrome oxidase

The cytochrome oxidase content of crude extracts of Azotobacter chroococcum (prepared as described below) was measured by the oxidation rate of ascorbate-reduced cytochrome c (Type III Sigma), as described by Wharton and Tzagaboff (1967). Cytochrome oxidase activity was quantitated in terms of "k" values/min obtained, or by the quantities of cytochrome oxidised per given time interval, using an $\Sigma_{mm(550)}$ of 27.7 for cytochrome c. Other details are given in the results.

4. Nicotinamide adenine dinucleotide-reduced (NADH) and nicotinamide adenine dinucleotide phosphate-reduced (NADPH) oxidase activities

These were determined by monitoring the changes in concentration of NADH and NADPH respectively, as indicated by changes in absorbance at 340 mμ, essentially as described by Robrish and Marr, (1962). Crude extracts used for these assays were prepared as described below.

5. Catalase

The catalase activity of Azotobacter chroococcum whole cells, grown in continuous culture, was estimated manometrically, or by utilisation of a Gilson oxygraph equipped with a Clarke-type oxygen electrode, to quantitate the production of oxygen from known quantities of H_2O_2. Reactant concentrations and conditions are given in the appropriate section of the results.

6. Peroxidase

Crude extracts (prepared as described below) were examined for peroxidase activity by monitoring the rate of oxidation of diaminobenzidine tetrahydrochloride (DAB) at 465 nm, as described by Fahimi and Herzog

(1973). 0.1 - 1 mg. of crude extract was added to 1 x 10^{-4}M DAB (in 0.1 M citric acid/sodium phosphate buffer containing 0.1% gelatin), and the reaction was initiated by adding approximately 12 mM H_2O_2 to the assay mix. Quantitation was effected by comparing the oxidation of DAB produced by commercial horse radish peroxidase (HRP) and that produced by the crude extract.

Cell-free Nitrogenase assay

Assays of crude cell-free extracts for nitrogenase were carried out essentially as described by Drozd and Postgate (1970). 1 litre of cells was collected at 4°C (in an ice filled polystyrene container) and the cells were harvested by centrigation at 28,000 g for 10 min at 4°C. The cell pellet was suspended in 100 ml. 25 mM Tris-HCl buffer pH 7.4, and disrupted by sonication for 2 min at 4°C, alternating 1 min periods of sonication with 1 min cooling at 4°C, using an Ultrasonic Insonator (Model 1000, New York Inc., Farmingdale, Ultrasonic Systems) at maximum output. The sonication was effected under a stream of N_2 which was passed through a Deoxy cartridge (Engelhard Industries, Union, N.J. 07083), to remove traces of O_2. Bovine SOD (when present) was added to a final concentration of 100 µg/ml. immediately after sonication. The sonicated cell preparation was then centrifuged at 38000 g for 20 min at 4°C, and the resulting supernatant was used as the cell free extract for assay of nitrogenase activity by the acetylene reduction technique as outlined for assay of the nitrogenase activity of whole cells described in the preceding section. The flask contents (except $Na_2S_2O_4$ and the cell extract) were added to a 25 ml. "Suba" seal stoppered flask and evacuated and refilled with O_2-purged argon. Acetylene was

added, and the contents brought to atmospheric pressure. Dithionite and the enzyme extract (both of which were evacuated and flushed with argon as above) were added by syringe to start the reaction, which was terminated by adding approximately 0.1 ml. conc. H_2SO_4 to the flask contents. Samples were incubated at 30°C with shaking at 100 rpm in a H_2O bath. Other relevant details are given in the appropriate section of the results and discussion which follows.

To ascertain the role of SOD (or other enzyme activities indicated) SOD was added to one sample and not to the other, with both samples being exposed subsequently to 20% O_2. In such instances exogenous SOD may have been omitted from the cell mix prior to effecting sonication of the cells.

Preparation of crude extracts from Azotobacter chroococcum whole cells for enzyme assays (SOD, cytochrome oxidase, NADH, NADPH Oxidases)

A litre of cells from the respective continuous cultures was harvested at 4°C overnight. The collected cells were centrifuged at 10,000 x g for 15 min, then washed twice with 50 mM phosphate buffer containing 1 mM EDTA. The washed cells were sonicated immediately, or stored at -75°C until subjected to sonication. The cell suspensions (thawed if previously frozen) were sonicated intermittently for a total time of 5 min at 4°C, alternating 1 min periods of sonciation and cooling at 4°C. The resulting sonicate was centrifuged at 37000 x g for 15 min at 4°C; the supernatants were collected and the residues were resuspended to the initial volumes; both residue and supernatant were examined as necessary for enzymic activity, as previously described.

Xanthine oxidase activity of A. chroococcum whole cells.

The oxidation of xanthine by Azotobacter chroococcum whole cells taken from continuous cultures grown under the conditions stated, was determined manometrically as described essentially by Magee and Burris (1956), who showed that whole cells of A. vinelandii oxidised xanthine. The procedure was also repeated using a Gilson oxygraph fitted with a Clarke type oxygen electrode, as described previously. A 10 ml. cell sample was centrifuged at room temperature at 5,000 g for 10 min and resuspended to a total volume of 2.5 ml., in 0.05 M phosphate buffer with 1 mM EDTA pH 7.4. Cell suspension (0.1 ml.) was added to 1.2 ml buffer and 0.2 ml. of 0.05 M xanthine in 0.05 M NaOH in the reaction vessel of the oxygraph, and oxygen uptake measured. The endogenous O_2 uptake was also determined.

The examination of the whole cells for xanthine oxidase was prompted by the use of xanthine and xanthine oxidase to generate O_2^- exogenously, thus it was necessary to see if A. chroococcum could oxidise exogenous xanthine at appreciable rates.

Localisation of catalase and superoxide dismutase in Azotobacter whole cells.

Samples of Azotobacter were taken from the respective cultures and washed 4 x (with 50 mM potassium phosphate buffer pH 7.8 containing 1 mM EDTA) by centrifugation at approximately 2000 x g for 45 min at 4^oC. The 'slime' layer above the cell pellet was subsequently removed, with minimal disturbance to the cell pellet. The cells were then subjected to osmotic shock and lysis according to the method reported

initially by Robrish and Marr (1962). Washed cells were placed in an equal volume of 3M glycerol for 10 min at room temperature. The glycerol cell mix was slowly injected by syringe into 10 volumes 10 mM Tris buffer pH 7.4 with 5 mM $MgCl_2$, with constant stirring. Deoxyribonucleotidase (DNAase) was then added to a final concentration of 1 µg/ml, and the entire mixture was left at room temperature for 20 min. The cell mix was then centrifuged at 10000 x g for 15 min at $4^{o}C$; the supernatant so collected was later added to subsequent washings of the resulting cell pellet, which was now dubbed the 'large particle' fraction. The supernatant and washes from the preceding steps were combined and centrifuged at 100000 x g for 2 hrs at $4^{o}C$, the resulting pellet was washed with buffer as above, and was named the 'small fraction'. The washings from both large and small particle fractions were pooled to form the 'soluble fraction'. All fractions were then examined for catalase and superoxide dismutase activity, before and after being subjected to sonication as described in the appropriate sections of the Materials and Methods. Fractions were prepared for electron microscopic examination as described subsequently.

Electron microscopy

Sub-cellular fractions, i.e., "large and small particle" fractions, were prepared as described in the section under the heading "localisation of catalase and superoxide dismutase in Azotobacter whole cells". The fractions were prepared for electron microscopic examination by the following procedure:

A. Fixation (Hess, 1966)

1. The respective cell fraction was suspended in a fixation mixture containing 5 ml. cacodylate buffer pH 7.4, 3.5 ml. distilled water, 1.2 ml. 25% glutaraldehyde, and 0.3 ml acrolein. The fractions were fixed in the mixture described for 4 hours at $4^{o}C$.

2. The fixed fractions were washed twice with 0.1 M cacodylate buffer pH 7.4 by centrifugation as stated in the procedure for obtaining the fractions.

3. The washed and centrifuged pellets (fractions) were mixed separately with warm, filtered agar, which was then spread on a glass slide and allowed to harden, being subsequently cut into small cubes (3 - 5 mm).

4. The agar cubes were then washed twice with 0.1 M cacodylate buffer pH 7.4 by suspension in buffer for 15 min each wash.

5. The cubes were then fixed for 4 hrs. in 1% osmium tetraoxide in 0.1 M cacodylate buffer pH 7.4 at $4^{o}C$.

6. Samples were then washed 4 x (15 min each wash) in 0.1 M cacodylate pH 7.4.

7. The washed samples were then placed overnight in 0.5% aqueous uranyl acetate, and subsequently washed once in distilled water.

B. Dehydration

Dehydration of the fractions was effected by standing them in increasing concentrations of ethyl alcohol - 50%, 70%, 90% for 10 min. each, followed by standing in two changes of absolute ethanol for 30 min. each.

C. Embedding

Embedding was effected in vinyl cyclohexene dioxide-based epoxyresin (ERL-4206) as described by Spurr (1969). The embedding mix, referred to subsequently as "Spurr", was prepared by mixing, in the order listed, the following components:-

10 gm ERL-4206

6 gm. diglycidyl ether of propylene glycol (DER 736)

26 gm. nonenyl succinic anhydride (NSA)

0.4 mg. dimethylaminoethanol (S-1, DMAE)

The first three components were thoroughly mixed and allowed to stand for 15 - 20 min to allow entrapped air bubbles to surface. The DMAE was then added. The samples were initially placed in a small volume of absolute ethanol to which had been added an equal volume of Spurr, and allowed to stand (after thorough mixing) for 30 min. The volume of Spurr added was doubled, and the mix stood for 30 min., after which the Spurr was decanted and fresh Spurr added. The decanting and standing was performed twice, followed by standing in Spurr for 2 hrs. and final standing in Spurr overnight.

D. Polymerization

Gelatin capsules were partially filled with Spurr and an agar cube containing the respective sample was added. The capsules then stood at room temperature for 12 hrs., after which they were then polymerised in a vacuum oven at 60^{o}C for 12 hrs. Silver-grey sections were subsequently cut from the cooled capsule, using glass knives mounted on a Reichert OM U2 ultramicrotome (Stockholm, Sweden).

Sections were picked off on 300 mesh copper grids, and post-stained in lead citrate, as recommended by Reynolds (1963), under an atmosphere of nitrogen for 3 min. The lead citrate stain was prepared by adding 1.33 gm. lead citrate, and 1.76 gm. sodium citrate, to 30 ml. distilled water. The resulting mix was shaken intermittently for 30 min., then 8 ml. 1N NaOH was added to dissolve the precipitate formed. The post-stained grids were washed in distilled water, air-dried, then examined and photographed using a model EM 6B (AEI) electron microscope (Harlow, Essex England).

ENZYME LOCALISATION OF POLYACRYLAMIDE GELS

1. Superoxide dismutase

This enzyme was localised on polyacrylamide gels using somewhat modified versions of the procedures reported initially by Fridovich and Beauchamp (1971),and by Salin and McCord (1974). Varying quantites (20 - 100 µg protein) of crude extracts of cells from continuous cultures, prepared as for enzyme assays, were added to 7.5% polyacrylamide gels prepared as outlined by Davis (1964). Electrophoresis was effected for the time periods specified in the results, using Pharmacia equipment and Tris glycine buffer pH 8.4. After electrophoresis, the cylindrical gels were removed and soaked in a reaction mix consisting of the following:

50 mM potassium phosphate pH 7.8

1 mM EDTA (Ethylenedinitrilotetracetic acid)

20 mM TEMED (Tetramethylethylenediamine)

30 µM Riboflavin

25 µM NBT (nitroblue tetrazolium)

The gels were incubated individually in glass test tubes, containing the above reaction mix, in the dark for 40 min. The gels were then removed from the tubes and reaction mix and exposed to an incandescent light source until maximum development was achieved. The SOD activity was indicated by clear zones (achromatic) in the otherwise uniformly deep-blue gels. Band width and clarity were apparently influenced by the amounts of active enzyme present in the crude extracts applied to the gels. Corresponding gels were stained for protein with Coomassie blue as recommended by Davis (1964), who also described the procedure for destaining of the gels.

2. Catalase and/or peroxidase

The procedure followed to demonstrate the enzyme activities on 7% polyacrylamide gel was as described by Gregory and Fridovich (1974). 20 µg crude extract were added to the gels, prepared as recommended by Davis (1964). Electrophoresis was effected as described previously for SOD activity localisation. The gels were then soaked in the dark for 45 min in a mixture consisting of 2 ml. 5 mM potassium phosphate pH 7.0 containing 1 mg. diaminobenzidine hydrochloride (DAB hydrochloride) and 0.1 mg horseradish peroxidase (HRP). HRP was omitted from the reaction mix to differentiate between catalase and peroxidase. The gels were subsequently rinsed twice with 5 ml. glass distilled water, and were then immersed in 20 mM H_2O_2 until staining was complete. The gels were stained uniformly brown except for achromatic regions indicating the presence of catalase. Peroxidase activity was indicated by the development of a band when HRP was omitted from the localisation mix. Peroxidase activity bands

were also differentiated from catalase by the latter having achromatic centers whereas peroxidase bands were darkly stained throughout.

PARTIAL PURIFICATION OF SUPEROXIDE DISMUTASE

5 x 20 litres of batch grown Azotobacter chroococcum, grown in 1% mannitol medium with 20% oxygen (air), were harvested with a Sharples steam-driven centrifuge to yield approximately 100 gm. cells. The cells were then suspended in 500 ml. 50 mM potassium phosphate pH 7.8 containing 1 mM EDTA, and disrupted in 200 ml. batches at 4°C, using an Insonator set at maximum output. The cells were subjected to sonication for 5 min., alternating 1 min periods of sonication with 1 min. periods of cooling at 4°C. The resulting sonicate was centrifuged at 13,200 x g for 60 min. at 4°C. Purification of the superoxide dismutase subsequently was effected, with minor modifications, using the methods outlined by Keele, McCord and Fridovich (1970); and by Yamakura (1976), as follows:

1. Potassium chloride (KCl reagent analytical grade) was added to the supernatant to a final concentration of 0.1 M, and the resulting precipitate was removed by centrifugaition at 13,200 x g for 15 min. at 4°C.

2. The resulting supernatant was heated, in 200 ml. batches, to 60°C for 3 min. followed by rapid cooling at 0°C.

3. Streptomycin sulphate was added to a final concentration of 2.5%, and the resulting precipitate was removed by centrifugation (as in step 1) after allowing the mixture to stand for 30 min.

4. Ultra-pure, granular ammonium sulphate (Schwarz-Mann, Orangeburg, N.Y. 10962) was added to the supernatant (from step 3), to achieve 50% saturation.

The resulting mixture was then stirred gently at room temperature for 1 hr., after which the precipitate produced was removed by centrifugation at 13,200 x g for 15 min. at 4°C.

5. Further additions of ammonium sulphate were made to bring the final concentration in the step 4 supernatant to 75%, with the resulting suspension being stirred at room temperature for 1 hr.

6. The 'step 5' precipitate was collected by centrifugation (as in step 4), and suspended in 2 mM potassium acetate pH 5.5, followed by dialysis at 4°C for 96 hrs. against 20 volumes 2 mM potassium acetate pH 5.5; the dialysis buffer was replaced every 12 hrs.

7. The precipitate produced during dialysis was removed by centrifugation as previously noted, and the resulting supernatant was dialysed against 5 mM potassium phosphate pH 7.8 for 60 hrs. at 4°C with the dialysis buffer being changed every 12 hours.

8. The dialysed extract from the preceding step was adsorbed into a 2.5 x 25 cm. DEAE cellulose (DEAE 32) column previously washed and equilibriated with the dialysis buffer. The DE-32 column was eluted with dialysis buffer.

9. The collected fractions were analysed for protein by recording the absorbance at 280 mμ, and for SOD activity by the method of Buchanan and Lees (1976).

10. Fractions were concentrated by flash evaporation at 30°C using an Evapomix (Buchler Instruments, Fort Lee, N.J., U.S.A.)

11. The concentrated fractions (from step 10) were adsorbed into a 1.5 x 25 cm. Bio-gel HTP (hydroxylapatite, Bio-Rad) column washed and equilibriated as recommended by the manufacturer. Elution was

effected with a gradient of 2 - 150 mM potassium phosphate pH 6.1. The collected fractions were examined for protein and SOD content as previously noted.

12. Pooled fractions, as indicated in the results, were concentrated by flash evaporation as noted previously.

The purification scheme and the attendant results are detailed in the appropriate section.

CHARACTERISATION OF PARTIALLY PURIFIED SUPEROXIDE DISMUTASE

The partially purified superoxide dismutase was characterised by the following:

1. Molecular weight determination
2. Reactivity with various chemicals
3. Sensitivity to cyanide
4. Absorption spectrum
5. Electrophoretic behaviour.

1. Molecular weight determination

The molecular weight of the partially purified SOD from Azotobacter was determined by Sephadex gel filtration and by electrophoresis in polyacrylamide gradient gels. Subunit molecular weight was determined according to Weber and Osborn (1969).

a) Sephadex gel filtration

Sephadex G75 superfine was washed, equilibriated, and packed into a 2.5 x 45 cm. glass column as recommended by the manufacturer (Pharmacia Fine Chemicals, Piscataway, New Jersey), using 0.15M

potassium phosphate buffer pH 6.1 throughout the procedure, described below:-

i) The void volume of the column was determined by elution with buffer of a 1 mg/ml. solution of Blue Dextran 2000 (Pharmacia) applied to the column using a sample applicator (Pharmacia).

ii) Twenty mg. of each of the standards - ribonuclease, chymotrypsinogen, ovalbumin, and aldolase - were dissolved in 1 ml. eluent buffer, then applied to the column after the mix was allowed to stand for 10 min.

iii) Elution was effected, using 0.15 M potassium phosphate pH 6.1., as subsequently reported in the results. The elution volume for each standard was determined.

iv) The partially purified Azotobacter chroococcum SOD, from the Biogel purification step described previously, was then applied to the column, and the void volume of the protein determined, using the assay procedure of Buchanan and Lees (1976) to measure the SOD content of the eluted fractions.
The molecular weight of the SOD was determined from a semi-logarithmic plot of 'Kav' values (on the linear scale) against corresponding molecular weights on the logarithmic scale. 'Kav' values were determined using the relationship

$$Kav = \frac{Ve - Vo}{V_t - Vo}$$

where Ve = elution volume for SOD

Vo = elution volume for blue Dextran 2000

V_t = total bed volume

Other details not herein mentioned accompany the data presented subsequently in the results.

b) Polyacrylamide gradient gel electrophoresis

20 - 50 µg of each of the standards ovalbumin, aldolase, chymotrypsinogen, ribonuclease, and bovine SOD (Sigma), dissolved in 5 mM potassium phosphate pH 7.8, were added to separate wells on a polyacrylamide gradient gel slab (PAA 4/30 Pharmacia) of 4 to 30 percent polyacrylamide. The partially purified Azotobacter SOD was obtained from a Bio-gel HTP column previously described. Electrophoresis was performed as stated in the results, using Pharmacia gel electrophoresis equipment with Tris-glycine buffer pH 8.3 as buffer. The gel slabs were removed from the casettes on completion of electrophoresis, and separate gels were stained for protein (with Coomassie blue) and for SOD, using the same enzyme localisation mix as was used to demonstrate SOD on cylindrical gels as described previously in this section. The position of the protein band localised by staining associated or identified with the SOD activity was ascertained from a comparison of gels run under the same electrophoretic conditions and stained subsequently for protein and for SOD activity. Destaining was performed electrophoretically.

The gradient gels used were cast in a solution containing 1 g/litre Na_2 EDTA, 1g/litre Boric acid, 2.5g/litre ammonium sulphate, and 0.2 g/litre sodium azide. The slabs were cast in cassetes 82 x 82 x 4.9 mm.

c) Sodium dodecyl sulphate (SDS) acrylamide electrophoresis - determination of subunit molecular weight.

The above-mentioned standards - ovalbumin and others - and the partially purified Azotobacter SOD were incubated in the presence of β-mercaptoethanol and SDS in 10 mM sodium phosphate pH 7.0 for 2 hrs

at 30°C, with shaking at 110 rpm, in a water bath. The dialysis step was omitted (after incubation) and the respective proteins dissolved in buffer, were applied to the cylindrical gels. The gel buffer was as specified by Weber and Osborn (1969). Electrophoresis was performed as stated in the results. Staining, and destaining, was as described (Weber and Osborn, 1969).

The mobilities of the proteins indicated were determined using the expression -

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before destaining}}{\text{distance of dye migration}}$$

The subunit's molecular weights were determined by plotting the mobilities of the protein standards against the known molecular weights expressed on a semi-logarithmic scale, as recommended by Weber and Osborn (1969).

Secretion of SOD by plate colonies of Azotobacter chroococcum

Agar plates were prepared from 2% agar mixtures of normal mannitol growth medium and inoculated with Azotobacter chroococcum taken directly from continuous cultures grown with air under nitrogen fixing conditions with normal concentrations of mannitol (1%). The SOD localisation mix, previously described for localisation of SOD on polyacrylamide gels, was added to the agar plates before these had cooled, and the plates were rotated to ensure mixing of the contents.

Inoculated plates were incubated at 30°C for 72 hrs., and subsequently exposed to an incandescent light source. Photoreduction of the NBT component of the localisation mix present in the plates produced a uniform blue colour throughout the plate except for areas associated with significant quantities of SOD requisite to inhibit the light-catalysed 'blueing' as indicated by achromatic zones around the bacterial colonies growing on the plates. Inocula were omitted from the control plates, which were treated similarly to the test plates in all other respects.

PRODUCTION OF O_2^- BY ACTIVELY GROWING AZOTOBACTER

Production of exogenous O_2^- by actively growing Azotobacter chroococcum was estimated from the O_2^- specific reduction of known quantities of acetylated cytochrome c, as confirmed by inhibition of the reduction noted by exogenous bovine SOD. The acetylated cytochrome c was prepared as described previously (Azzi et al., 1975).

Duplicate 20 ml. samples were taken from the respective continuous cultures and centrifuged at 5000 g for 10 min at 4°C. The centrifuged cells were resuspended (to 2.5 ml. volume) in mannitol medium as described previously, with 2% mannitol. A known quantity of acetylated cytochrome c was added, and the total volume of the flask contents adjusted to 5 ml. 100 µg bovine SOD was added to the control flasks. The 25 ml. flasks and contents were subsequently shaken in a 30°C H_2O bath at approximately 100 rpm, and 0.25 ml samples were subsequently withdrawn at the time intervals stated from both the control and the experimental flasks.

The samples were diluted to 1 ml., centrifuged at 5000g at room temperature for 10 min. to remove the cells, and the absorbances of the resulting supernatants were recorded at 550 mμ. These supernatants were subsequently reduced completely with dithionite ($Na_2S_2O_4$) and the resulting absorbances recorded. O_2^- production was estimated from the differences in absorbances at 550 mμ between the initial absorbance noted and that obtained after reduction with dithionite. The reduction noted which was specifically attributable to O_2^- was obtained by comparing the above-mentioned absorbance differences in both the control and experimental samples. The quantities of acetylated cytochrome c reduced were calculated from the extinction coefficient Σ_{mM} 550 mμ = 27.7.

An alterantive procedure was provided by adding cell samples from the respective continuous cultures to 25 μM acetylated cytochrome c in borate buffer pH 9.0 with 1 mM EDTA in a total volume of 1 ml. Reduction rates were then monitored *in situ* at 550 mμ, and O_2^- specific reduction was indicated by the effect of exogenous SOD on the rate of reduction (Δ OD_{550}/min or Δ cyt. c/min) observed.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

1. Method of Assay for Superoxide dismutase

The assay procedure has been described (Buchanan and Lees, 1976). Fig. 9 shows the rate of O_2^- mediated reduction of acetylated cytochrome c, (Ac.c) and the inhibition of this reduction by SOD, which formed the basis of the assay procedure. The inhibition produced by SOD was maximal at about pH 9, (Fig. 10). SOD inhibition of the rate of O_2^- mediated reduction of Ac. c as influenced by temperature is indicated in Figs. 10, 11. The observed inhibition fell markedly above 45°C (Fig. 11). On the basis of these results, it was suggested that the activity of superoxide dismutase could be estimated from the inhibition of O_2^- mediated reduction of Ac. c at pH 9.0, and at 29°C. 1 unit of activity was defined as that which produced an inhibition of 25% in the observed rate of reduction at pH 9.0, under the conditions specified, i.e., the reduction of 25 μM Ac. c by O_2^- generated by xanthine-xanthine oxidase reaction, (Fig. 12).

In respect to the procedures herein reported, it was noted that pH values higher than 9.0 gave lower assay values for a given SOD concentration, possibly attributed to competition between SOD and OH^- ions for O_2^- (Rigo, Viglino, and Rotillo, 1975) since radical attack of SOD lysyl residues (as a possible reason for the effect of pH was deemed unlikely at pH 9.0 (Barra, Bossa, Calabreese, Rotillo, Roberts and Fielder, 1975). SOD is reportedly stable at temperatures up to 70°C (McCord and Fridovich, 1969b) thus the

marked decline of SOD efficacy above 45°C here observed was unexpected. Variations in the xanthine oxidase concentrations did not produce marked differeces in the inhibition produced by SOD, a known concentration of which was observed to yield similar degrees of inhibition (33 - 34%) when reaction mixes contained 2×10^{-5}g, or $12 \times 10g^{-5}$, xanthine oxidase enzyme at an Ac.c concentration of 25 µM.

In the assay procedure as described, SOD competes with Ac.c for O_2^-, with Ac.c being favored when present at high concentrations as indicated by the decreased inhibition produced by SOD at elevated concentrations of Ac.c. (Fig. 12). Non-linear plots were obtained, as similarly reported by Misra and Fridovich (1971). A linear plot was produced when the concentrations of both SOD and Ac.c were low; under the conditions so specified, the inhibition by up to 0.5 µg SOD/ml in the presence of 25 µM Ac c, was linear.

The inhibition of the rate of reduction Ac. c, equated to the SOD activity present under the conditions so specified, could be estimated from the expression $((R_1-R_2)/R_1) \times 100\%$, where R_1 was the rate of reduction of Ac. c in the absence of SOD and R_2 the rate of reduction in the presence of SOD. A plot of $\frac{R_1}{R_2}$ against the concentration of SOD was linear, (Fig. 13)as has been found with other assay procedures previously used (Giannopolitis and Ries, 1977) attesting to the validity of the procedure herein described.

The addition of EDTA to the reaction mixes might have increased the reliability of the assay described by sequestering metal ions to

promote decomposition of the H_2O_2 produced during the dismutation process, which is of some importance since H_2O_2 may advsersely affect SOD activity and functioning (Beauchamp and Fridovich, 1973; Asada et al., 1975).

The assay procedure here described provided several advantages comparison to other methods previously used. The optimum pH for the reaction (pH 9.0) was readily achieved by dissolving borax (sodium tetraborate $Na_4 B_2 O_7$) in water, and the reaction was monitored at approximate room temeprature, obviating the necessity for rigid temperature controlling devices. The reaction between O_2^- and Ac. c is highly specific (Azzi et al., 1975), thus confers on the present procedure a reliability not hitherto found in other procedures (McCord and Fridovich, 1969, Beauchamp and Fridovich) utilising the reduction of cytochrome c, or tetrazolium dyes, both of which are influenced by enzyme activities other than SOD (cytochrome oxidases etc.) which are often active constituents of biological extracts. This present procedure is therefore applicable directly to the monitoring of SOD content in crude extracts of biological material without resorting to the use of inhibitors (cyanides etc.) which themselves may act on the SOD being measured, thus lessening the reliability of such a procedure.

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Figure 9 The rate of reduction of Ac.c (Δ 550 nm/min) by O_2^- in the presence (●) and absence (O) of SOD at different pH values. Total Ac.c concentration: 40 μM; SOD: 0.5 μg; temperature 29°C. Final volume of the reaction mix was 1 ml.

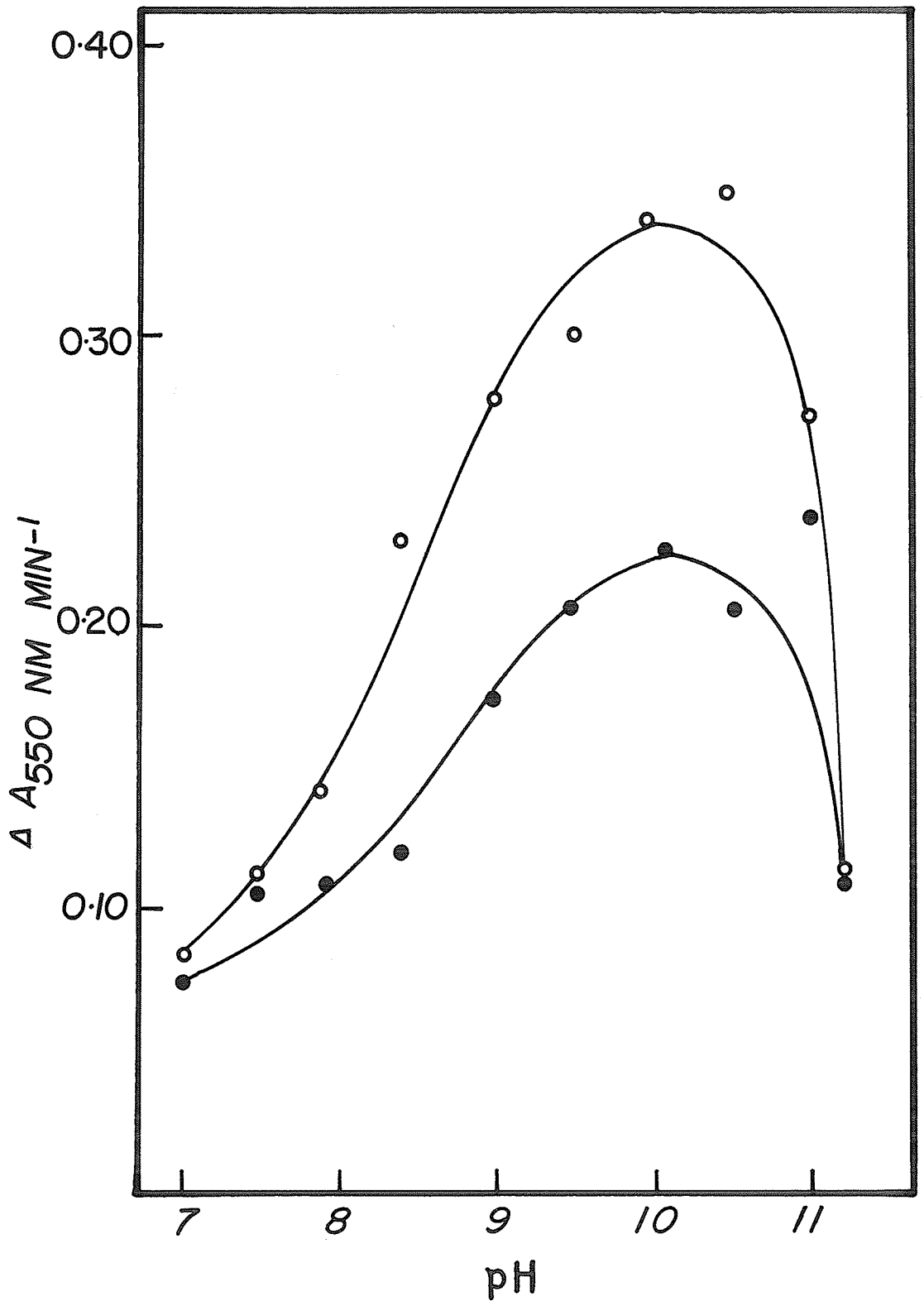
0·40
0·30
0·20
0·10
Δ A550 NM MIN-1
7
8
9
10
11
pH

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Figure 10 Percentage inhibition of Ac.c reduction by O_2^- in the presence of 0.5 µg SOD at different pH values (● , temperature of 29°C), and different temperatures (○ , pH of 9.0). Total Ac.c concentration was 40 µM.

Final volume of the reaction mix was 1 ml.

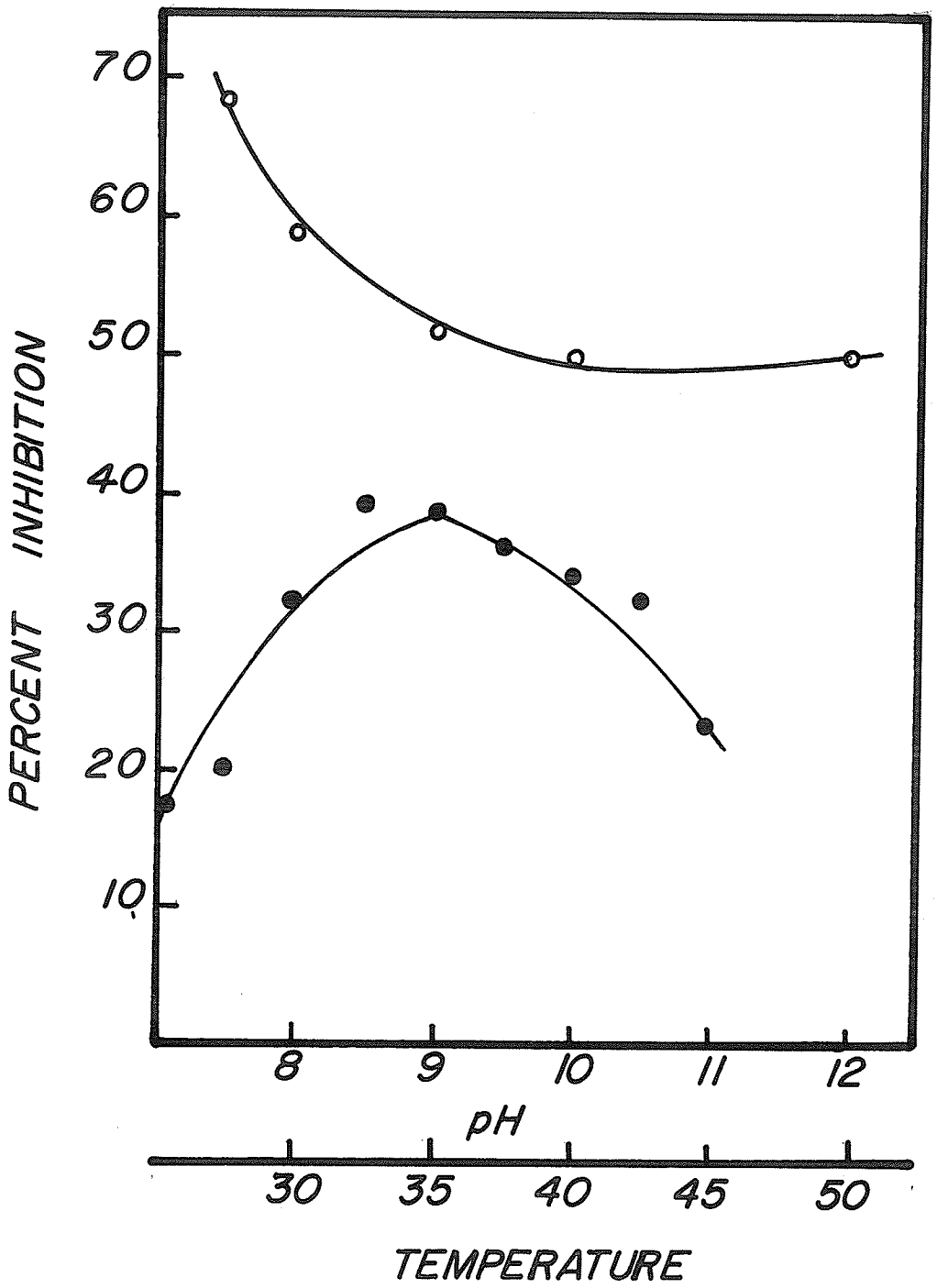
PERCENT INHIBITION
70
60
50
40
30
20
10
8
9
10
11
12
pH
30
35
40
45
50
TEMPERATURE

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Figure 11 Rate of reduction of Ac.c (Δ A550 nm/min) by O_2^- in the presence (●) and absence (o) of SOD, at different temperatures. Total Ac.c concentration: 40 μM; SOD: 0.5 μg; pH of 9.0. Total volume of the reaction mix was 1 ml.

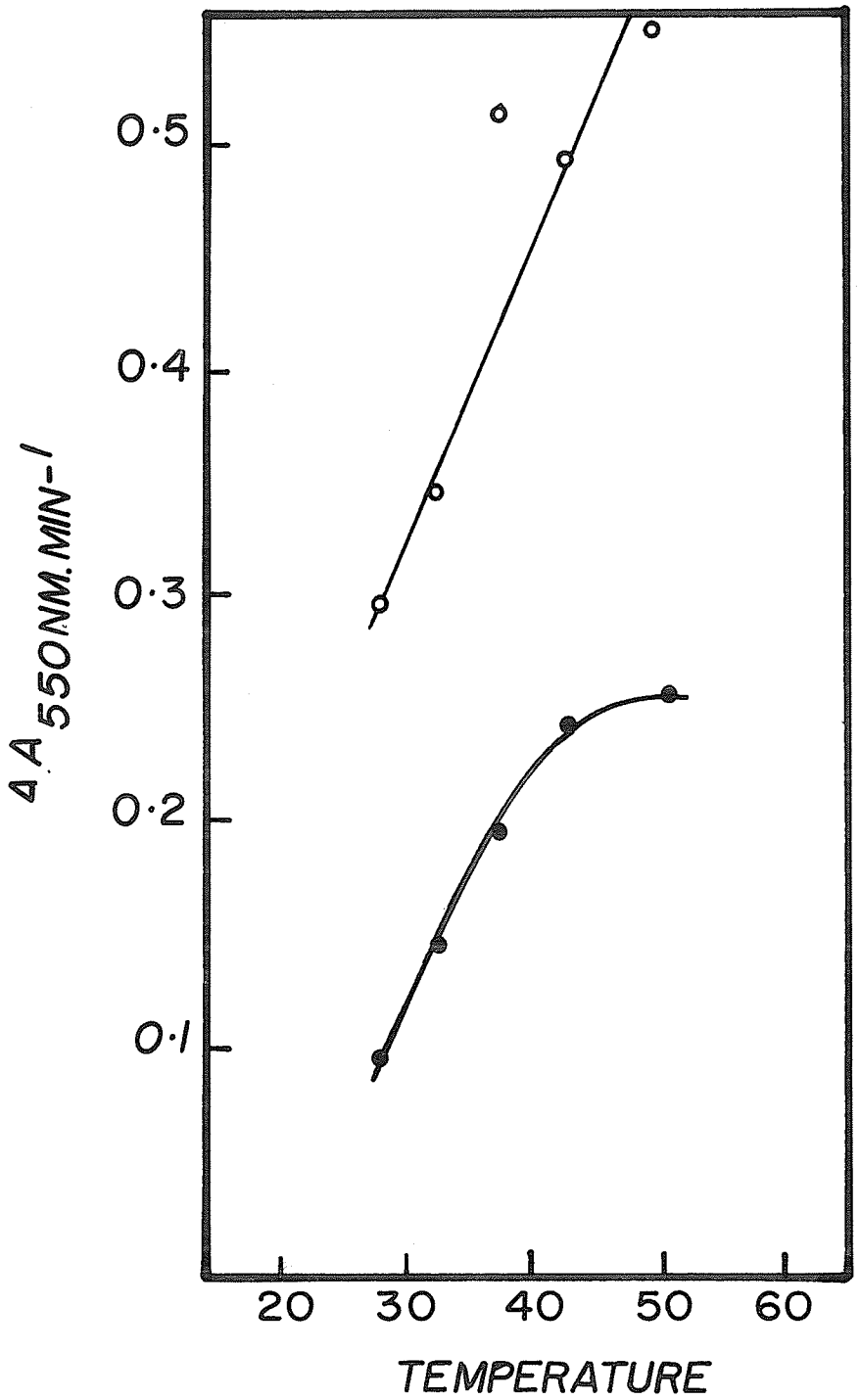
0·5
0·4
0·3
0·2
0·1
$4A_{550NM.MIN^{-1}}$
20
30
40
50
60
TEMPERATURE

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Figure 12 Percentage inhibition of Ac.c reduction by O_2^- at pH 9.0, and 29°C, at different total Ac.c concentrations in the presence of different amounts of SOD. Total Ac.c concentration -

○ - 25 μM

□ - 40 μM

△ - 50 μM

Total volume of the reaction mix was 1 ml.

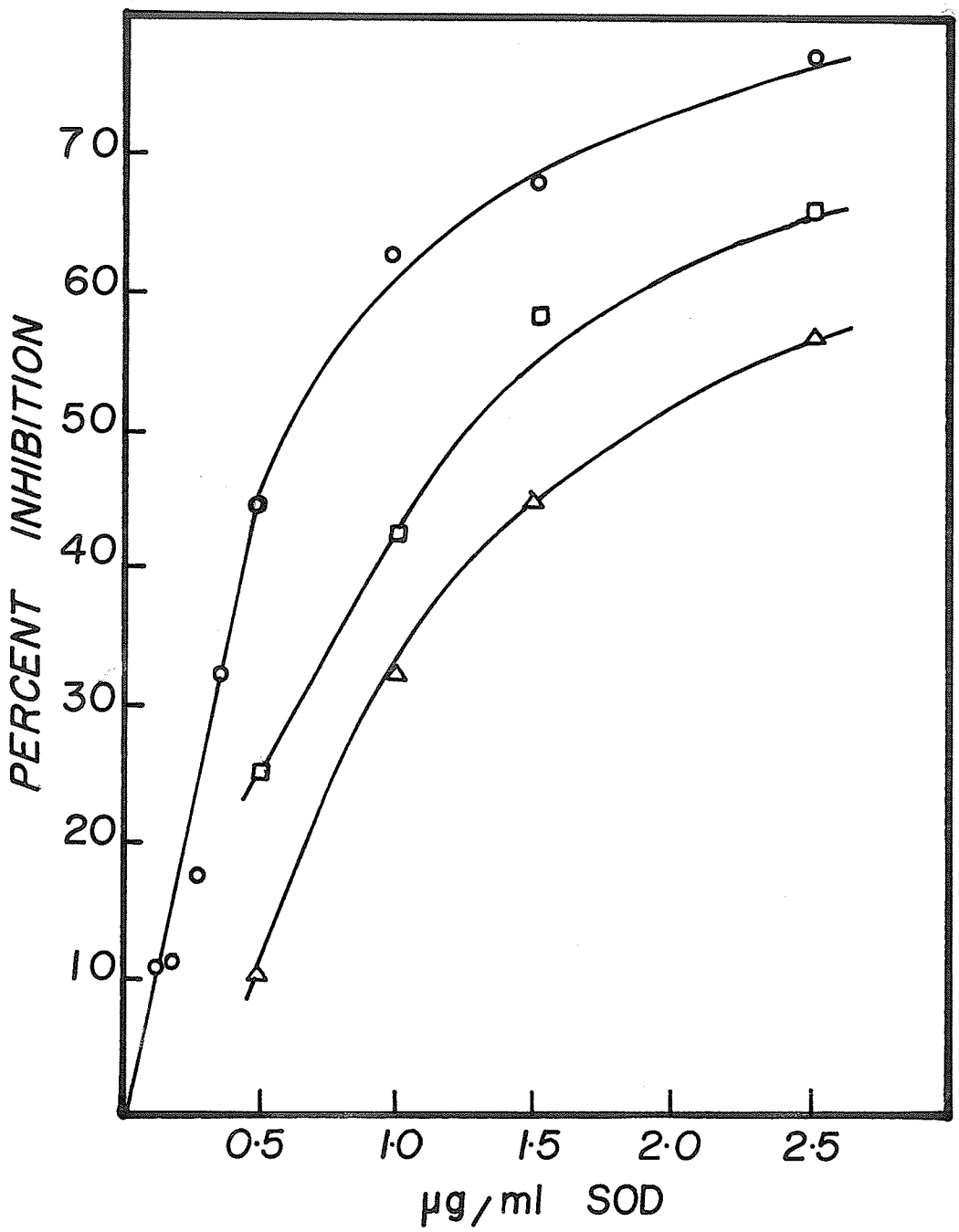
PERCENT INHIBITION
70
60
50
40
30
20
10
0·5
1·0
1·5
2·0
2·5
µg / ml SOD

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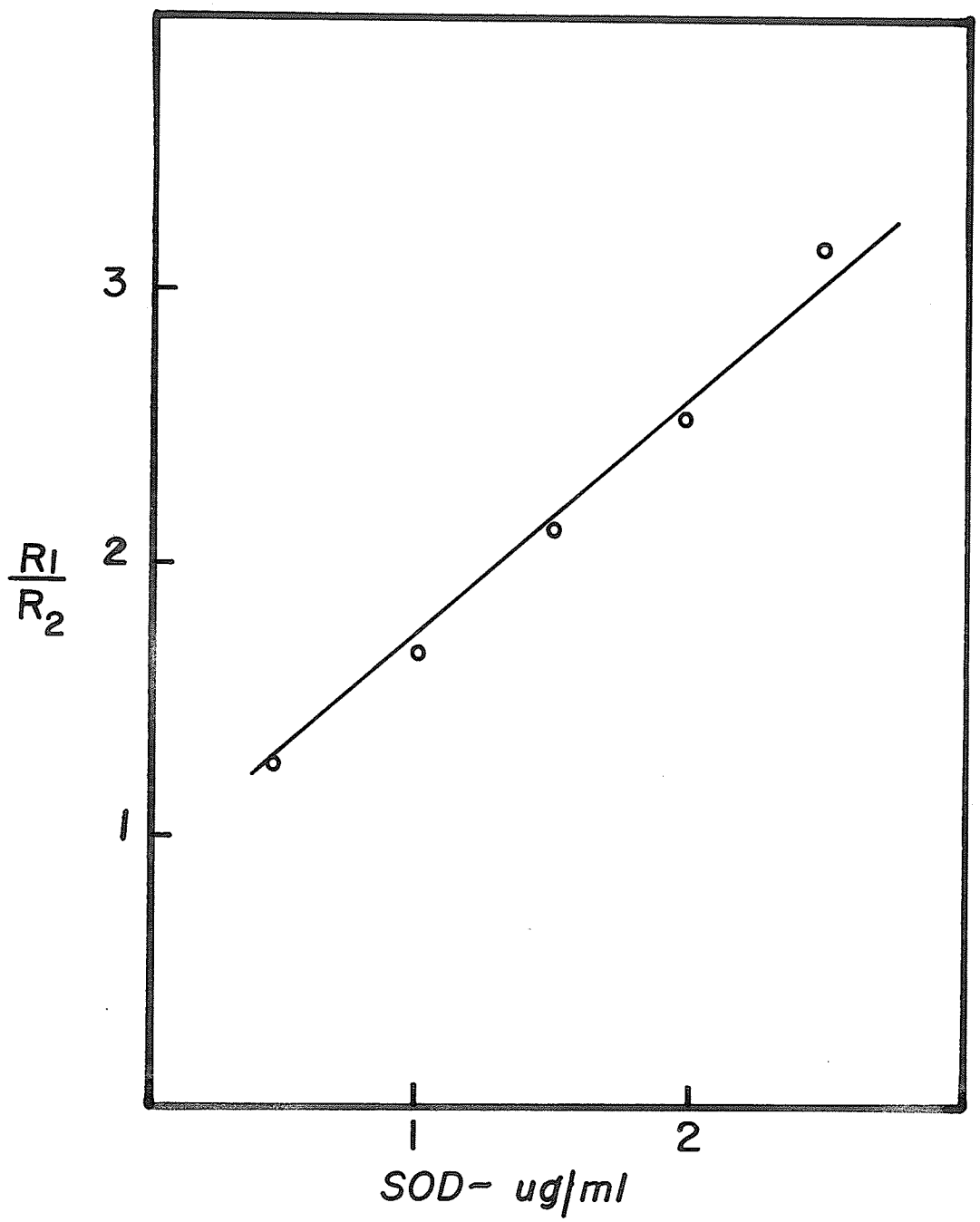
Figure 13 Relative reduction rates of acetylated cytochrome c by O_2^- in the presence/absence of SOD vs SOD concentration.

R_1 - rate of reduction of Ac.c

R_2 - rate of reduction of Ac.c in the presence of SOD

The assay system consisted of 40 µM Ac.c, 100 µM xanthine; 40 µg xanthine oxidase, in borate with 1 mM EDTA as in the assay procedure described. The reaction was monitored at 30°C, and at pH 9.0.


3
2
1
R1/R2
1
2
SOD- ug/ml

2. Enzyme activities associated with extracts of continuous cultures grown with different concentrations of oxygen.

The activities of the enzymes listed below were determined in crude extracts prepared from A.chroöcoccum grown in continuous culture, as described in the Materials and Methods section.

i) Cytochrome oxidase

ii) NADH oxidase

iii) NADPH oxidase

iv) Superoxide dismutase

v) Catalase

i) Cytochrome oxidase

The results are presented in Table 3. It is apparent that far larger quantities of cytochrome oxidase were associated with the extracts prepared from cells grown with 5% O_2 compared to those grown with 20%, or 30% O_2. Cells grown with 20% O_2 had twice as much cytochrome oxidase as those grown with 30% O_2.

The reports in the literature to date contain contradictory reports as to the presence of the various cytochrome components in Azotobacter, and as to the effect of oxygen on these components. Harrison (1972), summarising the physiological effects of dissolved oxygen tension on growing populations of micro-organisms, concluded that in 50% of the organisms examined (including A. vinelandii) cytochrome levels were highest under low oxygen conditions, and that the apparent repression of cytochrome by oxygen may confer some definite advantage. Jones and Redfearn (1967), suggested that in A. vinelandii, the terminal cytochrome oxidases were possibly cytochromes

a_1, a_2, and o, though cytochrome o was present in small quantities. Support for cytochrome a_2 as the major functional oxidase was also advanced by Yates and Jones (1974), who also showed, in agreement with the findings of Sinclair and White (1970), that there was a marked increase in cytochrome content as a result of oxygen limitation, and that this increase probably reflected an attempt by the bacterial cell to maintain electron flow under conditions of limited acceptor availability. Ackrell and Jones (1970), showed that maximum levels of terminal oxidases were often found under conditions of reduced oxygen availability, as has been reported by Castor and Chance (1959). Ackrell and Jones (1970) found that cytochrome a_2 alone continued to increase during severe oxygen limitation, and suggested that this may have represented a final attempt to maintain some respiratory activity under extremely unfavorable circumstances.

It is thus apparent that the data herein presented is in general agreement with previous findings which suggested that higher concentrations of terminal oxidases are present in conditions of oxygen limitation, as would be expected in actively growing continuous cultures supplied with 5% oxygen. The significance of these findings may conceivably be related to the relative utilisation, as determined partially or otherwise, by the availability of oxygen, of the different branches of the respiratory system of Azotobacter, as outlined by Jones and Redfearn (1967). The possible role of these observations will be alluded to in the general conclusions presented subsequently, particularly as they relate to the two other respiratory chain components - NADH, and NADPH oxidases - here considered.

Table 3 Cytochrome oxidase content of crude extracts of A. chroococcum grown in continuous culture with different concentrations of oxygen.

Oxygen Concentration (Percent)	Rate of Cytochrome Oxidation (μM/min)	[a] Rate/min/mg Protein
5	14.5	1.45
20	4.3	0.20
30	4.8	0.10

Rates indicated were obtained by measuring the rate of oxidation of ascorbate-reduced cytochrome c, using an Σ_{mm} of 27.7 at 550 mμ. Cell samples from the respective continuous cultures were subjected to sonication for 5 min at 4°C, alternating periods of sonication with periods of cooling at 4°C. The crude extracts, prepared by centrifugation of the sonicate at 37000 x g for 15 min at 4°C were examined for cytochrome oxidase activity. Reaction mixes contained 0.8 ml. 10 mM potassium phosphate buffer pH 7.5, 0.1 ml 1% cytochrome c (ascorbate reduced), and 0.1 ml crude extract prepared as above.

a- Rate/min/mg protein refers to μmoles cytochrome oxidised/min/mg. protein present in the crude extracts.

ii), iii) NADH, and NADPH oxidases

Examination of cell extracts from the respective continuous cultures, grown as described, showed that significantly larger quantities of NADH oxidase activity (unit/mg protein) were associated with these extracts than were NADPH oxidase activities, over the range 5 - 30% oxygen. NADPH oxidase was highest in cell extracts from cultures grown with 5% O_2, with a 3-fold increase in this activity produced by growth with 30% O_2 compared to growth with 20% O_2. NADH oxidase likewise was more abundant in extracts from 5% O_2 cells compared to those from 20% O_2 grown cells, but highest levels of NADH oxidase activity were apparently associated with extracts of cells grown with 30% O_2. The results are summarised in Table 4.

The findings here presented have to be considered with reference to the composition and functioning of the respiratory chain of Azotobacter. Yates and Jones (1974) proposed that in terms of electron transfer activity, substrate availability and flexibility of function, the two most important respiratory chain dehydrogenases present in Azotobacter vinelandii were those which catalysed the oxidations of NADH and NADPH. These dehydrogenases were thought to regulate intracellular NAD(P)H/NAD(P) ratios, particularly under conditions where NADPH oxidation to produce reductive capacity (as at high pO_2) for nitrogenase activity was inhibited. Biggins and Postgate (1971) have demonstrated NADH dependent acetylene reduction, and Jones and Redfearn (1967), among others, have implicated NADH and NADPH in

electron transfer to nitrogenase in Azotobacter. Senior and Dawes (1971) suggested that in A. beijerinckia, the NADH-dependent synthesis of polyhydroxybutyrate was a potential method of exerting control over NAD(P)H/NAD(P) ratios under oxygen limited circumstances, in effect allowing the energy-producing cycles to operate and nitrogen fixation to continue.

Jones, Brice, Wright and Ackrell (1973), demonstrated that there was a 50% increase in the activities of the rate-limiting NADH, and NADPH activities,during an aeration induced lag period. Dalton (1974), reviewing dinitrogen fixation by free-living microorganisms, likewise concluded that respiration occurred via uncoupled NADH dehydrogenase activity in highly aerobic conditions. The increased NADH oxidase activity noted (in Table 4) at 30% O_2 compared to that found in 20% O_2, may reflect such a trend. In close agreement with the findings of Ackrell and Jones (1971), who showed that the most striking change associated with oxygen limitation was a 9-13-fold increase in activity of the cyanide sensitive, minor terminal oxidation pathways compared with a lesser increase in NADH dehydrogenase activity, Table 4 indicates that a 13-fold increase in NADPH oxidase activity at 5% O_2 compared to that present at 20% O_2, was obtained under the conditions duly specified. An approximate 3-fold increase was noted for NADH oxidase activity over the same O_2 shift, i.e., 5 to 20% O_2. It is known that there are at least two electron transfer pathways in Azotobacter (Yates and Jones, 1974; Jones and Redfearn, 1967), with NADH and NADPH forming separate initial components, thus the difference in activities here documented may reflect changes in the

relative importance of each pathway as prompted by increased availability of oxygen. It is inferred that the NADH mediated pathway assumes a larger share of the electron transfer functions at elevated oxygen tensions than does the NADPH initiated branch.

The above is also supported by the work of Ritchie, Senior and Dawes (1971), who showed (in A. beijerinckia) that cell free extracts readily oxidised NADH, but exhibited low levels of activity with NADPH. Table 4 shows that 5 - 25 times as much NADH oxidase as NADPH oxidase activity was associated with the cell extracts of A. chroococcum and that the difference was most marked at higher O_2 concentrations. The work of Wimpenny and Firth (1972) also supports the present findings, in that they showed that NAD(H) biosynthesis was quickly stimulated by aeration, which may be reflected here in the increased level of NADH oxidase at 30% O_2 compared to that at 20% O_2. The apparently anamolous increase in NADH oxidase activity at 5% O_2 compared to that at 20% O_2 may be explained by the observation of Parker and Scutt (1960), who showed that maximum respiration was effected at oxygen concentrations of 5% of less. The increased NADH oxidase level at 5% O_2 may be indicative of such a maximal respiratory rate.

iv), v) Superoxide dismutase, and catalase

These two enzyme activities are jointly examined in view of their role as members of the oxygen-protective triad of the superoxide dismutases, the catalases, and the peroxidases.

A non-cytochrome dependent peroxidase activity was not detected over the range of oxygen (5 - 30% O_2) supplied to the continuous cultures under the conditions described previously (see Materials and Methods), even with use of the highly sensitive DAB oxidation procedure of Fahimi and Herzog (1973). The absence of a non-cytochrome dependent peroxidase activity may not be unusual, in view of the reported bi-functionality of catalase, which, (as determined by substrate concentration, pH, and inhibitors), can act either as a catalase or as a peroxidase. (Aebi, 1974).

The level of catalase detected in the whole cells from the various continuous cultures increased markedly on raising the oxygen supply to levels higher than that normally present in air, twice as much catalase was found in cells from cultures grown with 30% O_2 compared to those grown with 20% O_2. Cells grown with 5% O_2 had equivalent quantities of catalase to that present in cells grown with 20% O_2 (see Table 5 and Fig. 14). Fig. 14 shows clearly that far greater quantities of oxygen were produced from correspondingly greater amounts of catalase associated with cells obtained from cultures grown with 30% oxygen, on comparison to those grown with 20% or 5% oxygen. Similarly, oxygen production from catalase-mediated decomposition of hydrogen peroxide was greater with cells grown with 20% O_2 compared to those grown with 5% O_2. These differences, under the conditions here examined, were more marked initially, i.e., 10 - 15 min after admixture of cells and peroxide, than subsequently (> 15 min), and initial O_2 production should be regarded as being more truly indicative of whole cell catalase activity than were the readings obtained after 15 min. It is to be

noted that the above differences in catalase activity, as indicated by O_2 production from H_2O_2, were not significant at time periods greater than 15 min. It is thus apparent that the catalase content of the respective cells was directly influenced by the oxygen concentration with which these cells were grown, and that a higher concentration of oxygen during growth was associated with higher levels of catalase. Catalase activity of Azotobacter chroococcum whole cells thus seemed to be inducible by oxygen. The significance of this finding will be discussed after considering the effect of oxygen on SOD content of cells under similar conditions, since the two enzymes - SOD, and catalase - were presumably directly and closely related in the physiological responses of A. chroococcum to dissolved oxygen here reported and examined subsequently.

In direct contrast to the apparent inducible nature of A. chroococcum whole cell catalase activity, the levels of SOD associated with cell extracts prepared from similar cells did not alter significantly in response to variations in the oxygen supplied to the continuous cultures. SOD in this organism was thus evidently not inducible by oxygen; approximately equivalent total quantities, on a unit dry weight basis, of SOD, were associated with the extracts prepared from cells grown with 5% 20%, or 30% O_2 (Table 5). The quantities of SOD associated with each mg. protein present in the crude extracts decreased on increasing the oxygen supply, approximately 5 times as much SOD/mg. protein being present in extracts from cultures grown with 5% O_2 compared to those grown with 30% O_2. This is in sharp contrast to the catalase activity associated with each mg. protein in these same extracts, in that the catalase content/mg. protein

increased dramatically on raising the oxygen supply from 5% to 30% O_2. A similar finding has been reported for Bacillus subtilis (Gregory and Fridovich, 1973a) who showed that catalase in this organism was inducible by oxygen. Conversely, superoxide dismutase in Escherichia coli was induced by oxygen (Gregory and Fridovich, 1973a), or by conditions which increased the intracellular concentration of SOD substrate, i.e., the O_2^- anion (Hassan and Fridovich, 1977a, 1977b). Yousten and Nelson (1976), have also shown oxygen induced synthesis of SOD in the catalase-less aerobe Bacillus popilliae, as have Yousten, Bulla, and McCord, (1973); and Costillow and Keele, (1972). SOD was also induced by O_2 in Streptococcus faecalis (Gregory and Fridovich, 1973b).

The importance of superoxide dismutase in conferring protection from the adverse effects of oxygen in bacteria has been documented, and was used to form the basis for a theory of obligate anaerobiosis, as determined by SOD distribution in various microorganisms (McCord, Keele, and Fridovich, 1971). Numerous other reports have shown its role in affording protection from 1) streptonigrin (Gregory and Fridovich, 1973a); 2) radiation damage (Petkau and Chelack, 1974; Petkau et al., 1975a and 1975b; Petkau et al., 1976; and Petkau and Chelack, 1976), and 3) enzyme inactivation in various biological systems (Lavalle et al., 1976).

Several other protective effects of SOD have been outlined as previously discussed in the historical review preceding discussion of the data here presented.

Table 4 NADPH, and NADH oxidase activities of Azotobacter chroococcum grown in continuous culture with varying oxygen concentrations

O_2 Concentration (percent)	NADPH Oxidase (unit/mg protein)	NADH oxidase (unit/mg protein)
5	1.30	6.1
20	0.10	2.4
30	0.34	8.7

The enzyme activities were determined from the rates of oxidation of NADH, or NADPH, at 340 mμ. Reaction mixtures contained 0.1 ml. 5 mM NADH, or NADPH; 0.8 ml. 10 mM potassium phosphate pH 7.5, and 0.1 ml. crude extract (prepared as in Table 3). The units stated represent a change in O·D at 340 mμ of 1.0/min in the concentration of NADH, or NADPH stated.

TABLE 5 Catalase,and superoxide dismutase activities, of whole cells,and crude extracts,of A.chroococcum, grown in continuous culture under varying oxygen supply,with attendant respiratory rates.

Percent oxygen	a Respiratory rate (mmoles CO_2 hr/mg. dry wt.)	b SOD activity x 10^2 (Units /mg. dry wt.)	SOD activity x 10^3 (/mg. protein)	c Catalase Activity (μm O_2 Evolved/min/ mg protein)
5	12.15	13.0	9.4	0.09
20	17.15	15.5	4.7	0.24
30	22.14	15.0	3.4	0.68

a Respiratory rate given as carbon dioxide (CO_2) output, as recommended in Hine and Lees, (1976).

b SOD (superoxide dismutase) activity found in crude extracts prepared as described in Materials and Methods

c Cells from the respective cultures were shaken (after 5 min equilibration at 30°C) at 80 rpm and at 30°C with 1 ml 35 mM hydrogen peroxide and 1 ml 1% mannitol growth medium. Cells, or hydrogen peroxide, were omitted from the respective controls. Readings given were corrected for controls.

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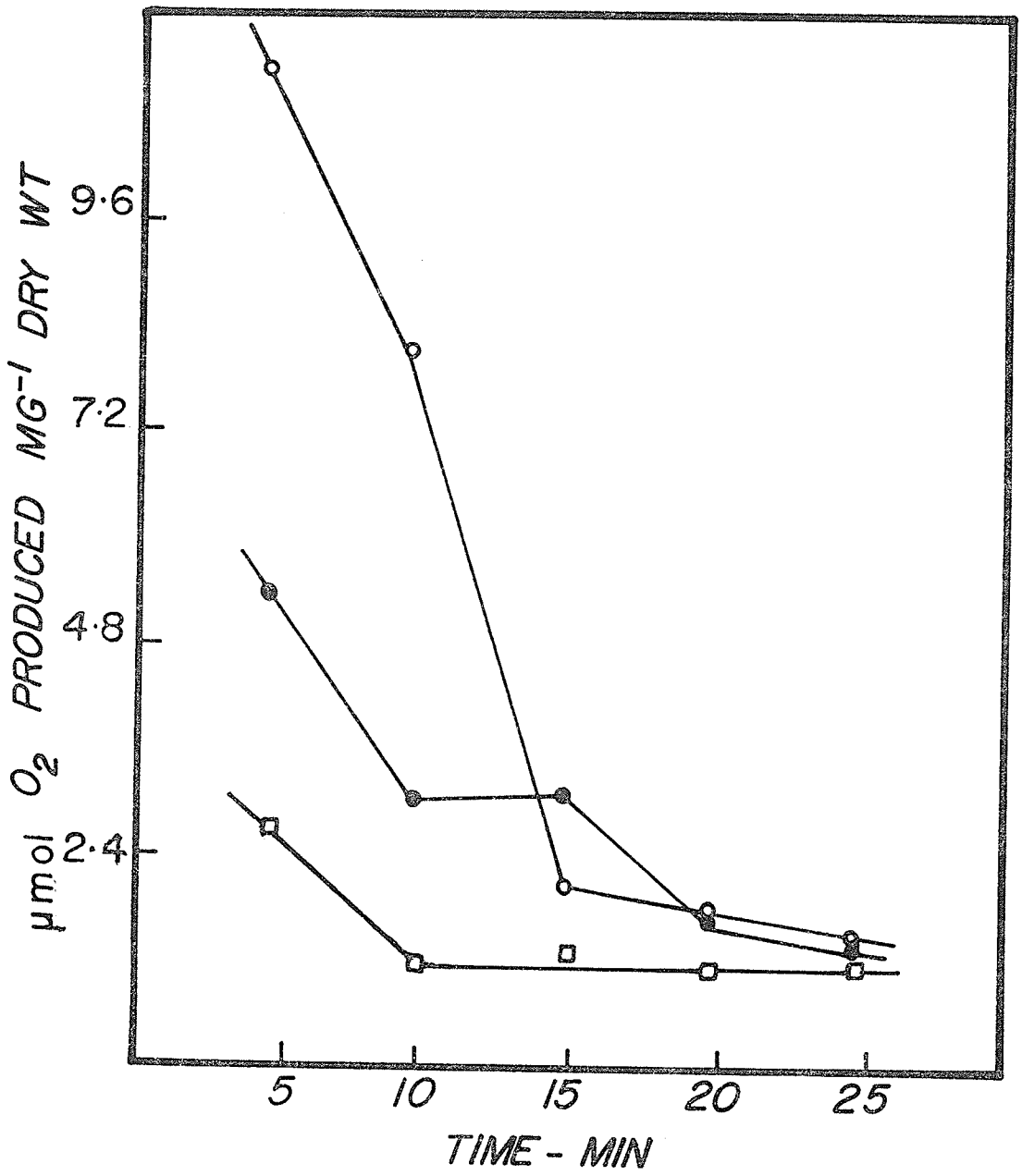
Figure 14. Catalase activity of whole cells from varying oxygen concentrations.

Reaction mixtures in the Warburg flasks contained 1 ml. 0.35 M H_2O_2, 1 ml cell suspension from the appropriate cultures, and 1% mannitol medium to a final volume of 3 ml. Controls were set up for the endogenous respiration of cells, and for the spontaneous decomposition of H_2O_2. Oxygen evolution was monitored at 30°C.

○ - 30% O_2

● - 20% O_2

□ - 5% O_2


μmol O2 PRODUCED MG-1 DRY WT
9·6
7·2
4·8
2·4
5
10
15
20
25
TIME - MIN

It might be expected that SOD in Azotobacter chroococcum should be inducible by oxygen, or by an increased respiratory rate, i.e., a greater rate of oxygen utilisation, since increased respiratory rates are presumably equated to increased production of O_2^- (Fridovich, 1975). Azotobacter chroococcum, as is evident from Table 5, displayed markedly greater respiratory rates at increased levels of O_2, the rate of carbon dioxide production at 30% O_2 being almost double that found at 5% O_2. Evidence to show that a lack of induction by oxygen need not detract from the protective role of SOD has been reported by Maral, Puget and Michelson (1977), who, in a survey of the levels of SOD, catalase, and peroxidase, in the erythrocytes of different animals, showed that very little variation in SOD occurred, and that there was no apparent correlation with respect to relative oxygen uptake in the different species of animals examined. Large variations in the levels of catalase and peroxidase were noted, and the relative constancy of superoxide dismutase levels was deemed indicative of its role in conferring protection from uncontrolled oxidative processes. The levels of SOD present in the bacterial cell, and the catalase content of A. chroococcum grown in continuous culture, showed little variation, as evidenced from an examination of these enzyme activities over a 3 day period. The results are shown in Table 6, and the relative constancy of these enzyme levels, might possibly indicate their role as major factors in the physiological response of A. chroococcum to dissolved oxygen.

The kinetic properties of the SOD enzyme, and the relative

Table 6 Daily levels of superoxide dismutase, in crude extracts, and catalase, in whole cells, of A.chroococcum grown in continuous culture.

Day	[a] Catalase Activity ($\mu m O_2 min^{-1}$)	Catalase Specific Activity ($\mu l O_2 min^{-1} mg^{-1}$ dry wt)	[b] SOD Activity ($unit\ ml^{-1} \times 10^3$)	Specific Activity-SOD ($unit\ mg^{-1}$ protein $\times 10^3$)
1	0.62	17.5	18	1.1
2	0.86	25.5	26	1.7
3	0.83	18.3	16	1.0

a Catalase activity was that detected in whole cells taken directly from continuous cultures, and was estimated by O_2 production from H_2O_2 as described in Materials and Methods, manometrically.

b Superoxide dismutase (SOD) activity was monitored by the method of Buchanan and Lees (1976), and was that detected in the crude extracts prepared as described in the Materials and Methods. The continuous cultures were grown with 20% O_2 under nitrogen fixing conditions as described previously (see Materials and Methods).

stability of H_2O_2, may be directly related to the responses noted in Azotobacter chroococcum. The rate constant for the reaction of O_2^- with SOD is close to 2×10^9 $M^{-1}sec^{-1}$ at pH 7.4 (Fridovich, 1975), and the O_2^- radical was thought to be incapable of accumulation in aqueous media because of this facile dismutation. The concentration of SOD inside cells reportedly vastly exceeds the steady state concentration of O_2^- (Fridovich, 1975, and Kellogg and Fridovich, 1977) pointed out the efficiency of the scavenging reaction of SOD. In addition, the reaction between H_2O_2 and O_2^-

$$H_2O_2 + O_2^- \rightarrow O_2 + OH^- + \cdot OH$$

commonly known as the Haber-Weiss reaction (Haber and Weiss, 1934) may also influence intracellular levels of these oxygen intermediates, in that SOD, by decreasing the steady state level of O_2^- would also decrease the overall reaction rate to allow the accumulation of more H_2O_2 (Beauchamp and Fridovich, 1970). H_2O_2 is also produced by O_2^- dismutation according to the reaction

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

and as such is probably the first relatively stable, relatively long-lived intermediate of the dismutation and reduction involved in oxygen metabolism (Fridovich, 1974). Consideration of all the factors referred to above thus provides a possible explanation for the inducible behaviour of catalase in Azotobacter chroococcum in contrast to the apparent constitutive behavior of the organism's SOD; the dismutation of O_2^- to H_2O_2, and the relative stability of the latter, may imply that the more opportune point of control (as related to

potential residence time intracellularly), may conceivably be at the level of control of H_2O_2 concentration. It is proposed also that the kinetic efficiency of SOD would require some specified concentration to achieve or confer protection, and that once this level was attained intracellularly, further synthesis of more SOD was unnecessary, hence a constitutive response was displayed.

The above conclusion is ably supported by the work of McCord et al., (1971), who presented data to strongly suggest that SOD was a factor of primary importance in enabling organisms to survive the challenge presented by the reative intermediate, O_2^-, resulting from the univalent reduction of molecular oxygen. Available evidence (McCord et al., 1971, Costilow and Keele, 1972, Yousten et al., 1973) tends to favor the conclusion that oxygen tolerance by an organism is more dependent on superoxide dismutase than on catalase, but the data herein presented strongly imply that catalase activity in Azotobacter chroococcum may be of equal (or somewhat greater) significance in the adaptive response of this organism to molecular oxygen. The question of relative significance of catalase or SOD may be irrelevant, in view of the proposals by Bray et al., (1974), that catalase may enhance SOD activity by preventing its inactivation by H_2O_2; or that the effect of SOD is to enhance catalase activity by the prevention of O_2^- mediated inactivation (Odajimi and Yamazaki, 1972).

3. Effects of exogenous O_2^- on Azotobacter chroococcum in continuous and batch cultures.

It is now accepted generally that metabolic utilisation of oxygen

by organisms produces O_2^-, thus since the primary purpose of the work here undertaken was to examine the responses of Azotobacter to oxygen, it was deemed apt to investigate the potential effects of O_2^-, generated exogenously, on the activities of A. chroococcum.

O_2^- was generated by the xanthine-xanthine oxidase system as outlined in the Materials and Methods, and examination of the effects of exogenous O_2^- was restricted to the following:-

a) whole cell nitrogenase activity

b) influence of ammonium in the growth medium on O_2^- mediated inhibition of whole cell nitrogenase activity.

c) oxygen uptake by batch and continuous culture samples.

d) viabilities

a) Whole cell nitrogenase activity

Enzymically generated O_2^-, exogenously supplied to whole cells (taken from continuous cultures of the organisms grown under varying concentrations of oxygen in the gas phase) inhibited total nitrogenase activity of these cells (Fig. 15). The degree of inhibition (of total ethylene generated) produced by exogenous O_2^- varied somewhat with the oxygen supplied to the respective cultures, being slightly higher (8 - 16%, dependent on time of assay as given in Fig. 15) in cultures grown with 30% O_2 compared to those grown with 20% O_2. Indications were that cultures having a lower respiratory activity were less sensitive to exogenous O_2^- mediated inhibition of whole cells nitrogenase activity than were cultures having higher respiratory activity (see Table 5 for the respective respiratory

activities). Tsim and Lees, in independent work done in this laboratory (manuscript in preparation), and in agreement with the reported effects of oxygen on nitrogenase activity (Drozd and Postgate, 1970), have shown that continuous cultures grown as described in this present report, had lower whole cell nitrogenase activities when grown with 30% O_2 compared to those grown with 20%, and with 5% O_2.

The effect of varying quantities of exogenous O_2^-, enzymically generated, on the nitrogenase activity of whole cell is indicated by Table 7. The inhibition produced increased significantly on increasing exogenous O_2^-, and could be lowered by increasing the cell density of test mixtures. A plot of relative nitrogenase activity (i.e., the activity present normally compared with that in the presence of exogenous O_2^-) against differing concentrations of O_2^- (Fig. 16) showed that O_2^- apparently acted as a competitive inhibitor of whole cell nitrogenase activity in Azotobacter chroococcum. At a high cell density, in the presence of a finite quantity of O_2^-, exogenously produced, a rate of nitrogenase reaction may be achieved approaching that of the uninhibited reaction.

Since available evidence (Khan, 1970; Singh, Greenstock, Jenks, Petkau, Raleigh and Singh, 1977; Krinsky, 1977) indicates that the superoxide ion may generate singlet oxygen and/or the perhydroxy radical in solution (in addition to producing H_2O_2 by dismutation) the effect of an organic peroxide and various radical scavengers on the nitrogenase activity of whole cells was examined. The results are presented in Table 8. The organic peroxide-ethyl hydrogen peroxide -

and hydrogen peroxide, both exogenously added to whole cells, produced marked inhibition of nitrogenase activity, though to differing degrees. The inhibiton produced by H_2O_2 was total, whereas a similar quantity of ethyl hydrogen peroxide produced approximately 65% inhibition. The enzymes catalase, and horse radish peroxidase (HRP), failed to prevent the inhibitions produced by H_2O_2 and ethyl hydrogen peroxide respectively, though HRP conferred minimal protection (9%) against ethyl hydrogen peroxide; the latter was a less effective inhibitor than H_2O_2 possibly due to its greater stability.

O_2^- can produce other reactive oxygen intermediates - singlet oxygen and the perhydroxy radical (Haber and Weiss, 1934) - thus the contribution of the hydroxy radical to the inactivation produced by exogenous superoxide was examined by use of benzoate and ethanol, which have been shown to act as radical scavengers (Beauchamp and Fridovich, 1970). The failure of these radical scavengers to effect more than minimal reversal - 2% in the case of sodium benzoate - is clear indication that the hydroxyl radical may not have been directly responsible, under the conditions here specified, for any significant portion of the inhibition produced by exogenous O_2^-. Benzoate is known to be a more effective inhibitor of ·OH, in accord with its greater rate constant for reaction with ·OH (Neta and Dorfmann, 1968).

Catalase failed to reverse the inhibition produced by H_2O_2, which points to the involvement of some reactive by-product of H_2O_2 disproportionation (Haber and Weiss, 1934, Fridovich, 1976), possibly singlet oxygen or some like species. The failure of benzoate and alcohol to confer protection would tend to rule out ·OH, but it is quite possible that the generation of ·OH from H_2O_2 is much more marked than

from the xanthine-xanthine oxidase mix here used to generate O_2^-, thus ·OH could possibly also play some role in H_2O_2 mediated inhibition of whole cell nitrogenase activity in Azotobacter chroococcum. There seems to be little doubt as to the involvement of O_2^- in this inhibition as reported herein, since the addition of as little as 50 µg bovine SOD to the reaction mixes containing the O_2^- generating xanthine-xanthine oxidase system prevented the inhibition of whole cell nitrogenase activity by O_2^-, producing rates of acetylene reduction virtually the same as in the uninhibited reaction in the absence of exogenous O_2^-. Evidence which indirectly supports an O_2^- mediated inhibition (or some like phenonema) of whole cell nitrogenase in Azotobacter chroococcum was presented by Lees and Postgate (1973) who showed that oxygen specifically affected nitrogenase or something closely associated with it, since the organisms remained viable after oxygen stress if growth in an ammonium supplemented medium was allowed.

The inhibitions observed were not generally attributable to the possible cytotoxic effects of the respective chemicals, as evidenced from the observed viabilities presented in Table 8. These observations will be subsequently evaluated as a potential explanation for the observed sensitivity of Azotobacter nitrogenase to oxygen.

b) The influence of ammonium in the growth medium on O_2^- mediated inhibition of whole cell nitrogenase activity.

Addition of NH_4^+ to the growth medium resulted in lowered rates

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Figure 15. Nitrogenase activity in cells grown under 20%, 30% oxygen and the inhibition produced by O_2^-.

○ - 20% O_2, untreated

● - 20% O_2, treated

□ - 30% O_2, untreated

■ - 30% O_2, treated

1 ml. cell suspension from the respective cultures was added to 1 ml 2 x 10^{-3} M xanthine and 200 µg xanthine oxidase, in a total volume of 2.5 ml. The reaction was carried out in 25 ml. suba-seal stoppered flasks. Untreated samples lacked the xanthine-xanthine oxidase O_2^- system. Nitrogenase estimated from ethylene production.

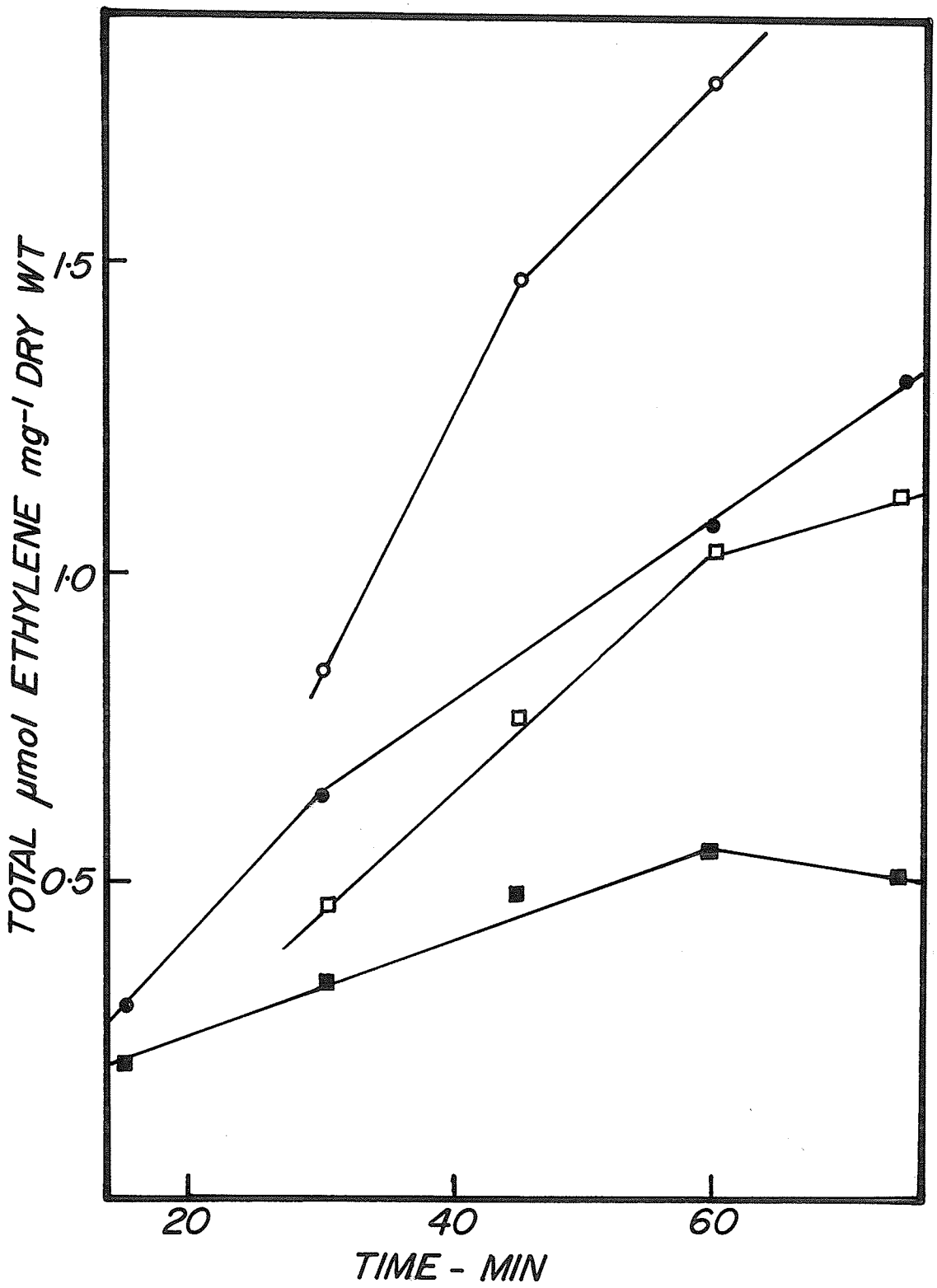
TOTAL µmol ETHYLENE mg-1 DRY WT
1·5
1·0
0·5
20
40
60
TIME - MIN

Table 7 The inhibition of whole cell nitrogenase activity by varying quantities of enzymically generated exogenous superoxide.

[a] O_2^- production (Δ O.D. 550 nm/ min)	Cell Density (mg. dry wt/ml.	Ethylene Produced (nm/ml/hr.)	[b] Relative Ethylene Production
0.004	0.5	219	0.9
	1.0	700	0.8
0.022	0.5	4	0.01
	1.0	84	0.09
0.054	0.5	2	0.008
	1.0	84	0.09
Control	0.5	237	1.0
	1.0	912	1.0

[a]
O_2^- production was equated to the rates of reduction of 25 μM acetylated cytochrome c at 550 nm produced by varying quantities of xanthine and xanthine oxidase.

[b]
Relative rates were obtained from a comparison of the control and O_2^- treated samples. Cell samples were taken directly from continuous cultures grown with air under nitrogen fixing conditions.

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Figure 16. The inhibition of nitrogenase by enzymically generated O_2^-.

O_2^- was generated by varying xanthine-xanthine oxidase mixtures such that 3 different rates of reduction of acetylated cytochrome c at 550 mμ were obtained. These rates were equated to O_2^- concentrations, and plotted against the relative reaction rates of acetylene reduction in untreated and O_2^- treated cells, which were taken from continuous cultures grown with 20% oxygen under nitrogen fixing conditions. S_1 (o) contained 0.5 mg/ml dry weight of cells, S_2 (●) contained 1 mg/ml. dry weight.

V - rate of uninhibited reaction

V_i - rate of inhibited reaction

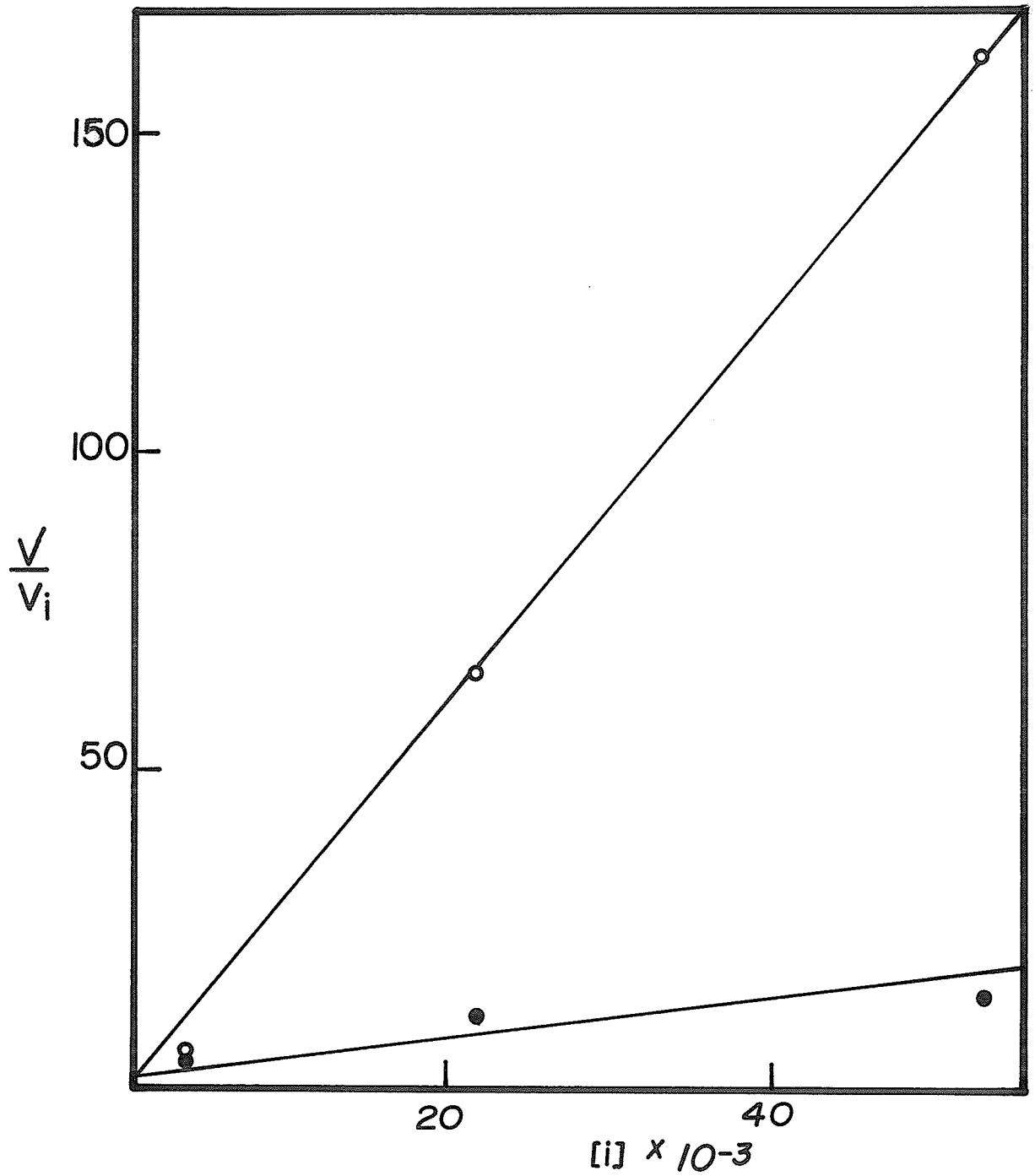
150
100
50
V/Vi
20
40
[i] x 10-3

Table 8 The effect of radical scavengers, catalase, and peroxidase on whole cell nitrogenase activity inhibited by O_2^- and peroxides; and the respective viabilities.

Treatment	[a] Ethylene Produced	Percent Inhibition	[b] Percent Viable
None	2300	-	98
O_2^-	30	99	66
H_2O_2	3	100	80
H_2O_2 + catalase	2	100	83
Ethanol + O_2^-	3	100	*
Sodium benzoate + O_2^-	40	98	*
Ethyl hydrogen peroxide	800	65	58
Ethyl hydrogen peroxide + horse radish peroxidase	1000	56	58

[a] Ethylene produced given in nmoles/mg/hr

[b] Viability was determined as described in Materials and Methods O_2^- (Superoxide anion) generated by 200 µg xanthine oxidase and 0.8 mM xanthine. 40 units catalase or horse radish peroxidase added to cells taken directly from nitrogen fixing continuous cultures supplied with air. 5 mM H_2O_2, or ethyl hydrogen peroxide; and 50 mM sodium benzoate or ethanol were added to samples indicated.

* Not done.

of acetylene reduction by cells taken from these cultures, a concentration of 1 mM NH_4^+ produced untreated reduction (no O_2^- added) rates approximately 70% lower than with growth under N_2 fixing conditions, (see Table 9). Raising the concentration of NH_4 from 4 mM to 20 mM with cultures grown on 10% O_2 resulted in untreated (no O_2^- added) rates of reduction some 45% less with 20 mM NH_4 than with 4 mM NH_4. This is in agreement with previously published work (Tubb and Postgate, 1973; Shah, Davis and Brill, 1972; and Mahl and Wilson, 1968) which indicated repression of nitrogenase activity by fixed nitrogen (NH_4^+). NH_4^+ grown cultures were also inhibited by exogenous O_2^-, irrespective as to whether the cells were grown with 10% or with 20% O_2 (Table 9). It should be noted that the degree of inhibition produced by exogenous O_2^- in cultures using fixed N_2 as NH_4^+ was less than in cultures fixing nitrogen. Similar quantities of exogenous O_2^- produced 99% inhibition of ethylene production in N_2 fixing samples, but only 80%in samples from cultures growing with fixed nitrogen as NH_4^+. Increasing the quantity of NH_4^+ in the medium for the same O_2 concentration (10% O_2) produced slightly greater inhibition by O_2^- - 90% with 20 mM NH_4, compared to 80% with 4 mM NH_4^+.

It is therefore apparent that smaller quantities of fixed N_2, as NH_4^+, below 20 μM NH_4^+, may confer some protection to whole cell nitrogenase from inactivation by exogenous O_2^-. This finding may explain or aid in clarifying the response of Azotobacter to oxygen noted by Dalton and Postgate (1969), who reported that phosphate

limited populations of A. chroococcum, using NH_4^+ instead of fixing atmospheric N_2, showed no comparable sensitivity to oxygen as did N_2 fixing cultures. Dalton and Postgate (1969) concluded that hypersensitivity to oxygen was not shown by populations that were not fixing nitrogen. The agreement here noted strongly implicates O_2^- as being responsible for, or having a major role in the effect of oxygen on nitrogenase of A. chroococcum, since in respect to the effect of NH_4^+ as related to oxygen sensitivity, the responses can be duplicated or approximated by exogenous O_2^-.

c. The effect of exogenous O_2^- on oxygen uptake by batch and continuous cultures of A. chroococcum

One of the most salient features of this present discourse is the attempt to show that the organism's responses to oxygen can indeed be mediated by O_2^-. Presumably therefore, the effects of O_2^- on Azotobacter chroococcum should be reflected in other activities - other than nitrogenase functioning - of this organism. This presumption provided the rationale for examining the effect of exogenous O_2^- on the oxygen uptake of cells from batch and continuous cultures.

Oxygen uptake rates in batch cultures were expectedly lower than in continuous culture samples. In batch cultures, cells with mannitol as carbon source displayed slightly higher rates of oxygen uptake than those with glucose as carbon source. Oxygen uptake, whether with mannitol or glucose as substrate, in samples from batch cultures, was inhibited by exogenous O_2^- (see Fig. 17). No apparent protection was

conferred by mannitol, despite its reported use as a radical scavenger (McCord and Fridovich, 1969). This result also tends to support the conclusion that the effect observed was attributable to O_2^- and not to other free radicals of like nature to those scavenged by mannitol.

In respect to the continuous cultures, oxygen uptake rates were lower in cultures supplied with 5% O_2, on comparison to cultures grown with 20% or 30% oxygen, which finding agrees with previously published work (Dalton and Postgate, 1969). Oxygen uptake in samples from 5% and 20% O_2 grown cells was inhibited by exogenous O_2^- to relatively the same degree (Fig. 18). A difference in the degree of inhibition produced may have been expected, but the result here obtained was not surprising, since under the conditions of growth described, the organisms were apparently not subjected to oxygen stress at 20% oxygen, as evidenced by maintenance of the culture medium dissolved oxygen tension at zero, and abundant growth without any indications of "washout" attributable to oxygen toxicity. The effect again was due specifically to O_2^-, since addition of 20 µg bovine SOD to whole cells prior to adding the xanthine-xanthine oxidase O_2^- generating system resulted in nearly normal oxygen uptake rates. Exogenous O_2^- thus inhibited the uptake of oxygen by Azotobacter chroococcum whole cells, an effect which was formerly attributed to elevated oxygen (Parker and Scutt, 1960), but which can now be explained on the basis of interaction with O_2^-, providing cogent support for its postulated role in A. chroococcum's response to oxygen.

Table 9 The effect of O_2^- on the nitrogenase activity of cells grown with varying ammonium and oxygen concentrations.

Percent Oxygen	[a] Ammonium	[b] Ethylene Production		[c] Percent Inhibition due to O_2^-
		[d] Untreated	O_2^- treated	
10	4	37	8	80
10	20	21	2	90
20	1	690	130	80
20	0	2300	-	-

[a] Ammonium stated added as ammonium sulphate in mM concentrations.

[b] Ethylene produced given in nm/mg.dry wt/hr.

[c] Percent inhibition given by $100 \times \frac{\text{Ethylene produced by untreated} - \text{ethylene produced with } O_2^-}{\text{ethylene produced by untreated}}$

[d] Untreated samples lacked the O_2^- generating system, as given in Table 8.

- Not applicable

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Figure 17. Oxygen uptake by batch grown cultures and the effect of O_2^-.

Reaction conditions are as given in Fig. 18. Cell samples were taken from batch cultures grown on mannitol or glucose as carbon source. O_2 uptake was measured in the presence of the respective substrate. Medium composition was as described in the Materials and Methods, with glucose replacing mannitol .

○ - O_2 uptake in untreated cells, grown with mannitol

● - O_2 uptake in O_2^- treated cells, grown with mannitol

□ - O_2 uptake in untreated cells, grown with glucose

■ - O_2 uptake in O_2^- treated cells, grown with glucose.

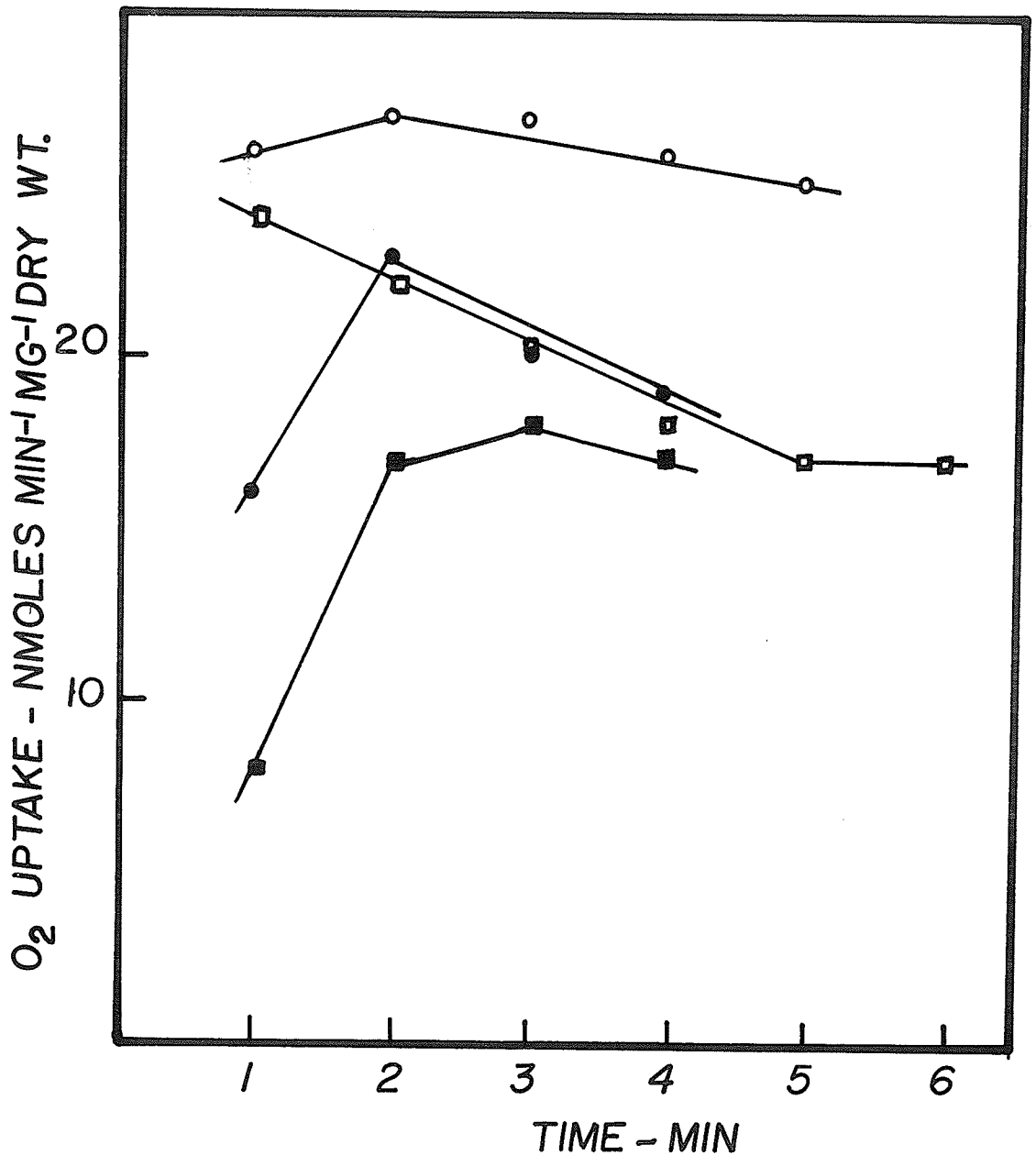
O_2 UPTAKE - NMOLES MIN^{-1} MG^{-1} DRY WT.
20
10
1
2
3
4
5
6
TIME - MIN

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Figure 18. Oxygen uptake in whole cells from continuous cultures grown with 5%, 20% oxygen, and the effect of O_2^- on these rates of oxygen uptake.

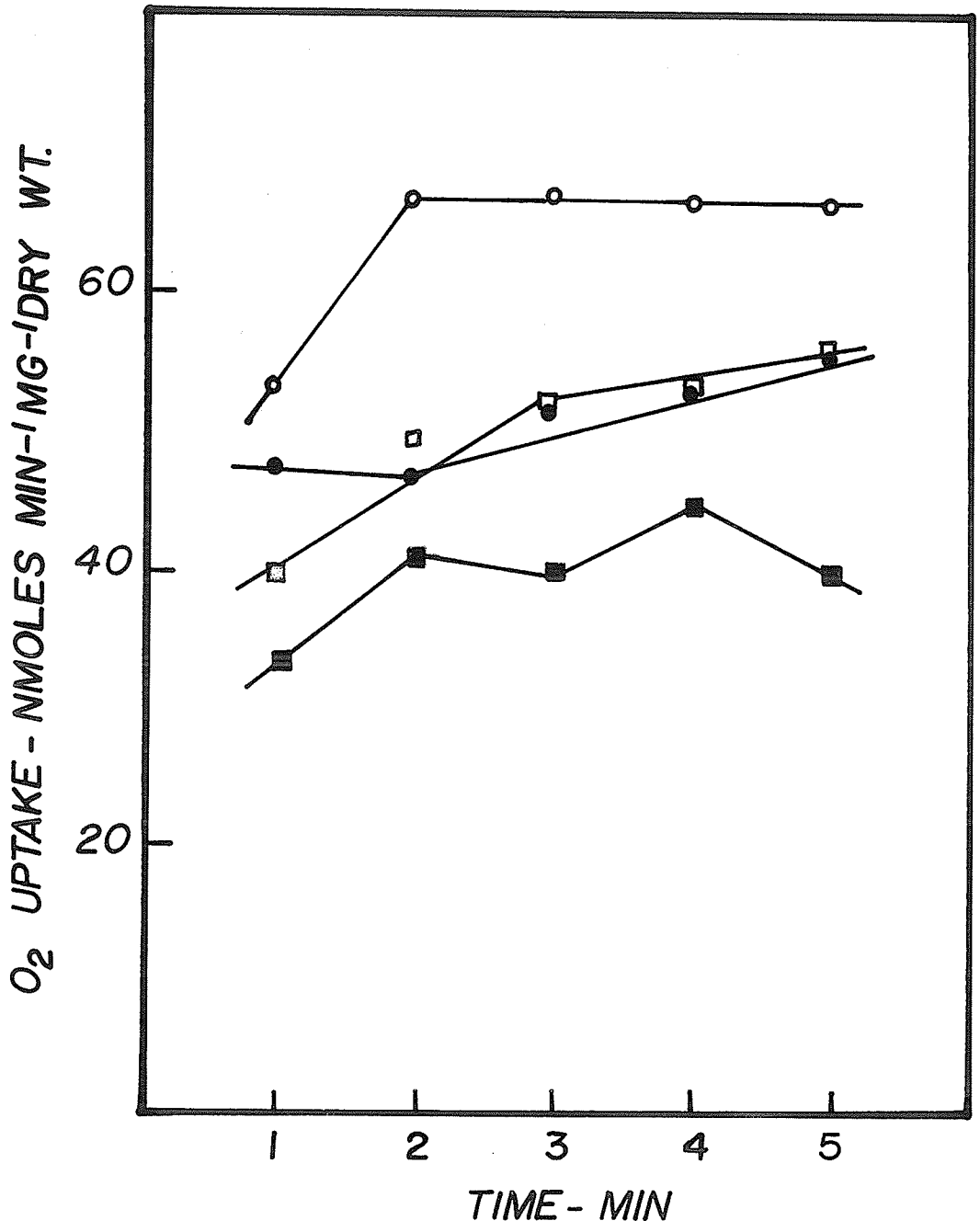
Uptake in untreated cells was measured by adding 0.4 ml. cell suspension from the respective cultures to 0.5 ml. 1% mannitol growth medium and distilled water to a final volume of 1.5 ml in the reaction vessel of a Gilson oxygraph. Treated cells contained 80 µg xanthine oxidase and 100 µM xanthine. Xanthine-xanthine oxidase mixtures in growth medium as above was used as control, results for O_2^- treated cells are the differences noted between the uptake by the xanthine-xanthine oxidase system and the same mixture containing cell suspensions.

○ - 20% O_2 untreated

● - 20% O_2, O_2^- treated

□ - 5% O_2, untreated

■ - 5% O_2, O_2^- treated.


O_2 UPTAKE - NMOLES MIN^{-1}MG^{-1}DRY WT.
60
40
20
1
2
3
4
5
TIME - MIN

a) Viabilities of Azotobacter chroococcum continuous culture samples on treatment with exogenous O_2^-, ethyl hydrogen peroxide and hydrogen peroxide

That exogenous O_2^- is toxic to A. chroococcum is undeniable from the information supplied by Table 8, treatment with O_2^- lowers the observed viability by some 32%. H_2O_2 is less toxic, apparently, than O_2^-, and minimal protection - some 3% - was provided by catalase, which may indicate that some other species (·OH and/or singlet oxygen) produced from H_2O_2 by the Haber-Weiss reaction (1934) may be the causative agent of the toxicity attributed to H_2O_2. It has to be assumed that minimal quantities of this "causative agent" were effective in producing toxicity, possibly through the well documented chain reaction sequences associated with free radicals (Pryor, 1976), or that under the conditions specified, generation of the primary causative agent was effective in producing the observed toxicity. Ethyl hydrogen peroxide was also toxic to A. chroococcum, thus O_2^-, H_2O_2, and organic peroxides influence both nitrogenase activity and viability in A. chroococcum. Since the observed effects were not completely lethal, it is possible that some synthesis of nitrogenase may have occurred after the inital contact with the toxic or inactivating chemical; and that the inhibition of nitrogenase activity produced by O_2^- was partially reversible; or that subsequent removal (by organism metabolism or the relative instability) of the chemicals referred to partially freed nitrogenase synthesis from inhibition to effect fixation of nitrogen requisite to the maintenance of viability in the absence of fixed nitrogen. It is interesting to note, in support of the above explanation, that remarkably similar results were

obtained by Drozd and Postgate (1970) who concluded that part of the inhibition produced by oxygen, in reference to cell-free extracts of A. chroococcum reducing acetylene, was not reversible. The relationship of these observations as they relate to suggesting a coherent theory for an understanding of the organism's sensitivity to oxygen will be subsequently examined when other confirmatory lines of evidence have been presented.

Oxidation of xanthine by A. chroococcum whole cells.

There was no apparent oxidation of xanthine by whole cells, when examined as described in the Materials and Methods. It is possible that significant quantities of xanthine did not enter the cells, or that the rate of oxidation of xanthine under the conditions described was negligible.

Cell free nitrogenase assays and the effect of SOD.

The results varied considerably on repeated attempts at obtaining reliable data. The sensitivity of the assay to even trace quantities of oxygen did not allow an acceptable demonstration of a protective effect being produced by added bovine SOD. It was not possible to distinguish clearly between the effect produced by trace quantities of oxygen and the protection presumably provided by adding SOD to the reaction mixtures. Use of the xanthine-xanthine oxidase system to generate the superoxide anion was not possible since the system requires O_2, which has to be eliminated from the nitrogenase assay system.

Macromolecular composition of A. chroococcum whole cells grown with varying oxygen supply.

The results are given in Table 10. They are in general agreement with previously published work (Hine and Lees, 1976; Lees and Postgate, 1973). The pHB content of the cells increased on lowering the oxygen supply to the cultures; protein and polysaccharide content of cells increased on increasing oxygen supply. These results thus serve to confirm those obtained previously in these laboratories (Hine and Lees, 1976).

Residual mannitol.

The residual mannitol present in the median decreased on increasing the oxygen supply to the continuous cultures (Fig. 19) as has been previously found (Hine and Lees, 1976). An increased rate of utilisation of mannitol at higher oxygen concentrations may reflect the respiratory protection of nitrogenase proposed by Postgate et al., 1973.

Intracellular location of catalase and superoxide dismutase in Azotobacter chroococcum

Table 10 shows the distribution of catalase, and of superoxide dismutase, in the respective cell fractions of A. chroococcum disrupted by the glycerol-DNAase procedure of Robrish and Marr (1962). Most of the total catalase activity was found to be associated with the "soluble" cell fraction, and virtually all of the total SOD activity (99%) was found in this fraction. Five times as much catalase, and eight times as much SOD, were present in the "large" fraction compared to that in the "small" fraction. These differences, though less striking, were also applicable on comparing the respective enzyme activities as related to the amount of protein present in the particular fraction. Equivalent quantities of SOD were associated with each mg. protein in the soluble and large fractions, with 100-fold less activity present in the small fragment. Sonication

of the large and small fractions revealed that some 3000 units total SOD were associated with the large fragment, compared to 720 units total activity detected in this same fraction before sonication, which apparently effected release of previously sequestered (enclosed) or membrane bound SOD activity.

Electron microscopic examination of the fragments, prepared and fixed as described in the Materials and Methods, showed that the unsonicated large fraction consisted essentially of cell envelopes substantially devoid of cytoplasm (Fig. 20). The sonicated large fragment was made up of vesicle-like structures and varying sizes of membrane fragments (Fig. 21). The small fragment consisted essentially of ribosomes and membrane fragments (Fig. 22), some associated to form small vesicles; sonication of this fraction produced further fragmentation so that far fewer vesicles were observed, lacking apparent internal contents when present (Fig. 23). A normal cell of A. chroococcum is displayed (Fig. 24) for comparison.

The results of the cell fractionation procedures, coupled to the observation afforded by electron microscopic examination, suggests quite cogently that most of the catalase and SOD activities of the A. chroococcum reside in the cytoplasm, and that some compartmentalisation of enzyme may occur within vesicle-like structures or cell envelopes. It is also possible that these enzyme activities were associated (with minimal tenacity) to membranes, and that the procedure used here effected virtually complete release.

Table 10 Macromolecular composition of Azotobacter chroococcum grown in continuous culture with varying concentrations of oxygen.

O_2 Supply	Dry Weight mg/ml	Protein	RNA	pHB	Polysaccharide
5%	0.51±0.04	30%	3.5%	5.4%	4.3%
20%	0.88±0.06	36.3	2.7	2.3	10.3
30%	1.16±0.13	42.5	2.8	2.1	15.5

All samples were taken from continuous cultures, grown under N_2 fixing conditions, with the oxygen supply stated. Cultures were maintained at a dilution rate of 0.2 hr^{-1}. The methods of assay are given in the Materials and Methods. Readings were taken for three consecutive days after the continuous cultures had stabilised, as indicated by relatively stable dry weights and CO_2 production. The macromolecular constituents are given as percentages of the dry weights for each sample.

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Figure 19 The variation of residual mannitol with oxygen supply to continuous cultures of A. chroococcum.

Samples were taken from the respective continuous cultures for 3 successive days. The samples were centrifuged at 5,000 g for 10 min. at room temperature, and the residual mannitol present in the supernatant was determined according to Neish (1950).

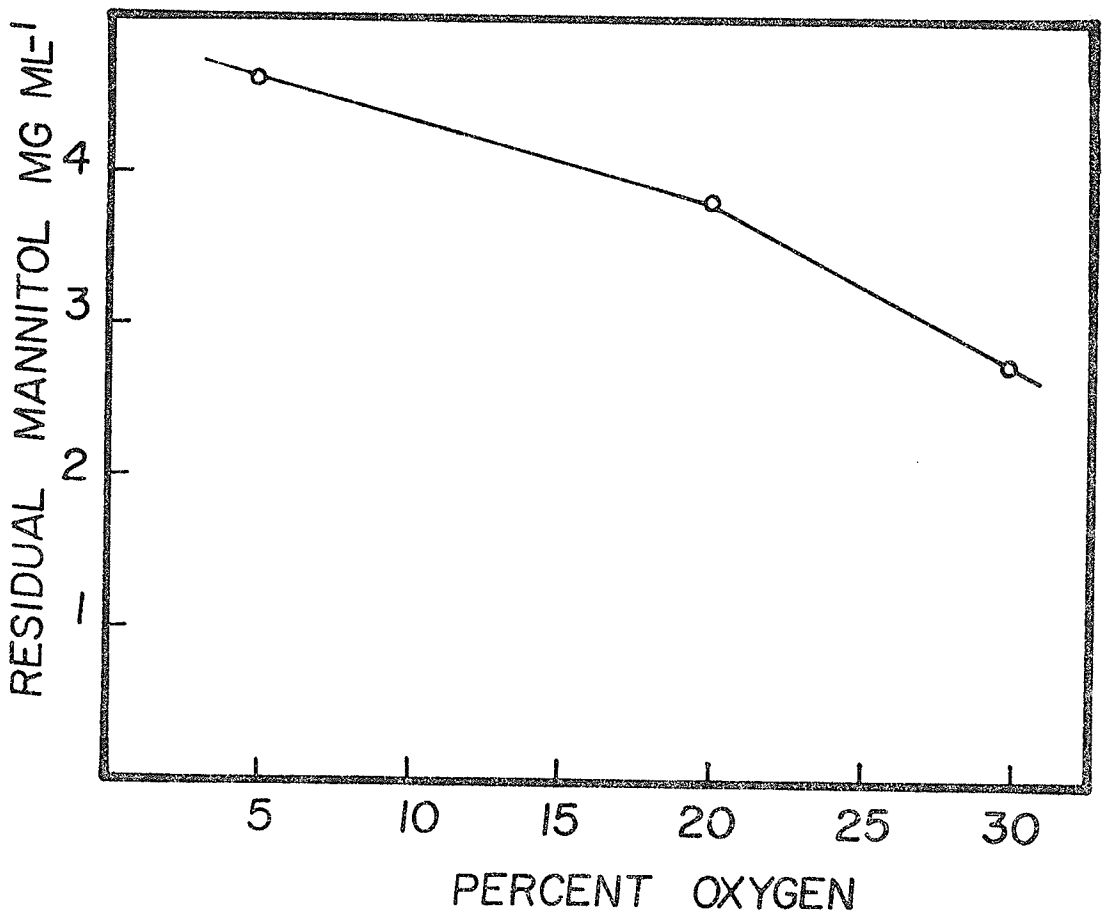
RESIDUAL MANNITOL MG ML-1
1
2
3
4
5
10
15
20
25
30
PERCENT OXYGEN

Table 10. Catalase and superoxide dismutase activities associated with cell fractions of Azotobacter chroococcum; intracellular location of the enzymes.

Cell Fraction	1 Catalase Activity			2 Superoxide Dismutase Activity		
	Total O_2 Produced (nm min^{-1})	% of Total in Fraction	O_2 mg^{-1} Protein (nm)	Total SOD (units)	% Total in Fractions	SOD Units mg^{-1} Protein
Soluble[a]	5750	88	25.2	90,000	99	109
Large[b]	660	10	15.1	720	0.8	106
Small[c]	143	2	8.7	112	0.1	11.8

The method of cell rupture is as described in the Materials and Methods. The cell fractions designated are as follows: [a]"soluble" - combined supernatants and washings; [b]"large" - washed cell pellet obtained from glycerol disrupted cells by centrifugation at 10000 g for 15 min at 4^oC; [c]"small" - pellet from centrifugation of above supernatants and washings at 100000 g for 2 hrs at 4^oC.

1 Catalase activity was estimated by O_2 production from 0.2 ml 0.35 m H_2O_2 in mannitol medium to a total volume of 1.5 ml in the reaction vessel of a Gilson oxygraph fitted with a Clarke type O_2 electrode.

2 Superoxide dismutase activity was determined by the method of Buchanan and Lees (1976).

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Figure 20 Electron micrograph - unsonicated large fraction of *A. chroococcum* cell

ce - cell envelope showing internal membrane network

c - cytoplasm in ruptured cell

v - vesicle

Details are as in Figure 21.

Magnification: 32,000

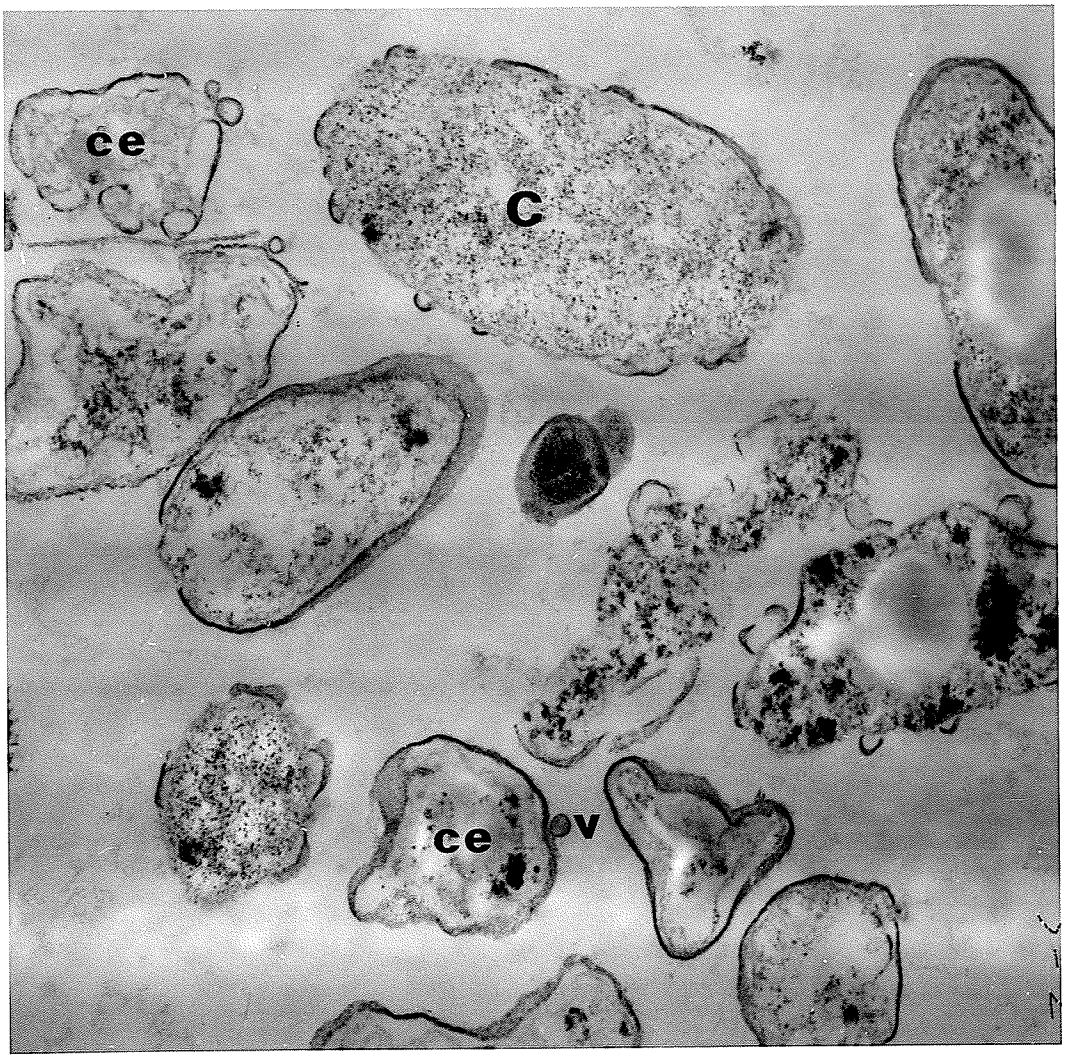
ce
C
ce
v

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Figure 21 Electron micrograph - sonicated large fraction of *A. chroococcum* cell.

me - membrane enclosure

v - vesicle

c - cytoplasm enclosed in membrane

The cell fraction was obtained from an osmotically shocked cell, and was prepared for electron microscopy as described in the Materials and Methods.

Magnification: 32,000

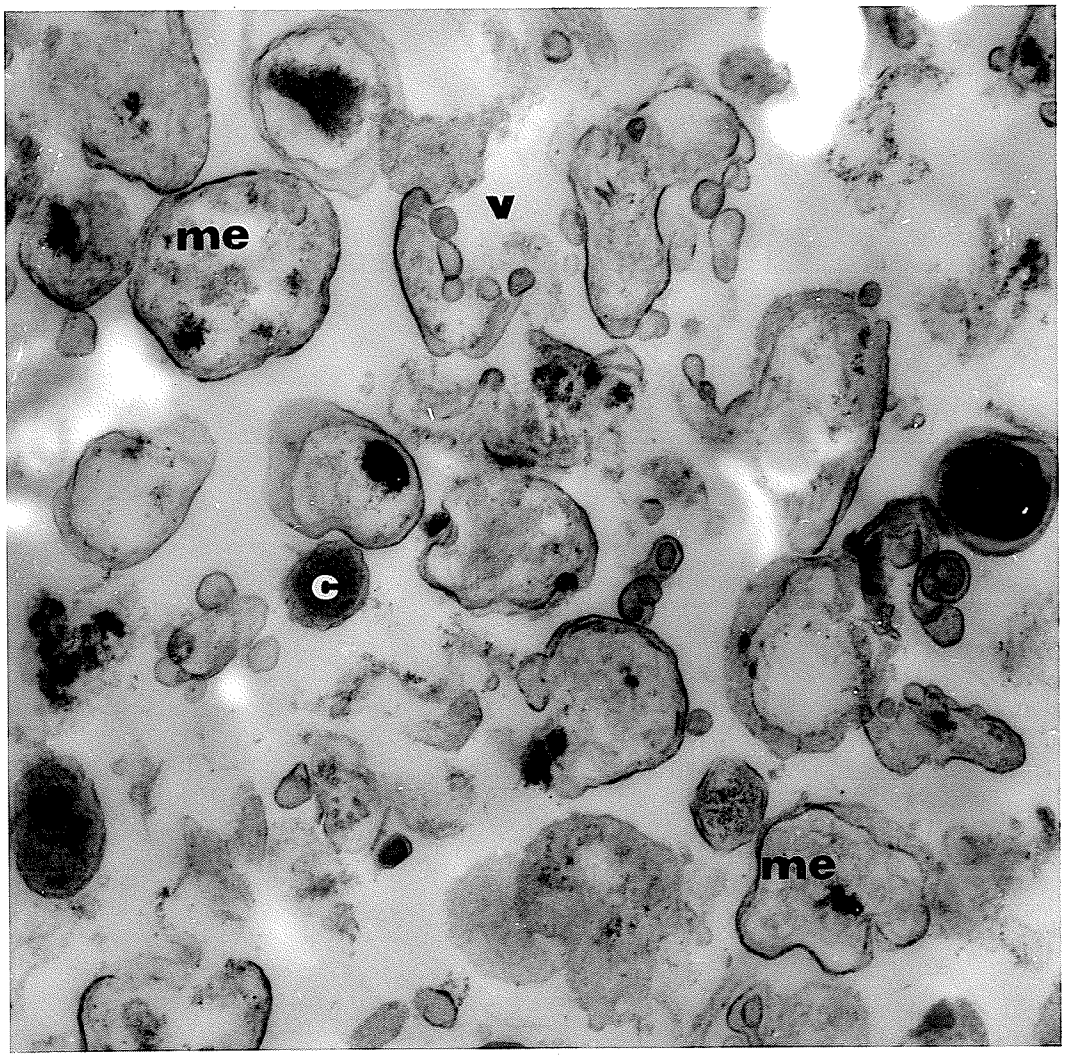
me
v
c
me

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Figure 22 Electron micrograph - unsonicated 'small fraction' of A. chroococcum cell.

me - membrane enclosure

v - vesicles

mf - membrane fragments

mec- membrane enclosed cytoplasm

The cell fraction was obtained from an osmotic-shocked cell, and was prepared for electron microscopy as described in the Materials and Methods.

Magnification: 40,000

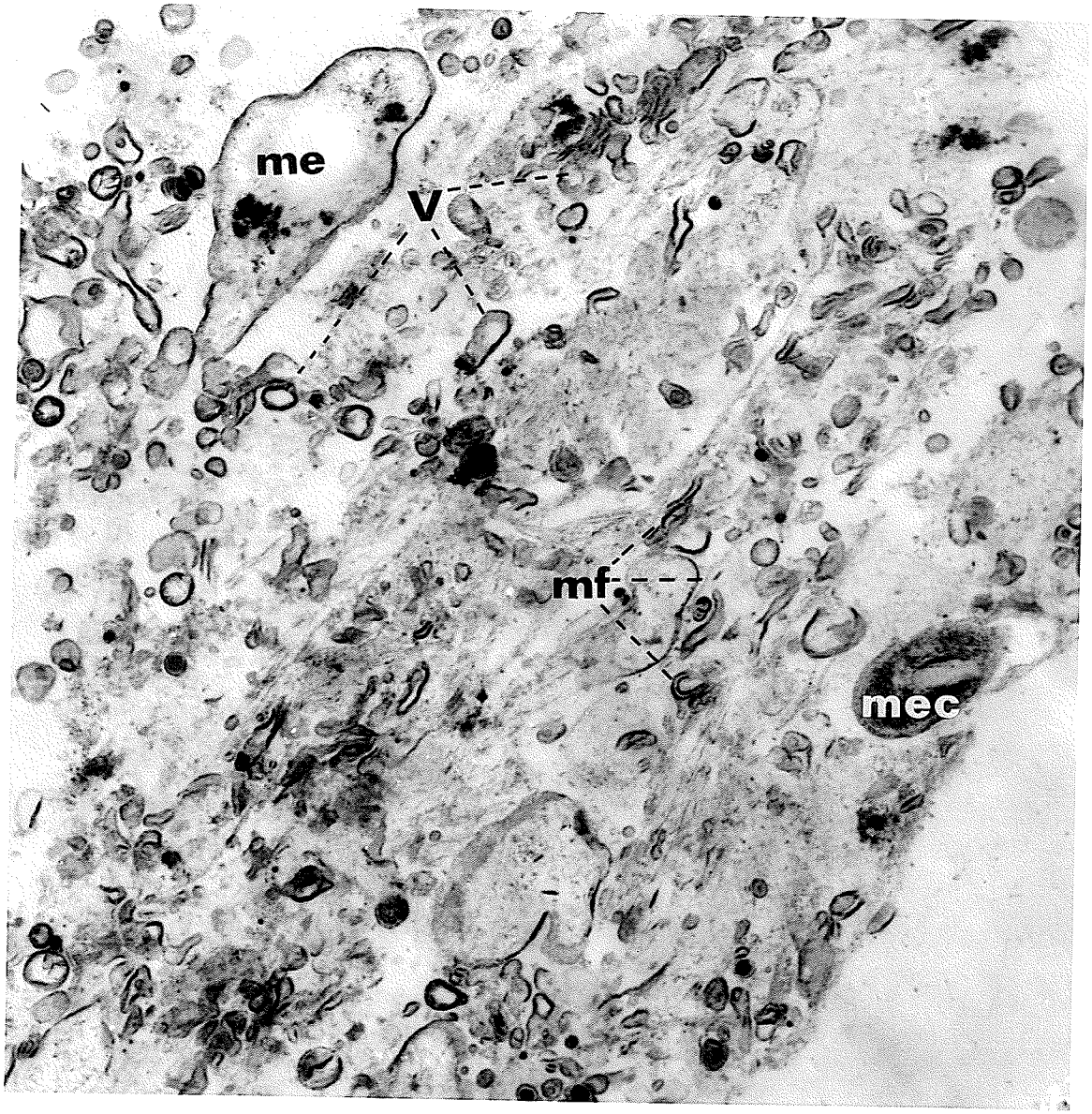
me
V
mf
mec

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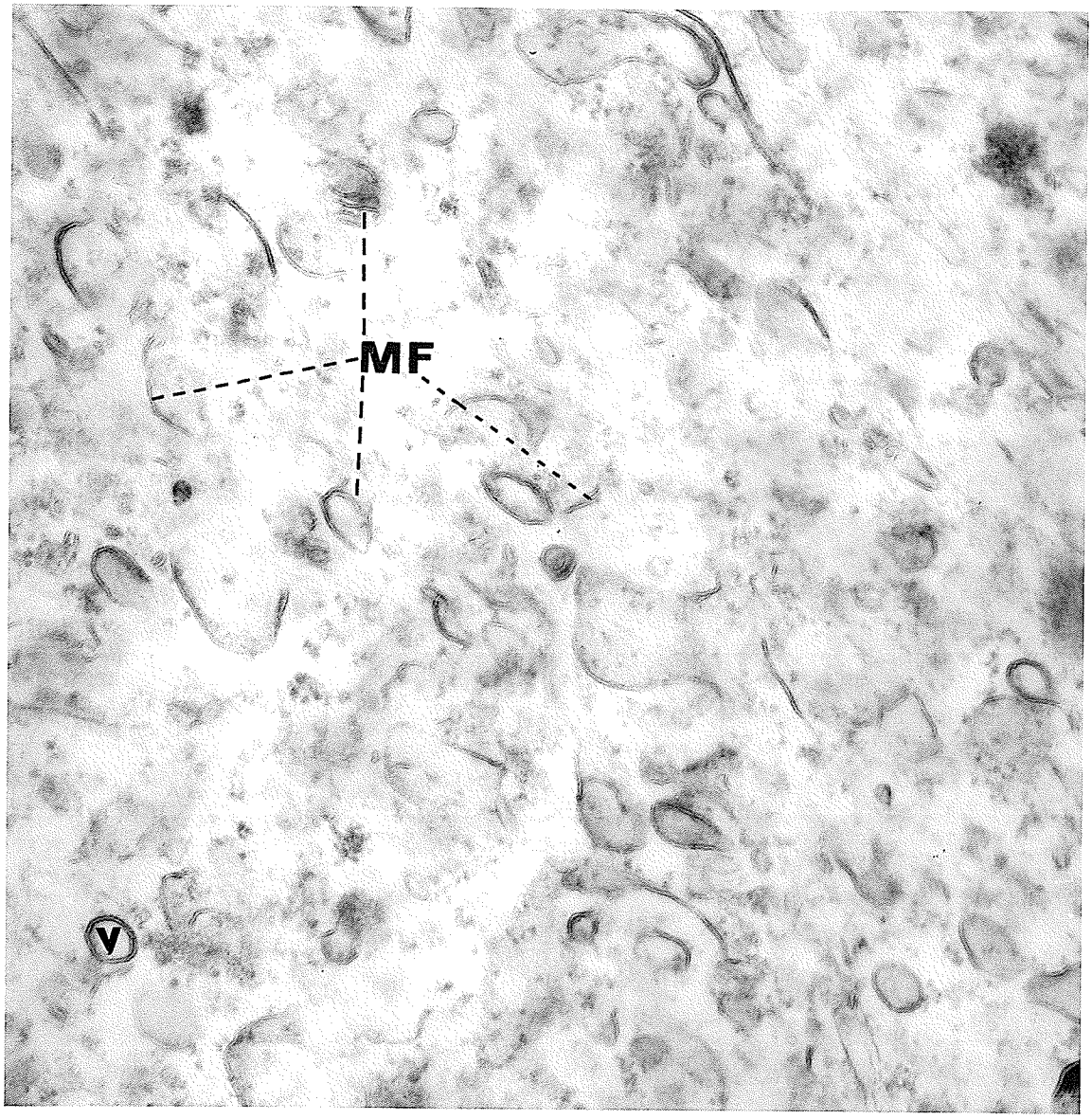
Figure 23 Electron micrograph - sonicated small fraction of A. chroococcum cell.

v - vesicle

mf - membrane fragments

Cell fraction and preparation as in Figure 22.

Magnification: 80,000.


MF
V

A model for the interaction between nitrogenase, catalase, and superoxide dismutase, as related to their respective intracellular locations

The literature to date relating to the possible intracellular location of nitrogeanse in A. chroococcum has been contradictory. The results have been extremely variable, and greatly influenced by the methods of cell rupture.

Stasny, Burns, Korant, and Hardy (1974), using a ferritin-conjugated antibody technique, reported localisation mainly around the cell periphery but also in an organised manner within the internal cytoplasm. Lees and Dawson (manuscript in preparation) have inferred a similar location, based on electron microscopic examination of cells cultured under varying oxygen and ammonium concentrations. The electron micrograph (shown in Fig. 24) of an A. chroococcum N_2 fixing cell shows "vesicles" arranged around the cell periphery and at random in the cytoplasm. A soluble nitrogenase has been reported by Oppenheim et al., (1970), and more recently Haaker and Veeger (1977) concluded that no direct association of nitrogenase with the cytoplasmic membrane could be observed, but did not rule out weak association with the cytoplasmic membrane. Reed, Toia, and Raveed (1974) purified azotophore membranes containing nitrogenase from A. vinelandii, whereas Pate et al., (1973) suggested the internal membrane structure of A. vinelandii was produced in response to oxygen availability rather than to nitrogen source. It should be noted that Reid et al., (1974) found similar quantities of nitrogenase in both the cell-free lysate (soluble fraction) and the azotophore

membrane fraction, which does imply that the enzyme may be partially associated with the soluble cell fraction.

The report presented here will contend that only some slight modification of the proposed model is necessary to accommodate either of the two possible locations suggested. The proposed model is based on the following:-

1. Superoxide dismutase and catalase are found to be mainly cytoplasmic, though some association with membranes may occur, as evidenced by enzyme release from membrane-formed "vesicles" on sonication.
2. The internal membrane network appears to be continuous with the periplasmic space, and discrete vesicles may be formed enclosing areas of cytoplasm in membrane structures.
3. Nitrogenase, catalase, and superoxide dismutase may be found within these areas of membrane-enclosed cytoplasm (vesicles) and may or may not be bound, loosely or otherwise, to membranes.
4. Exogenously generated O_2^- inactivates nitrogenase, and exogenously added bovine SOD confers protection to the whole cell. Kleiner and Kleinschmidt (1976) have shown that no inactivation of nitrogenase occurred when induction of encystment was studied under anaerobic conditions. Oxygen, or some product of its metabolism, may therefore be responsible for effecting the inactivation.

5. Endogenous SOD of the bacterial cell may be restricted largely to the cytoplasm on the inside of the membranous vesicles.

The proposed model, showing the above features, is diagrammed in Fig. 25. Figure 26 shows how exogenous O_2^- effects nitrogenase inactivation and exogenous SOD affords protection. The endogenous SOD of the bacterial cell is viewed as enclosed eventually by the membraneous network ramifying throughout the cytoplasm. The SOD located on the inside of the membrane does not allow protection from O_2^- generated enzymically outside of the cell, but exogenously added SOD can protect nitrogenase in the cytoplasm (internal to the membrane) by forming an O_2^- impenetrable barrier around the outside of such structures. In the absence of exogenous SOD, presuming that the cell's SOD does not move freely across the membrane, O_2^- interacts with the membranes to result in exposure of the previously protected nitrogenase, which is then inactivated. Figure 27, representative of several fields of view, shows an actual membrane-cytoplasm enclosure in an A. chroococcum cell. To account for the findings of Lees and Dawson (manuscipt in preparation, Fig. 28), catalase is seen as residing in the periplasmic space and is also apparently associated with the membrane vesicles. Catalase thus located would not provide protection against exogenous O_2^-, since the enzyme acting on this toxic species is SOD and not catalase, but conceivably protects against the peroxide produced by O_2^- dismutation.

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Figure 24 Electron micrograph of A. chroococcum whole cell showing internal membrane network and vesicles.

v - vesicle

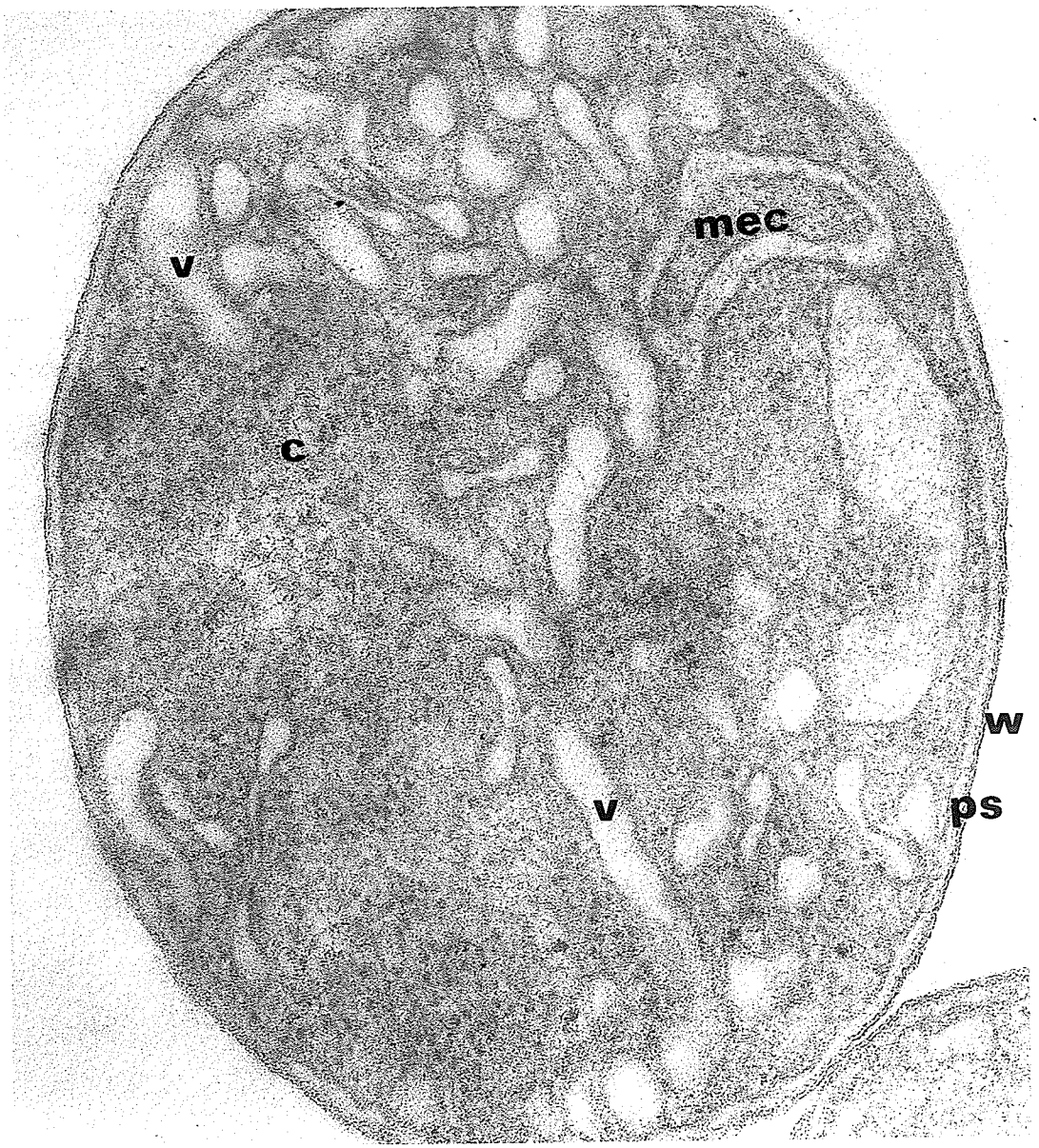
mec - membrane enclosed cytoplasm

w - cell wall

ps - periplasmic space

The cell sample was taken from a continuous culture grown under N_2 fixing conditions with 30% O_2. The electron micrograph was obtained as described in the Materials and Methods.

Magnification: 120,000


mec
v
c
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v
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Figure 25 Model for nitrogenase, catalase, and superoxide dismutase assembly in cytoplasm of A. chroococcum.

M - membrane

MT - microtubule linking vesicle and membrane

V - vesicle

C - cytoplasm-containing enzymes - enclosed by vesicle

● - nitrogenase

▨ - superoxide dismutase

Δ - catalase

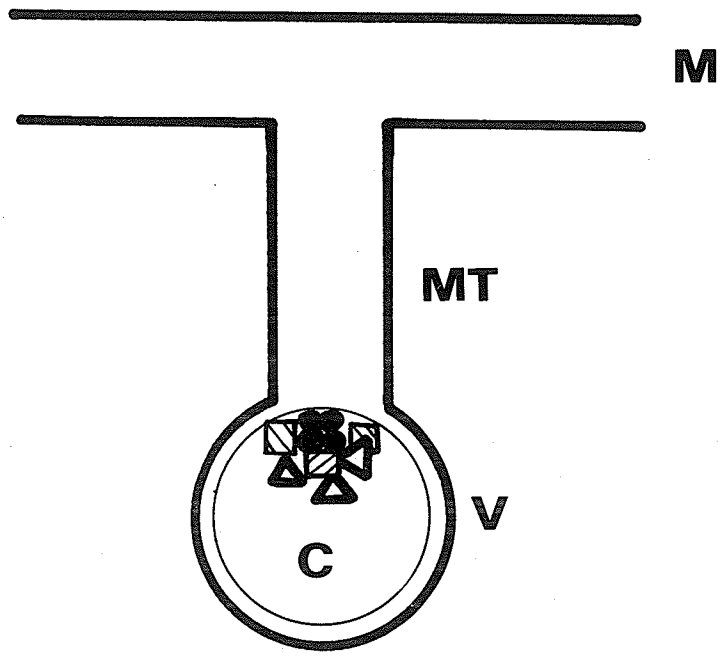
M
MT
V
C

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Figure 26 Exogenous O_2^- mediated inhibition of nitrogenase in the proposed model.

M - membrane

MT - microtubule

V - vesicle

C - cytoplasm

→ - points of attack by the superoxide anion (O_2^-)

- - - - superoxide dismutase location

The location of the endogenous SOD is represented in 1, that of the exogenous in 2. The endogenous enzyme may not be able to protect the nitrogenase due to the SOD location on the inside of the membrane. Exogenous SOD is visualised as forming an O_2^- impenetrable barrier around the vesicle, thus protecting the nitrogenase enclosed therein.

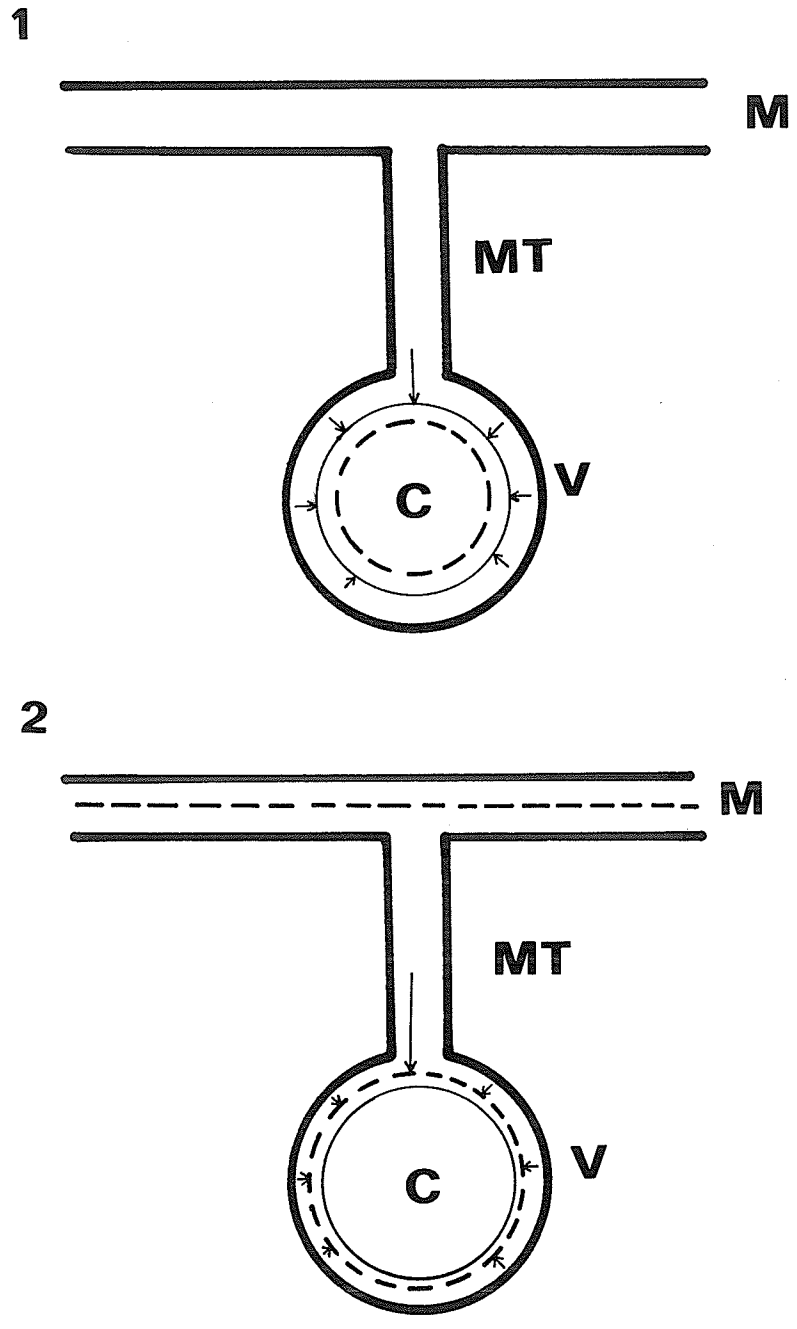
1
M
MT
C
V
2
M
MT
C
V

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Figure 27 Electron micrograph of A. chroococcum whole cell showing the continuity between the cell membrane and that of the internal vesicles.

mt - membrane microtubule continuous with the periplasmic space, leads to vesicle

ps - periplasmic space

m - membrane enclosed cytoplasm (possible site of nitrogenase, catalase, and SOD)

w - wall

c - cytoplasm

Cell sample from continuous culture grown with 5% O_2 under N_2 fixing conditions. Electron micrograph prepared as described in Materials and Methods.

Magnification: 120,000.

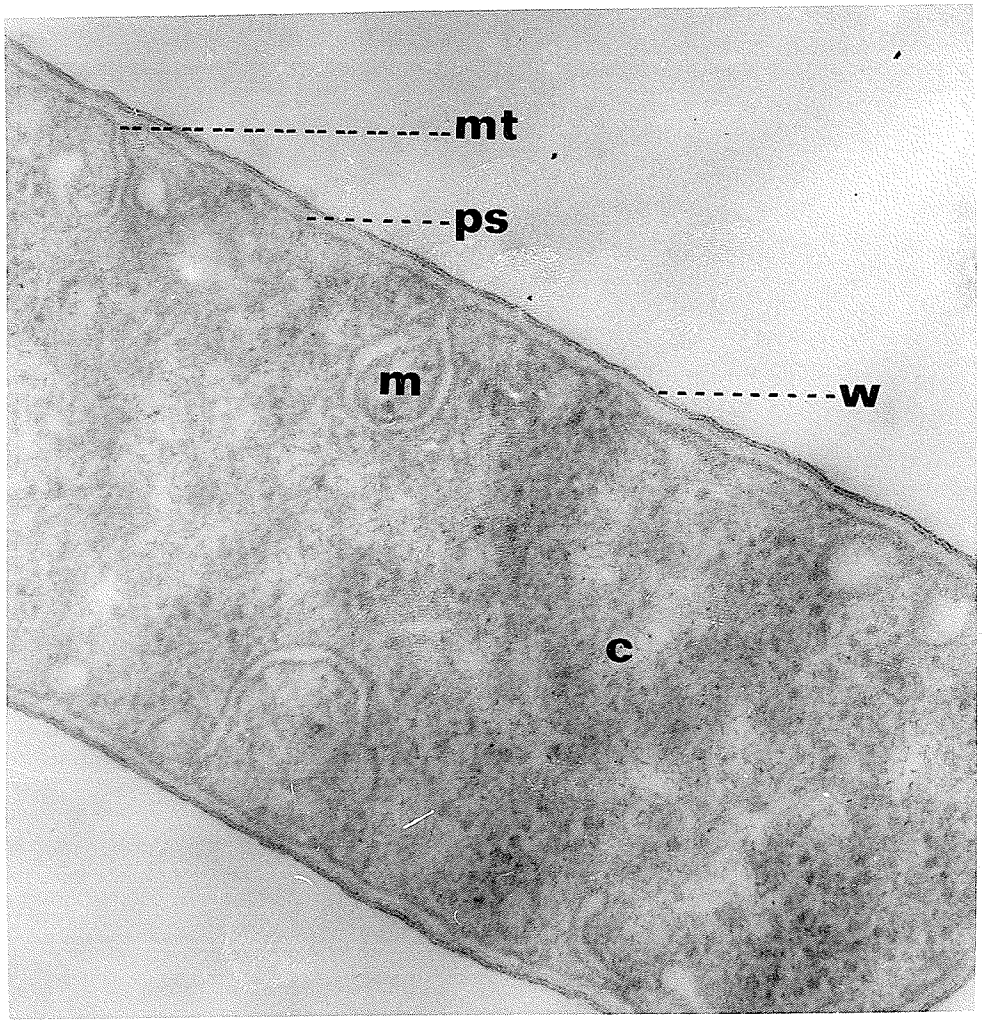
mt
ps
m
w
c

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Figure 28 Electron micrograph of A. chroococcum whole cell showing location of catalase.

w - wall

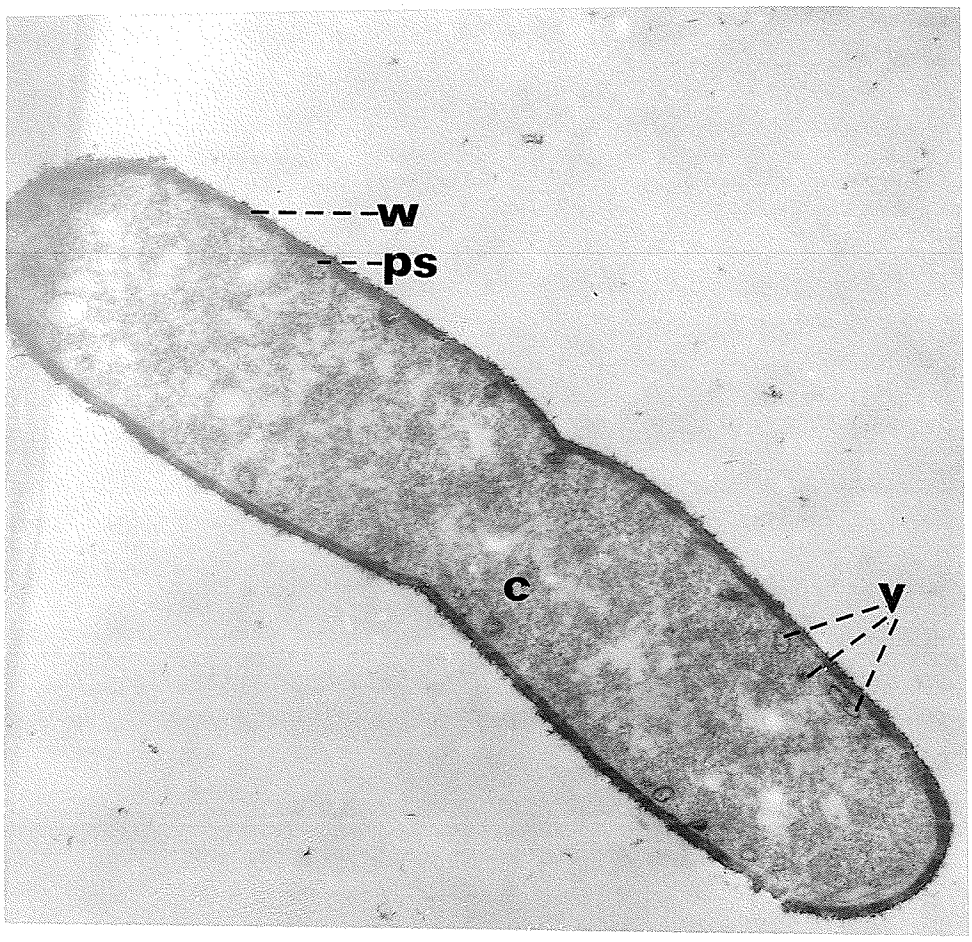
ps - periplasmic space, site of catalase activity

c - cytoplasm

v - vesicle

The cell sample was taken from a continuous culture of A. chroococcum grown under N_2 fixing conditions with 20% O_2. The histochemical procedure used for the localisation of catalase is given in Dawson and Lees (manuscript in preparation).

Magnification: 40,000.


w
ps
c
v

The nature of the cell fractions as revealed by electron microscopic examination agrees with that reported by Robrish and Marr (1962), and by Oppenheim et al., (1970). Similar cytoplasmic location of both catalase and superoxide dismutase has been implied by Takanaka and O'Brien (1975) for polymorphonuclear leucocytes; Rest and Spitznagel (1977) found that the potassium cyanide sensitive SOD of human neutrophils was cytoplasmic. A similar locale for an SOD type has also been reported for chicken liver and pig heart (Weisiger and Fridovich, 1973). In reference to bacteria, Britton and Fridovich (1977) have shown that all of the superoxide dismutase isosymes of Escherichia coli occur in the cell matrix. Copper-and zinc-containing superoxide dismutase have also been found in the cytosols of Neurospora crassa (Misra and Fridovich, 1972); Saccharomyces (Goscin and Fridovich, 1972); and in spinach (Asada, Urano and Takehashi, 1973). The occurrence of SOD and catalase in the cytoplasm of Azotobacter chroococcum is thus not unique, similar sitings for these enzyme activities having been found in both procaryotic and eucaryotic cells.

At this point, evidence having been presented to show a cytoplasmic location for SOD and catalase in A. chroococcum, and having demonstrated that exogenous O_2^- can effect inactivation of whole cell nitrogenase activity, it is most convenient to propose ideas reconciling these observations with what is reportedly known about the possible location of nitrogenase in A. chroococcum.

An Explanation of Respiratory and Conformational Protection by the Proposed Model

Postgate and colloborators, in numerous publications relating to Azotobacter nitrogenase (Drozd and Postgate, 1970; Dalton and Postgate, 1969; Lees and Postgate, 1973; Hill et al., 1973) have stated that nitrogenase may be protected from oxygen damage by respiratory control and by conformational modification. Can the theory proposed here explain these accepted pehnonema?

To provide a satisfactory explanation, the proposed model has to be expanded to consider the following documented observations -

a) O_2^- is attracted to and collects at membrane surfaces, (Petkau, 1971), and SOD confers protection to model phospholipid membranes (Petkau and Chelack, 1976).

b) O_2^- can participate in redox reactions with suitable cations sorbed to the membrane surface (Petaku, 1971).

c) damage to membranes has been attributed to O_2^- mediated or initiated peroxidation (Fong et al., 1973) of lysosomal membranes, liposome oxidation and erythrocyte lysis (Kellog and Fridovich, 1977), and the reactivity of singlet molecular oxygen with cholesterol in a phospholipid membrane matrix has been proposed as a model for oxidative damage to membranes (Suwa et al., 1977).

d) respiratory protection is characterised by increased O_2 utilisation (Dalton and Postgate, 1969) and significant production of O_2^- has been documented in respiring tissue (Fridovich, 1974); increased respiratory rates may thus be

equated to increased generation of O_2^-. Ackrell and Jones (1971) have shown that a considerable increase in whole cell respiratory activity was observed on changing from low to high aeration conditions. O_2 mediated respiratory increases thus have been documented for Azotobacter.

e) the respiratory enzymes and electron transfer components in Azotobacter are membrane bound (Scherings et al., 1977; Yoch and Arnon, 1972; Ackrell and Jones, 1971; and Haddock and Jones, 1977).

Considering the above, and referring to Fig. 26, the nitrogenase is protected under normal respiratory conditions, during which relatively small quantities of O_2^- produced diffuse rapidly from their production sites in the membranes and undergo dismutation by the SOD. This dismutation may be favored by the proximity between the membrane and the cell's SOD. In the presence of elevated oxygen tensions and consequent elevated respiratory rates, rapidly increasing generation and collection of O_2^- at the membranes would effect inactivation of nitrogenase. Inactivation of the Fe-protein in nitrogenase has been deemed to be partially mediated by O_2^- and H_2O_2, based on the effects of added SOD and catalase, (Mortenson, Walker and Walker 1976). Postgate (1974) has stated that the "switch on" process for nitrogenase did not involve protein synthesis de novo, but required some "signal". It is now proposed that the requisite signal is O_2^- (or some like species) which is

overly produced as a direct result of an increased utilisation of molecular oxygen. O_2^- is therefore inherently suitable as a proposed signal, since it is directly produced from oxygen, and the organism's responses are to oxygen stress resulting from an excessive supply of oxygen.

Accumulation of this negatively charged species (O_2^-) subsequently could create a condition whereby the nitrogenase complex was discharged by electrostatic repulsion from the membrane (possibly to become enclosed by superoxide dismutase and/or catalase molecules) representing the "switched-off" situation in conformational protection. Burris and Orme-Johnson (1976), and Eady et al., (1975) among others, state that the molybdenum-iron (Mo-Fe) protein of nitrogenase binds substrate, with the numerous Fe atoms serving as an electron sink for electron donation. The active nitrogenase complex therefore has a preponderance of negative charges. Pate et al., 1973, suggested that internal membrane control may be controlled by oxygen tension. As previously reported (Buchanan, 1977) SOD appears to be constitutive in A. chroococcum. Thus, if greater development of the internal membrane occurs in response to elevated oxygen, the defence against O_2^- provided by SOD becomes "thinly stretched out" over the markedly increased area, thereby allowing the previously protected nitrogenase to be attacked. The propoposals therefore suggest an "On-site, Off-site" model mediated by O_2^- prompted electrostatic repulsion. This model will be

subsequently referred to as the "On-site, Off-site" model (Fig. 29). Petkau and Chelack, (1976), in indirect support of the "On site-Off site" model have suggested that superoxide dismutase provides a framework for variable protection of cellular membranes, depending on whether the enzyme was associated with them and to what extent. Inadequate protection (as envisaged in the "On site-Off site" model here proposed) would render the membranes susceptible to damage from O_2^- mediated toxicity. The nitrogenase complex is functional, i.e., switched on in the "On site" position when associated with the membrane, and non-functional, i.e., switched off in the "Off-site" state when not directly associated with the membrane. Since the functioning of nitrogenase does require electron donation from membrane components (Jones and Redfearn, 1967; Scherings et al., 1977), removal of nitrogenase from the membrane (in the absence of some suitable intermediary) would effectively inhibit its operation. Haaker and Veeger (1977), have also shown that an energised cytoplasmic membrane is necessary for electron transport to nitrogenase, implying a possible requirement for some close structural or functional contact of nitrogenase and membrane.

It is conceivable that the nitrogenase complex may be bound to the cell membrane by binding to a specific receptor. Superoxide anion, or some like species, may act by changing the conformation of these receptor sites and rendering them incapable of binding

the nitrogenase complex. O_2^-, alternatively, or simultaneously, may also produce a marked lowering of affinity between binding site and enzyme complex with similar results.

Another explanation for the control of nitrogenase activity is that O_2^- may mediate an allosteric inhibition of the nitrogenase complex itself. Newton et al., (1976), in proposing a model for nitrogenase, suggested that a complexing of the Mo-Fe protein with the Fe-protein led to a configurational change such that electron transfer occurred between the exposed chromophore of the Fe-protein and the nearby electron-accepting site on the Mo-Fe protein. Simultaneously, a site on molybdenum is cleared for substrate coordination. Juxtaposition of a second metal might be responsible for the steric requirements observed for substrate reduction. Any (or all) of the above phenonema, which are mediated by electron transfer, offer attractive sites for interaction with O_2^-. Interaction of O_2^- and enzyme complex is viewed as effecting a serious disruption of the normal interaction of the components responsible for maintaining the necessary metal juxtaposition and protein conformation for full activity of the complex. An allosteric modification, prompted or mediated by O_2^-, is thus suggested.

The allosteric or other modifications which may be prompted by O_2^- are envisaged to occur only when the rate and quantity of O_2^- generation are such that production and perceptible accumulation occurs at or near the membranes, or when its rate of production

exceeds its rate of diffusion from the membranes. Such circumstances are presumably associated with oxygen stress and the accompanying increased respiratory rate that produce unusually large quantities of O_2^-.

The above proposals are at present largely speculative, and are based partly on data available at present. These proposals, if reasonable, should prompt an examination of O_2^- mediated steric or conformational alteratons of nitrogenase. It is not unreasonable to speculate that a process which depends largely on electron transfer, as does nitrogenase, should be subjected to the influence of such a charged species as O_2^-.

Nitrogen sensitivity to oxygen may thus be due to interaction with O_2^-, as explained thus: 1) Structural damage to the membranes surrounding nitrogenase, O_2^- interaction with membrane lipids may alter the integrity of these membranes to expose the previously protected nitrogenase to attack by O_2^- or other species; 2) Alteration of nitrogenase components by interaction with O_2^-. Nitrogenase components contain numerous acid labile sulphurs through which reduction occurs (Burris and Johnson, 1976). Any significant alteration in these clusters could produce inactivation, which acquires direct significance in view of the documented interaction of sulphur groups with O_2^- (Barron, 1955, Lavalle et al., 1973); 3) Interference of electron flow to nitrogenase. In usual preponderance of changed species (O_2^-, on some other) in the locale of the components could seriously disrupt electron flow to nitrogenase, precluding activity of the complex, since its function depends on efficient electron transfer between the various components.

Indubitably, other ideas may be advanced to explain the observations here presented, but as far as has been ascertained, this is the first

attempt at a different explanation for the effects of oxygen on Azotobacter's physiology, which brings together the classical ideas of respiratory and conformational protection and the more modern role of the oxygen scavenging and protective mechanisms which have been shown to be mandatory for an aerobic existence. It is indeed apt that such a reconciliation should be effected via Azotobacter, which is characterised by its unusually high rates of oxygen utilisation.

The remaining discourse will show the effects of increased oxygen, and of ammonium on the nitrogenase activity of A. chroococcum whole cells in continuous culture. Features of the partially purified enzyme will also be described.

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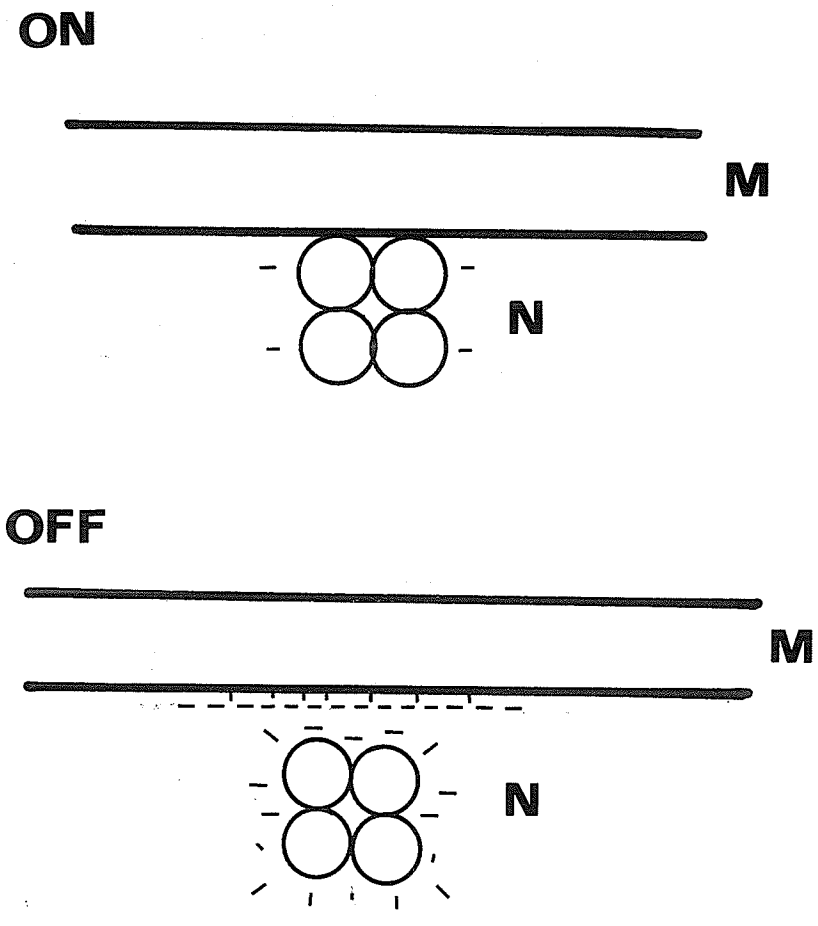
Figure 29 The "on-site, off-site" model for the O_2^- mediated control of nitrogenase activity.

- negative charges produced by accumulation of O_2^- at membrane

M - membrane

N - nitrogenase complex

The nitrogenase complex is functional when in contact with the membrane (on), and non-functional when not in contact with the membrane (off). Accumulation of O_2^- at and around the membranes produces electrostatic repulsion of the negatively charged nitrogenase, which loses contact with the membrane to assume the non-functional conformation. It is possible that the "off-site" nitrogenase may be associated with superoxide dismutase and/or catalase, equivalent to the conformationally protected enzyme. Allosteric modifications in the complex may also facilitate or accompany the O_2^- mediated inactivation.


ON
M
N
OFF
M
N

The Effect of Increased Oxygen Supply, and of Ammonium, on the Nitrogenase Activity of Whole Cells in Continuous Culture

The results are presented in Figs. 30 and 31. Fig. 30 generally indicates an initial drop in nitrogenase activity (i.e., nmoles C_2H_4 produced /hr/mg) within the first 2 hrs, after which an increase was noted (up to 8 hrs.). Subsequently, the nitrogenase level dropped, in approximately 5 days, to levels comparable to that present before the oxygen supply was increased from 5% to 20% O_2. Similar oscillations in the levels of various enzymes, as effected by elevated oxygen tensions, have been documented (Harrison, 1972; Dalton and Postgate, 1969). The increased nitrogenase activity noted after 2 hrs may reflect an overall change in cell metabolism, as evidenced from an increased CO_2 output and a greater utilisation of mannitol, in accordance with the increased O_2 supply. The dry weight of the continuous culture likewise oscillated, and on restablising, the nitrogenase activity as related to the unit dry weight of cells, had decreased, indicating a lowered efficiency of nitrogen fixation at elevated oxygen tensions. The respiratory index was higher (1.4) at 20% O_2 compared to that at 5% O_2 (1.0). Fig. 31 clearly shows an immediate inhibition of whole cell nitrogenase activity by added NH_4^+, in keeping with the now generally accepted idea of the effects of NH_4^+ on nitrogenase (Dalton and Postgate, 1969). It should be noted that the levels of activity detected on subsequent stabilisation were significantly lower than those obtained previously under nitrogen-fixing conditions. Nitrogenase synthesis in A. vinelandii is reportedly completely suppressed by

25 μM NH_4^+ in both batch and continuous cultures (Kleiner, 1974), but as reported by Drozd, Tubb, and Postgate (1972), NH_4^+ at concentrations well below the concentrations which repress nitrogenase synthesis depress the nitrogenase activity of intact bacteria. The results here reported support the conclusions arrived at by Drozd et al., 1972, and are in general agreement with those of Dalton and Postgate, (1969).

The nature of the responses elicited by added NH_4^+, and by increased oxygen supply, seem to indicate that some mutant strain of bacteria - better able to grow under the altered conditions - was being selected. Evidence to support this comes indirectly from the observation that the cultures reverted (over several days) to a comparable "steady state" level to that which existed before varying the conditions. This assumption does not rule out other regulatory and adaptive responses which may also be responsible for producing the behavioural responses observed. Dalton and Postgate (1969) presented evidence to show that continuous cultures at low growth rate favored the selection of slow growing mutants, and suggested that fast-growing variants arose by the reverse process. Harrison (1972), discussing mechanisms of cell responses, stated that overlap of the various control mechanisms may occur, and that several types of responses can occur simultaneously. A response through a time scale of many generations indicated selection of mutants. Thus the results presented here may reflect a combination of several response types.

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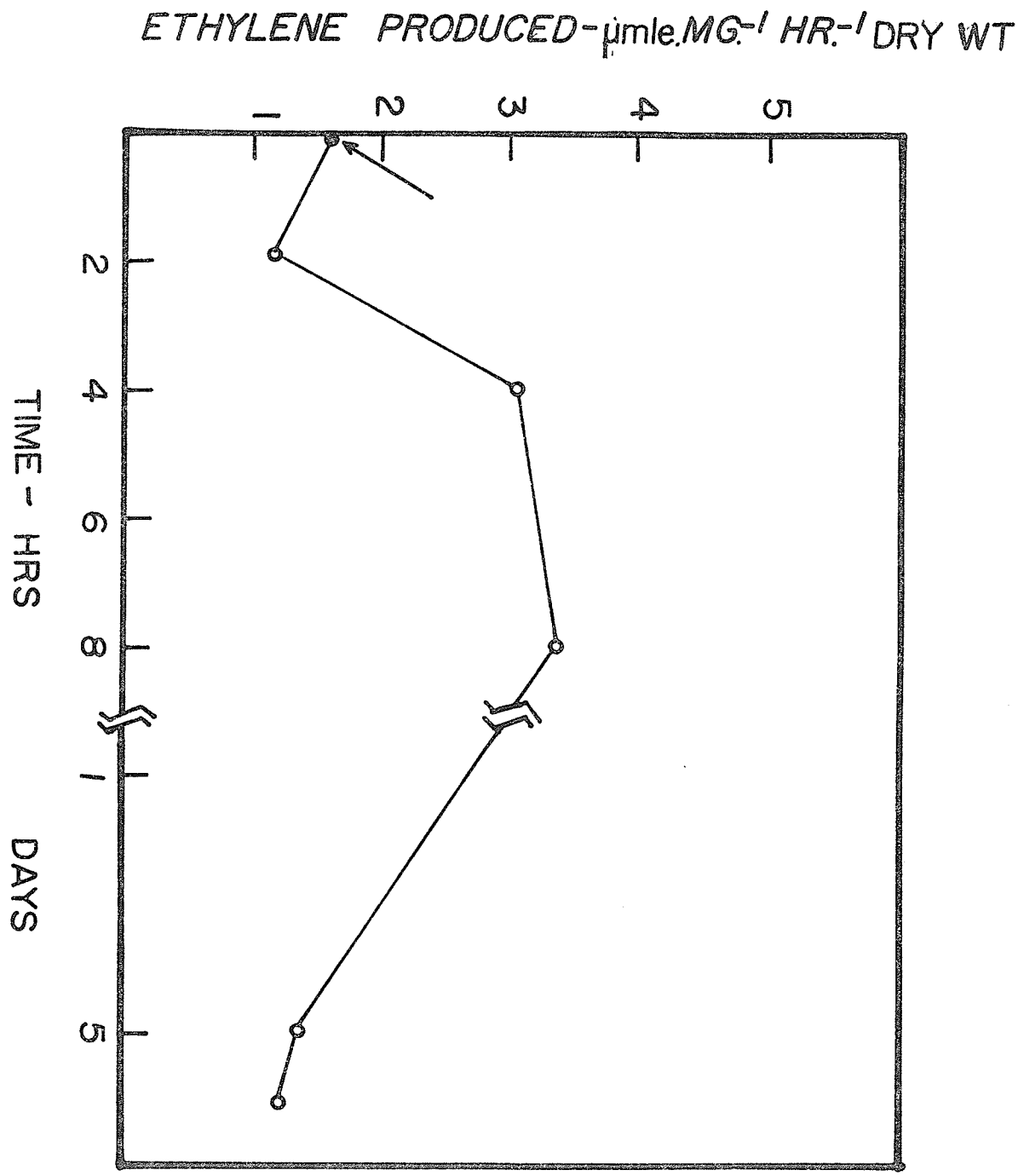
Figure 30 The effect of an increased O_2 supply on the whole cell nitrogenase activity of A. chroococcum in continuous culture.

(●) - nitrogenase activity with 5% O_2

(O) - nitrogenase activity with 20% O_2

Cultures were grown in 1% mannitol B_6 medium with vortex stir rate. The oxygen supply was suddenly switched from 5% O_2 to 20% O_2 at the point indicated by the arrow. The nitrogenase activity of cell samples taken from the continuous culture was estimated as in Figure 31.


ETHYLENE PRODUCED - µmle. MG.-1 HR.-1 DRY WT
1
2
3
4
5
TIME - HRS
2
4
6
8
1
DAYS
5

LEAF

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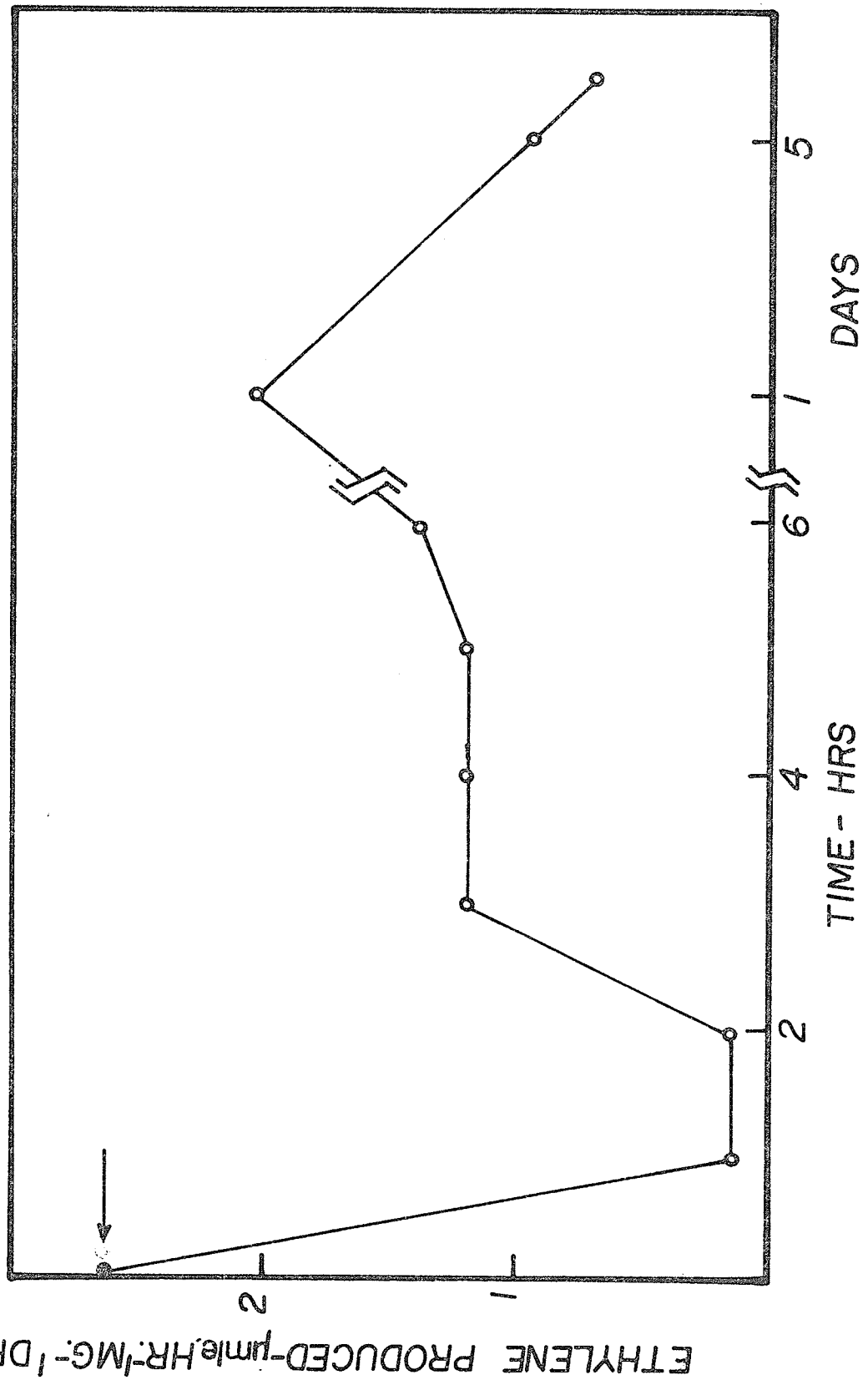
Figure 31 The effect of ammonium on the whole cell nitrogenase activity of A. chroococcum in continuous culture.

(●) - nitrogenase activity under N_2 fixing conditions

(○) - nitrogenase activity in the presence of NH_4^+

Cultures were grown under 20% at vortex stir rate in 1% mannitol B_6 medium. 1 ml. samples of the culture were taken at the times stated and assayed for nitrogenase as in the Materials and Methods. NH_4^+ was added as $(NH_4)_2 SO_4$ as indicated by the arrow, to a final concentration of 4 mM.


ETHYLENE PRODUCED-μmle.HR-1MG-1DRY WT
2
1
TIME- HRS
2
4
6
1
DAYS
5

Secretion of Superoxide Dismutase by Plate Cultures of Azotobacter Chroococcum, and Whole Cell Production of O_2^-

SOD apparently was not secreted by plate cultures of A. chroococcum under the conditions described in the Materials and Methods. The nitro-blue impregnated plates were uniformly blue coloured on exposure to an incandescent light source, and no achromatic zones were observed around the colonies on the plate, as would be expected if secretion of appreciable quantities of SOD was occurring. The experiment was prompted by the protection conferred to nitrogenase inactivation by exogenous SOD (Buchanan, 1977), thus it was thought that some secretion of SOD might occur in an attempt by the organism to protect its interior.

O_2^- production was monitored by the rate of reduction of acetylated cytochrome c (Ac c) as outlined previously (see Materials and Methods). In the procedure involving a comparison of the rates of Ac c reduction by whole cells in the presence (or absence) of 100 µg bovine SOD, there was clear indication of an increased rate in the absence of bovine SOD exogenously added to the cells, (see Fig. 32). Actively growing cells of A. chroococcum therefore apparently produce O_2^-.

In experiments comparing the rates of Ac c reduction by whole cells grown with varying oxygen concentrations, cells grown with 20% O_2 reduced Ac c at a rate of 3.2×10^{-7} moles min^{-1} whereas 2% O_2 grown cells effected reduction at a rate of 2×10^{-7} moles min^{-1}. The results therefore tend to verify an increased rate of O_2^- production at elevated oxygen tensions.

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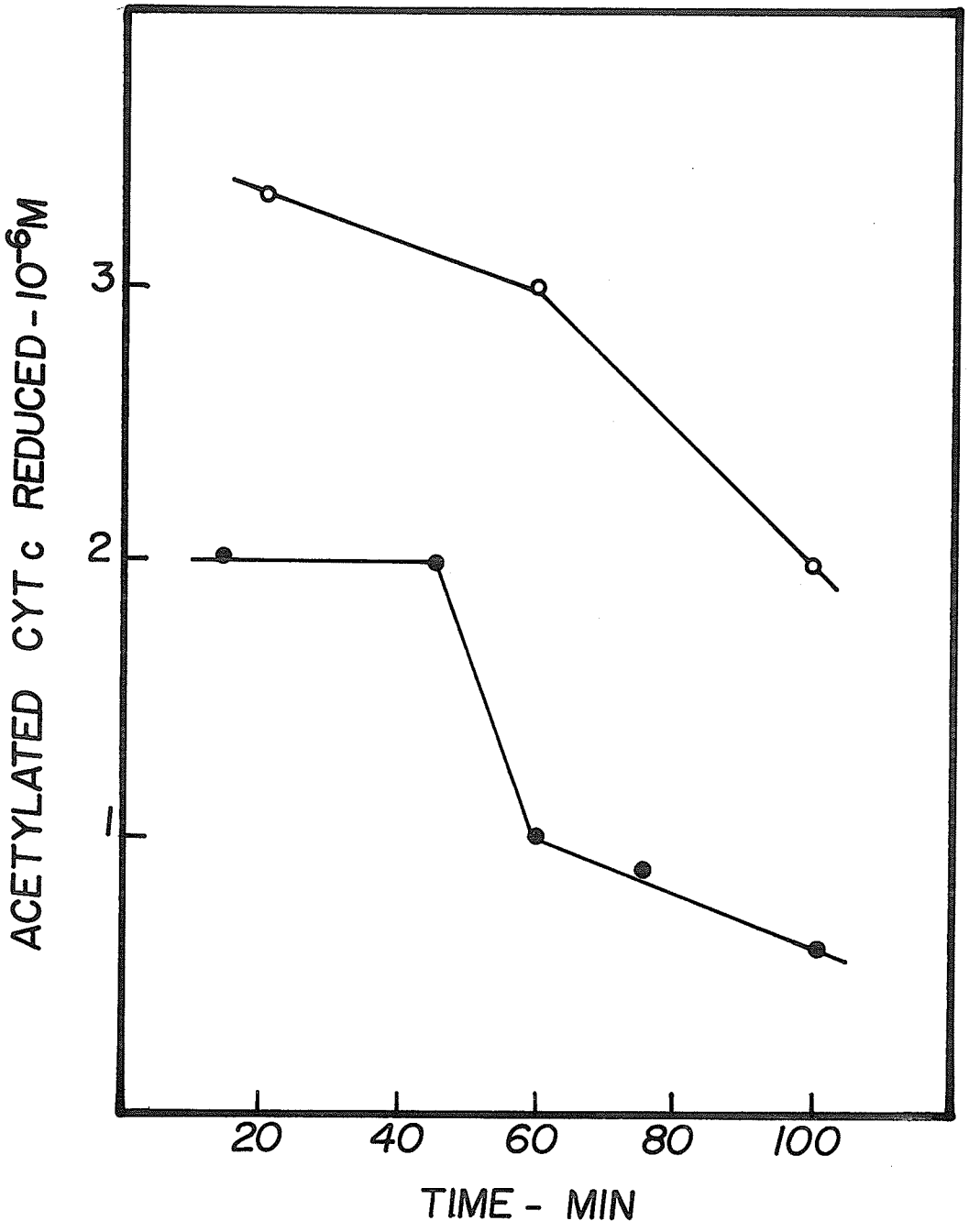
Figure 32 Production of O_2^- by A. chroococcum whole cells.

(O) - Rate of Ac.c reduction in the absence of SOD (control)

(●) - Rate of Ac.c reduction in the presence of bovine SOD. (Test)

Cell samples were taken directly from a continuous culture grown at vortex stir rate with 20% O_2. Cells (20 ml each) were centrifuged at 5000 g for 10 min at 4°C, and the resulting pellet was resuspended to 2.5 ml. in mannitol medium. 125 µM Ac.c was added to each flask. 100 µg bovine SOD was added also to the control flask. 0.25 samples were withdrawn from both the control and test flasks at the times given. Each sample was diluted to 1 ml. with glass distilled water, then centrifuged as previously. The absorbance of the supernatants at 550 mµ was noted, and the reduction of Ac.c due to O_2^- was established by comparing reduction rates in the control and test, as given in the Materials and Methods.


ACETYLATED CYT c REDUCED - 10^{-6}M
3
2
1
20
40
60
80
100
TIME - MIN

Purification and Characterisation of Superoxide Dismutase from Azotobacter Chroococcum

A. Purification

Superoxide dismutase from batch cultures of A. chroococcum was partially purified as described in the Materials and Methods. The results of the purification procedure are given in Table 11. The elution patterns from DEAE cellulose (DE32) and hydroxylapatite (Bio-gel HTP) are given in Figs. 33 and 34 respectively.

B. Characterisation

1. Types and occurrence

Two electrophoretically separable enzyme activities were obtained on polyacrylamide electrophoresis of supernatant crude extracts from continuous cultures. The two activities were not sensitive to inactivation by cyanide, or by treatment with chloroform and ethanol, a procedure which has been used to distinguish SOD types. Batch-grown cultures likewise displayed two activity bands. There was some difference noted between the extracts prepared from cells grown with 5% O_2 compared to those grown with 30% O_2, when both supernatant and resuspended residues were examined. The supernatant from cells grown with 30% O_2 showed two bands, as did the resuspended residues of the same cells. A total of three electrophoretically separable bands was evident when both supernatants and residues were examined, since the single band observed in supernatant from 5% O_2 grown cells had a similar mobility to one of the

three bands present in the residue. A similar finding was noted for cells grown at 30% O_2 (Fig. 35). The single activity in the supernatants of 5% O_2 grown cells was apparently sensitive to cyanide (Fig. 36); all three bands observed in 5% O_2 residues were not sensitive to cyanide. One of the two bands associated with the 30% o_2 supernatant was cyanide-sensitive, whereas none of the bands present in the resuspended residues were abolished by cyanide. It is apparent that at least two different SOD types - based on sensitvity to cyanide - can be found in crude extracts of A. chroococcum. Several types of SOD may be found in the same organism; E. coli was shown to contain cyanide-insensitive SOD types containing manganese or iron (Keele et al., 1970); several different enzyme types have been obtained from a variety of sources which included algae, mosses and seed plants (Asada et al., 1977). Giannopolitis and Ries (1977) have also shown that several different SOD enzymes may exist, and bovine SOD has been found to consist of 2 size isomers (Fridovich, 1975). Fe- and Mn-containing SOD types have also been found in Plectonema boryanum (Asada et al., 1975).

2. Thermal stability of SOD in Azotobacter chroococcum. Heating at 40°C and at 60°C for 10 min. did not inactivate the SOD present as verified by polyacrylamide gel electrophoresis of the heated extract. Boiling inactivated the enzyme (Fig. 37). The enzyme from A. chroococcum is therefore quite heat resistant. Similar stability at elevated temperatures has been noted for the manganese enzyme from Thermus aquaticus (Sato and Harris, 1977), and for the eucaryotic Cu-Zn enzyme (Fridovich, 1975).

Table 12 Purification of superoxide dismutase from Azotobacter chroococcum

Purification Step	Protein (mg ml^{-1})	Total Protein (mg)	Activity (units ml^{-1})	Total Activity ($x10^4$)	Specific Activity (units mg^{-1} protein)
Crude extract	19.8	8910	7625	343	385
KCl-Heat treatment	11.3	4237	6250	234	553
Streptomycin SO_4	10.7	4280	2500	100	233
50% $(NH_4)_2$ SO_4	5.0	2100	1250	52.5	250
75% $(NH_4)_2$ SO_4	16.5	495	5312	16	321
Dialysis	9.6	384	6250	25	651
DE.32 chromatography	0.19	2.1	3600	4	18,950
Bio-gel HTP chromatography	0.06	0.42	1500	1	22,060

Activity of the relevant fractions was measured according to Buchanan and Lees (1976). Other details are given in the Materials and Methods.

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Figure 33 Purification of SOD from A. chroococcum: elution pattern from DE-32 column.

(O) - Protein

(●) - SOD activity (units/ml.)

(↔) - pooled fractions

The precipitate produced by 75% $(NH_4)_2\ SO_4$ was dialysed for 60 hours against 5 mM phosphate pH 6.8, and the resulting extract was placed on a 2.5 x 25 cm. DE32 column. Enzyme fractions were eluted from the column at $4^{o}C$, with the dialysis buffer. Fractions were assayed for protein by absorbance at 280 mμ, and for SOD according to Buchanan and Lees (1976).

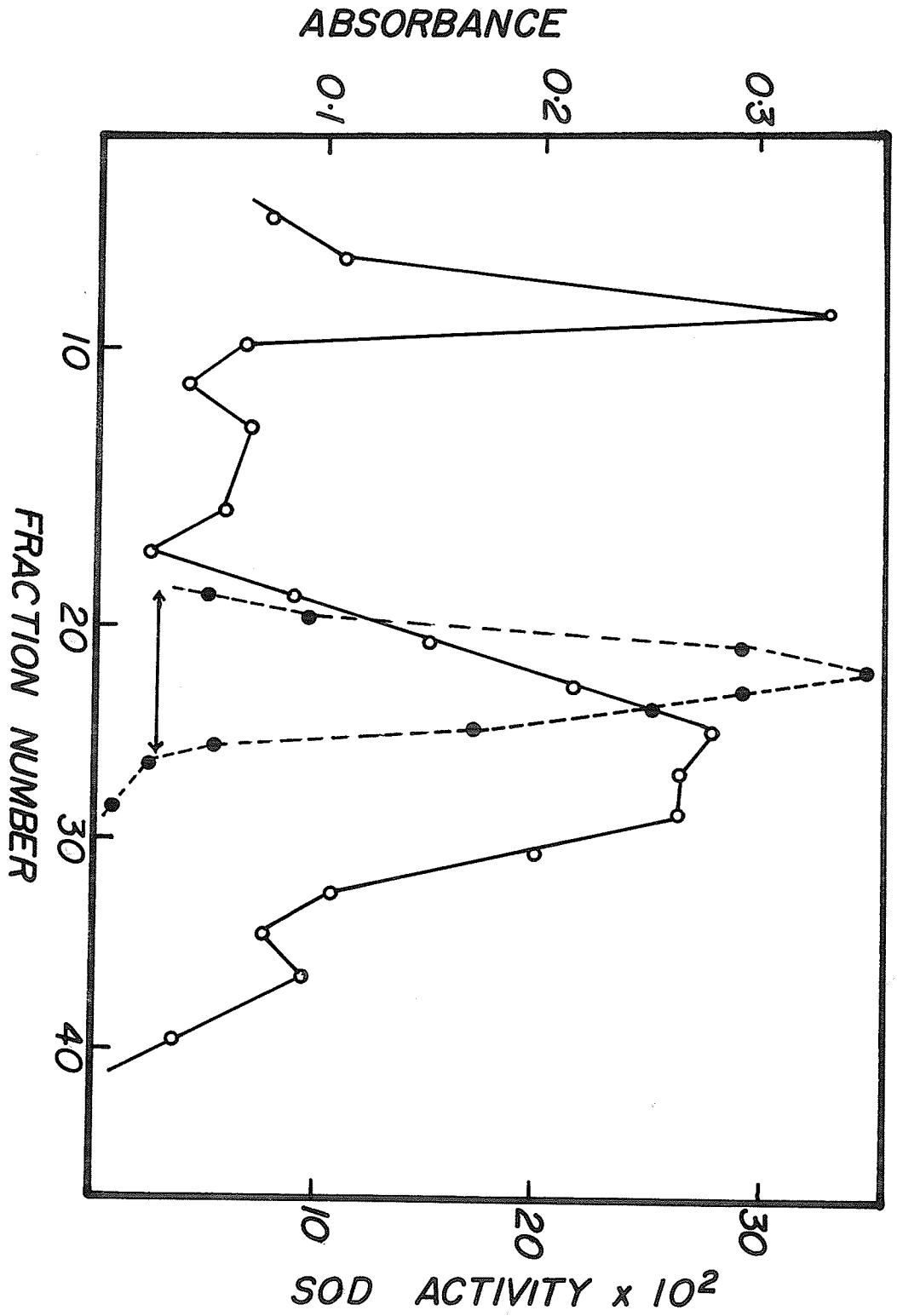
ABSORBANCE
0.1
0.2
0.3
10
20
30
40
FRACTION NUMBER
10
20
30
SOD ACTIVITY x 10²

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Figure 34 Purification of SOD from A. chroococcum: elution from Bio-gel HTP column

(O) - protein

(●) - SOD activity (units/ml)

(↔) - pooled fractions

Fractions collected from a DE32 column (Fig. 33) were pooled and concentrated to ~2 mg/ml. protein. The protein extract was added to 1.5 x 25 cm. column packed with Bio-gel HTP, and elution was effected at $4^{o}C$ with a gradient of 2-150 mM potassium phosphate, pH 6.1. The collected fractions were assayed for protein by absorbance at 280 mμ, and for SOD activity by the method of Buchanan and Lees (1976).

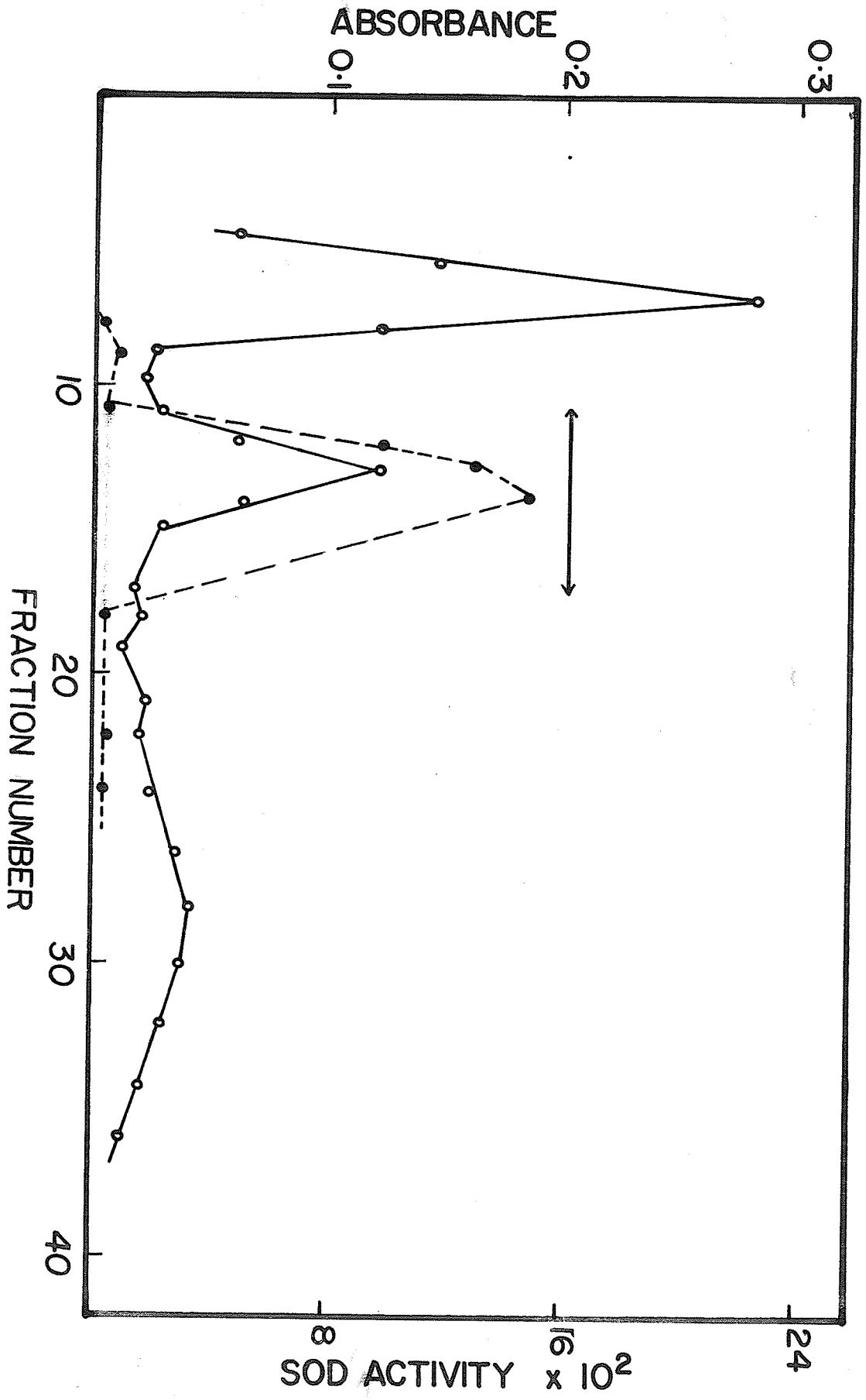
ABSORBANCE
0·1
0·2
0·3
10
20
30
40
FRACTION NUMBER
8
16
24
SOD ACTIVITY x 10²

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Figure 35 Gel electrophoresis of crude extracts of A. chroococcum showing SOD in supernatants and resuspended residues.

a - SOD activity stain

b - protein stain

1 - residue from cells grown with 30% O_2

2 - supernatant from cells grown with 30% O_2

3 - residue from cells grown with 5% O_2

4 - supernatant from cells grown with 5% O_2

Bars (-) on the gels indicate SOD bands

The cell samples were taken from continuous cultures grown with the O_2 supply stated, under N_2 fixing conditions. Electrophoresis was performed for 1½ hours at 200 volts and 3mA/gel in a water-cooled Pharmacia 2000 electrophoresis apparatus. Other details are as stated in the Materials and Methods. Differences in mobilities between samples are due to relatively larger quantities of protein in the residues compared to that present in the supernatants.

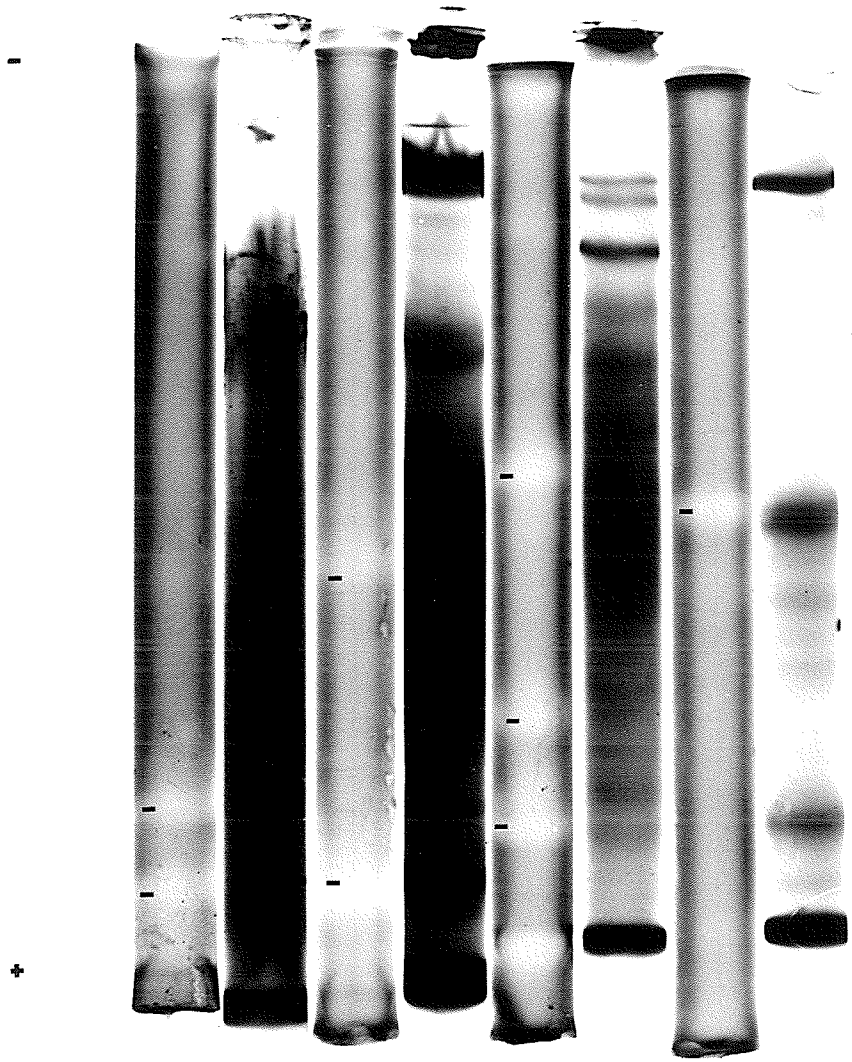
1a
b
2a
b
3a
b
4a
b

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Figure 36 Gel electrophoresis of A. chroococcum crude extracts to show sensitivity of SOD to cyanide.

1 - residue from cells grown with 30% O_2, 1 mM NaCN added to extract before electrophoresis

2 - as in 1, but with CN^- omitted

3 - supernatant from cells grown with 30% O_2, 1 mM CN^- added

4 - as in 3, CN^- omitted

5 - residue from cells grown with 5% O_2, 1 mM CN^- added

6 - as in 5, CN^- omitted

7 - supernatant from cells grown with 5% O_2, 1 mM CN^- added

8 - as in 7, CN^- omitted

Cell samples were obtained from continuous cultures grown under N_2 fixing conditions, with oxygen supplied as stated. Other details were as in Figure 35.

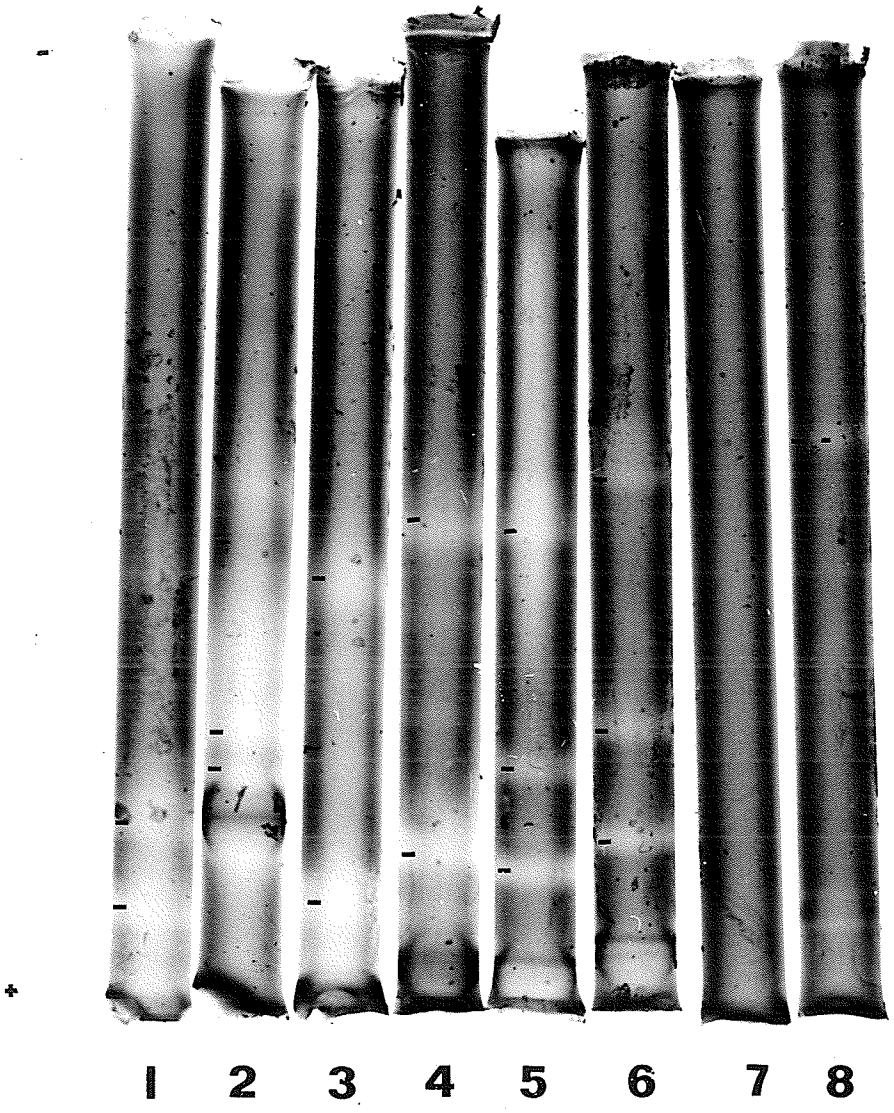
1 2 3 4 5 6 7 8

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Figure 37 Gel electrophoresis of A. chroococcum crude extract to show thermal stability of SOD present.

a - SOD activity stain

b - protein stain

The crude extract was treated as indicated;

1 - room temperature

2 - 40°C for 10 min.

3 - 60°C for 10 min.

4 - boiling H_2O bath for 10 min.

Electrophoresis was carried out at 100 V and 6 mA/Tube for 2 hours as described in the Materials and Methods, which also gives other details not here stated.


1a
b
2a
b
3a
b
4a
b

3. Molecular weight determination

The molecular weight of A. chroococcum SOD, using the standards aldolase, ovalbumin, chymotrypsinogen, and ribonuclease, was determined by two methods, namely; i) electrophoresis on polyacrylamide gradient gels (PAA 4130) prepared as described in the MATERIALS AND METHODS ii) Sephadex G-75 gel filtration.

i) Electrophoresis on PAA4130

The pattern of separation obtained by electrophoresis is given in Fig. 38. The distances moved by each protein was as expected from their molecular weights. A semilogarithmic plot of mobility vs molecular weight is given in Fig. 39. The mobility of the A. chroococcum SOD was somewhat greater than that of the bovine SOD, corresponding to a molecular weight of 31,000.

ii) Sephadex G-75

The elution profile of the protein standards from Sephadex G-75 superfine is given in Fig. 40. The elution volume of the A. chroococcum SOD, as indicated by the SOD content of the eluted fractions, is given in Fig. 41. An elution volume of 91.0 ml. was determined for the A. chroococcum SOD, corresponding to a molecular weight of 35,000 (Fig. 42).

The molecular weight of the A. chroococcum SOD was therefore estimated at 33 000 ± 2 000 from the results.

The results obtained are in the same range as has been found for SOD in other organisms, where a molecular weight of 40,000 has been determined for the manganese SOD from several bacteria (Keele et al. 1970, Vance et al. 1972, Brock et al. 1976).

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Figure 38 Slab gel electrophoresis of protein standards and partially purified A. chroococcum.

Slab gels (PAA 4/30) were prepared and supplied by Sigma, as described in the Materials and Methods.

a - aldolase

o - ovalbumin

b - bovine SOD

c - chymotrypsinogen

s - A. chroococcum SOD

The SOD activities were photochemically detected (see Materials and Methods). Fifty ug of each of the protein standards,and of A. chroococcum SOD were added to seperate wells in the gel slab. Electrophoresis was carried out at 250 volts for 2.5hrs,using the equipment described in the Materials and Methods.

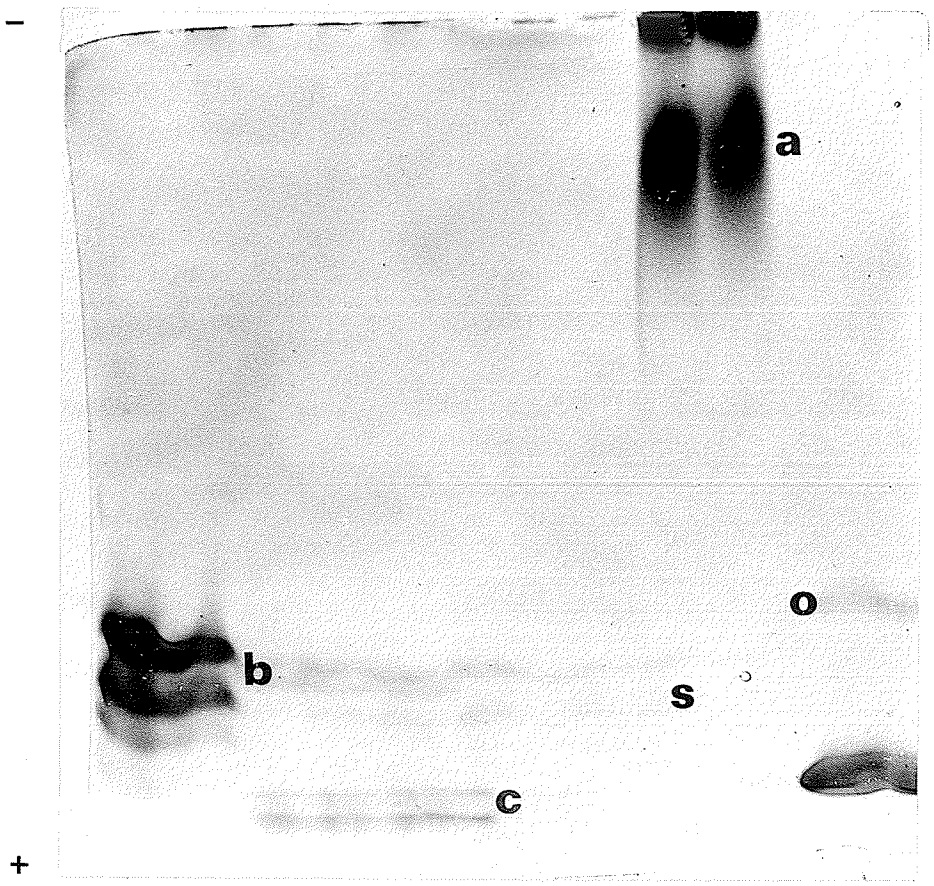
−
a
o
b
s
c
+

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Figure 39 Semilog plot of molecular weights vs mobility on PAA 4/30 slab gels. The relative mobilities of the standards, and the partially purified A. chroococcum, SOD are presented in Figure 38. Labelling as in Figure 38. The arrow indicates the mobility of the A. chroococcum SOD.

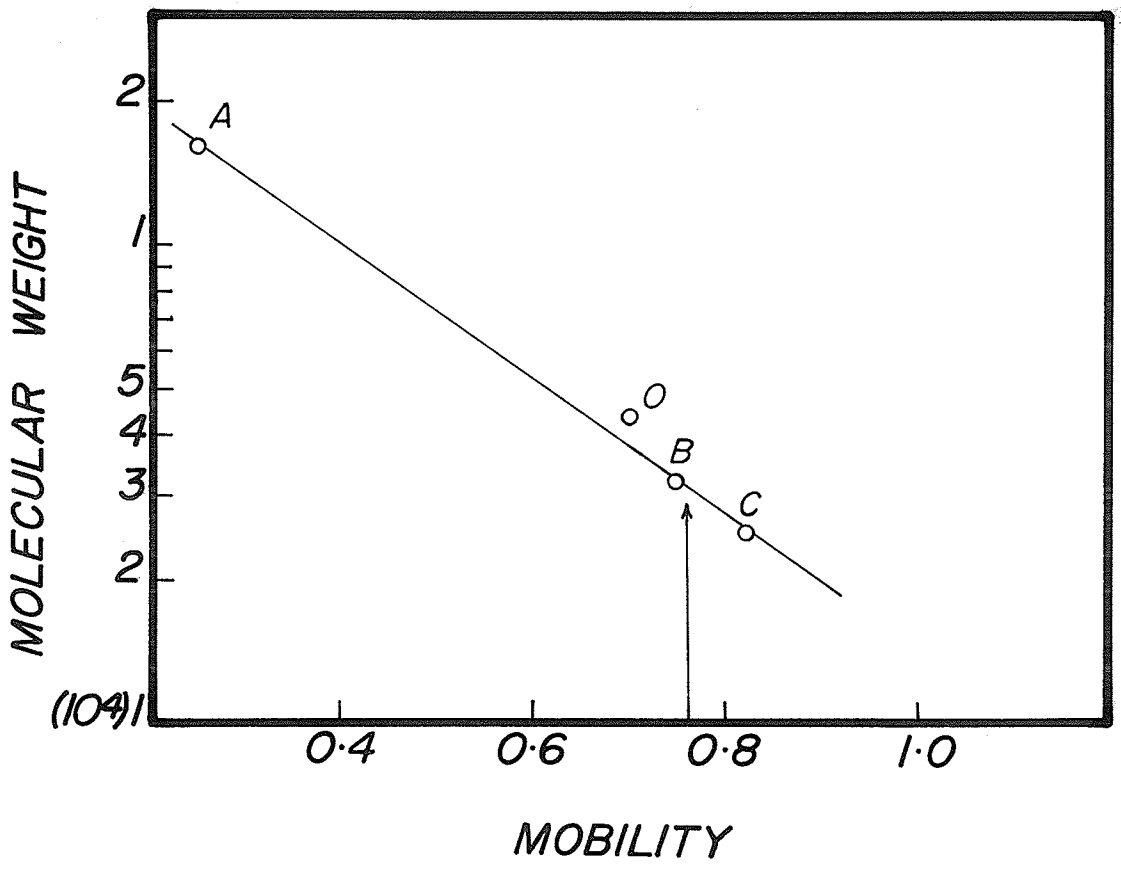
MOLECULAR WEIGHT
2
1
5
4
3
2
(10⁴)1
A
O
B
C
0·4
0·6
0·8
1·0
MOBILITY

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Figure 40 Molecular weight determination of A. chroococcum SOD: elution profile of protein standards chromatographed on Sephadex G-75 Superfine.

a - aldolase

o - ovalbumin

c - chymotrypsinogen

r - ribonuclease

1 ml. of a protein mixture containing 20 mg each of aldolase, ovalbumen, chymotrypsinogen, and ribonuclease (Pharmacia calibration kit CN 01) dissolved in 0.15M potassium phosphate pH 6.1, was added to a 2.5 x 45 cm. column. Elution was effected at 4^{o}C with phosphate as above. Protein was estimated by absorbance at 280 mμ. Bed volume was 180.0 ml, and the void volume 62.2 ml. A flow rate of ~20 c.c./hr, at a hydrostatic pressure equivalent to 50 cm., was effected during elution.

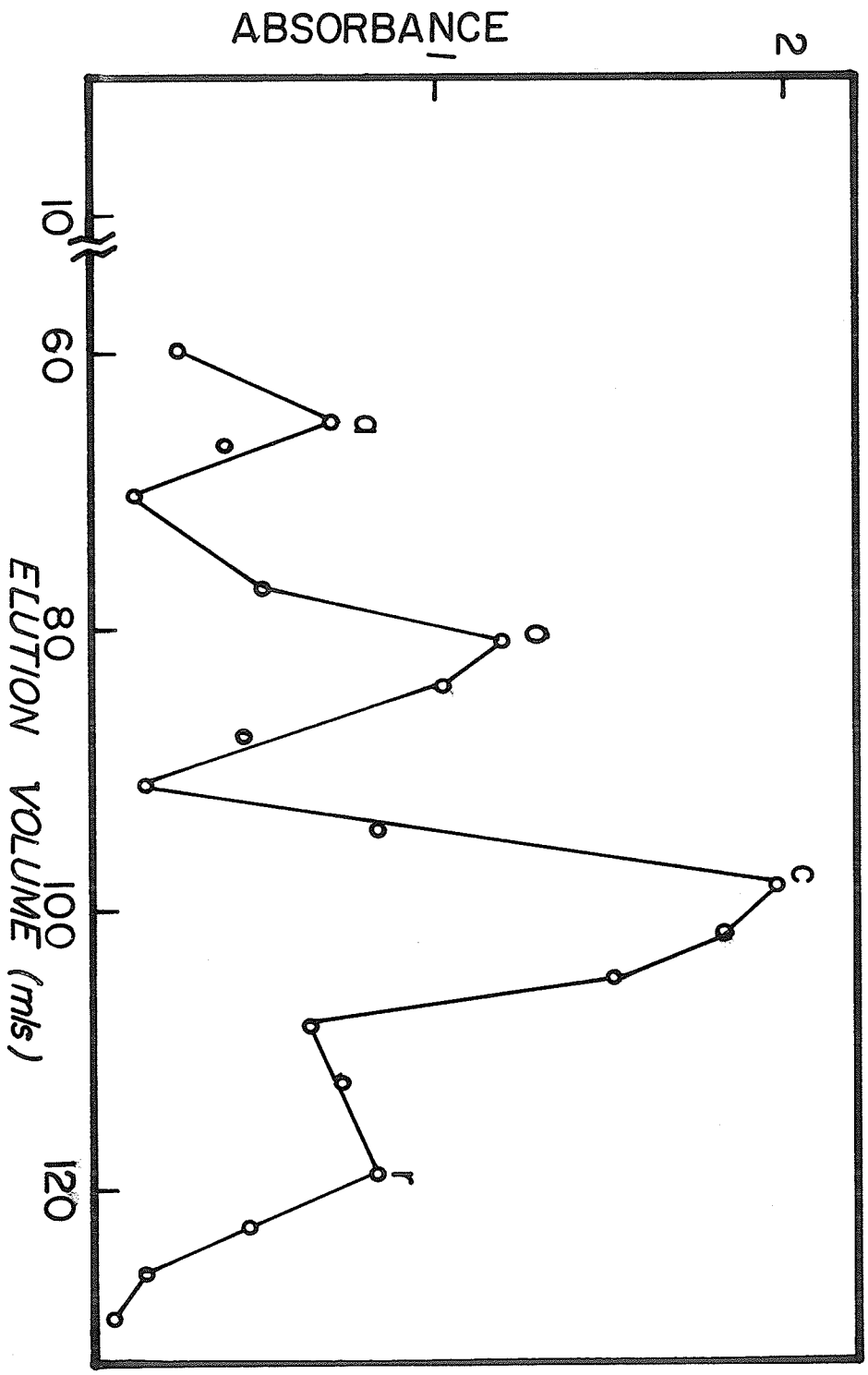
ABSORBANCE
1
2
10
60
80
100
120
ELUTION VOLUME (mls)
a
c

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Figure 41 Molecular weight determination of A. chroococcum SOD: elution profile of SOD chromatographed on Sephadex G-75 superfine.

(○) - protein (absorbance at 280 mμ)

(●) - SOD activity (units/ml., according to Buchanan and Lees 1976)

The arrow indicates the elution volume of the A. chroococcum SOD. The enzyme was eluted with 0.15 M potassium phosphate pH 6.1, at $4^{o}C$.

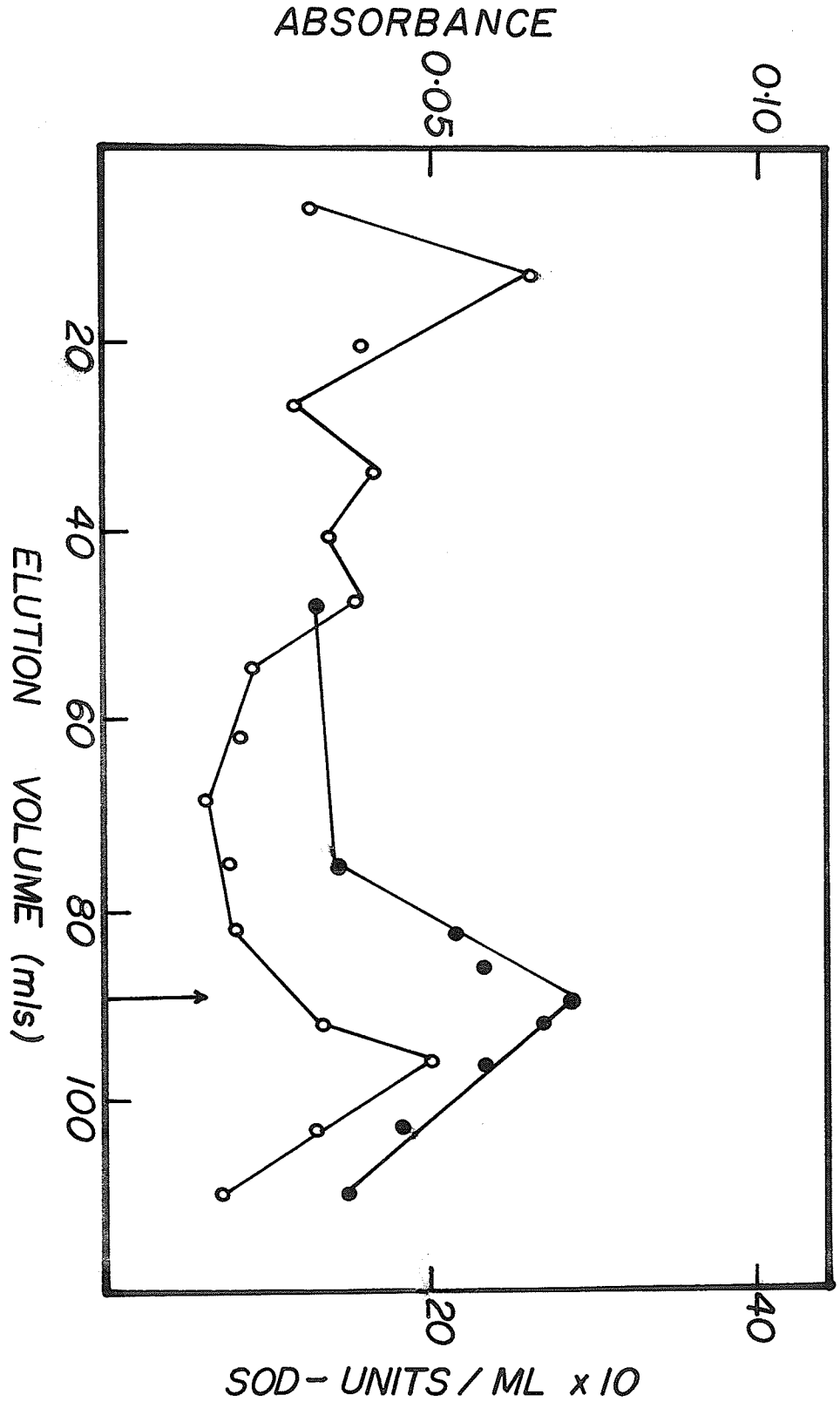
ABSORBANCE
0·05
0·10
20
40
60
80
100
ELUTION VOLUME (mls)
20
40
SOD - UNITS / ML x 10

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Figure 42 Molecular weight determination of SOD from A. chroococcum.

Selectivity curve for calibration of protein standards chromatographed on Sephadex G-75 superfine.

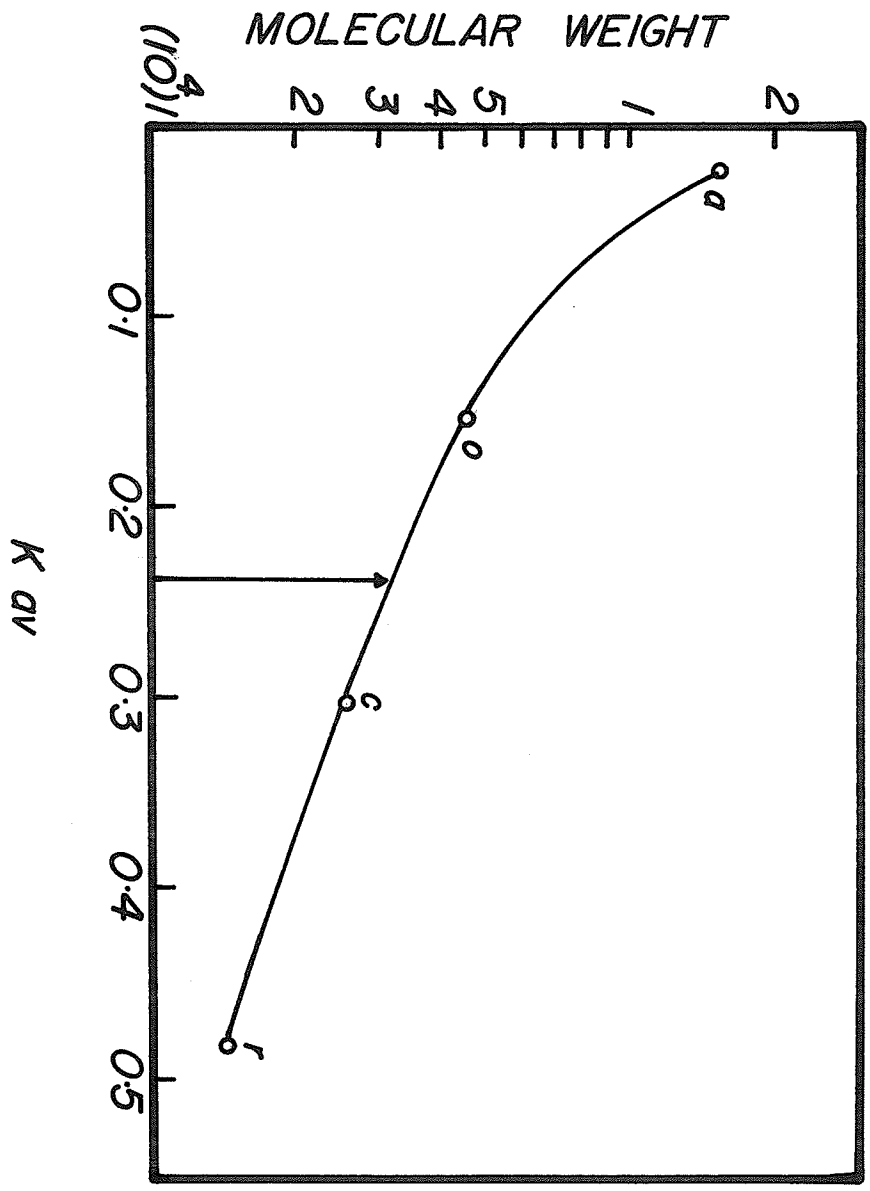
a - aldolase

o - ovalbumin

c - chymotrypsinogen

r - ribonuclease

The elution profile from the Sephadex G-75 column is given in Figures 40, 41. The arrow indicates the value obtained for the Azotobacter SOD; Kav values were obtained from Fig. 40.


MOLECULAR WEIGHT
(log)
4
2
3
4
5
1
2
0.1
0.2
0.3
0.4
0.5
K av
a
b
c
r

4. Absorption Spectrum

The wine red A. chroococcum SOD (partially purified) displayed an absorption spectrum similar to that of the Mn-containing enzyme found in other procaryotes (Vance, Keele, and Rajogopalan, 1972), as is evident from examination of the spectra (Figs. 43, 44).

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Figure 43 Characterisation of A. chroococcum SOD:
u.v. spectrum.

Details are as in Figure 44.

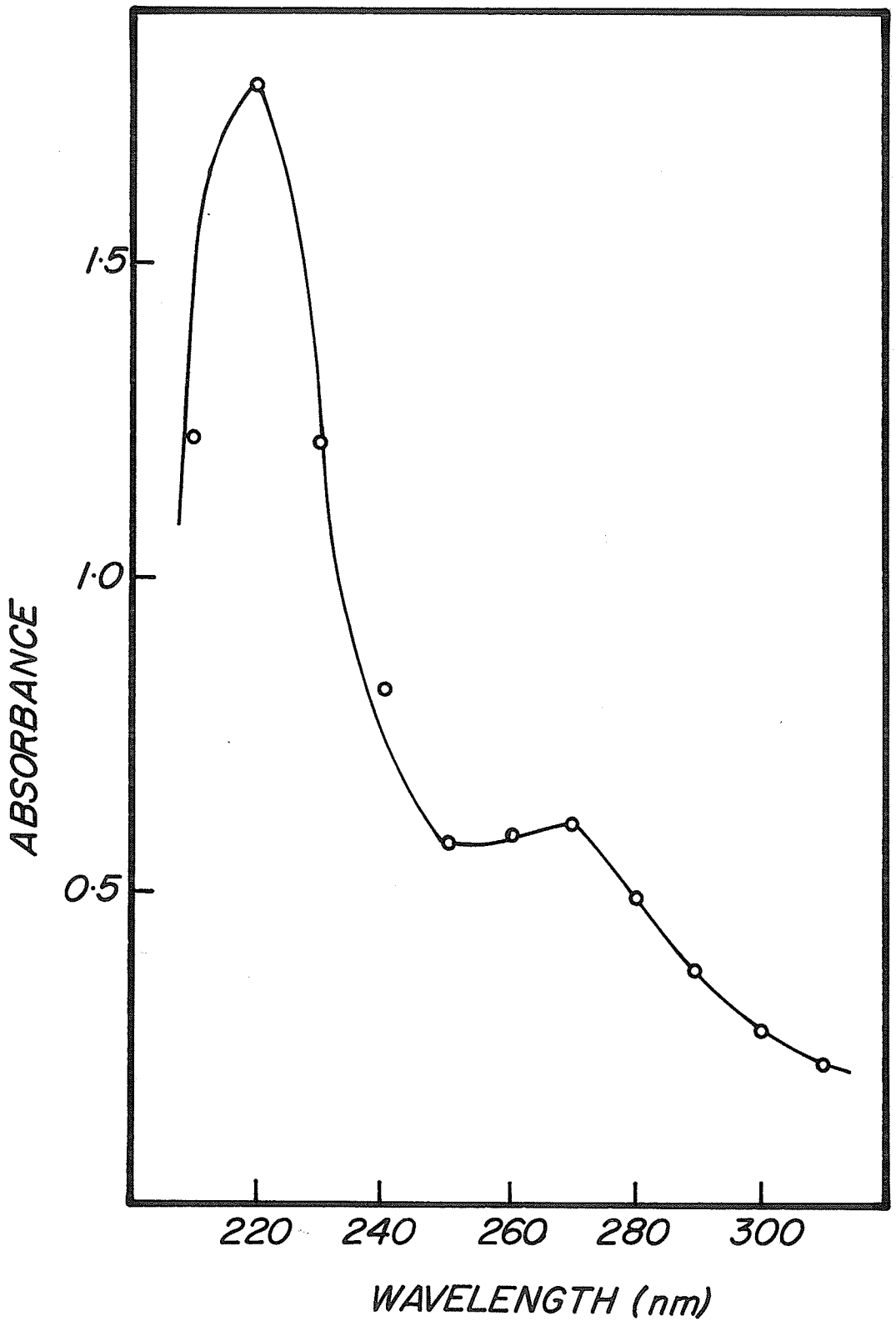
1·5
1·0
0·5
ABSORBANCE
220
240
260
280
300
WAVELENGTH (nm)

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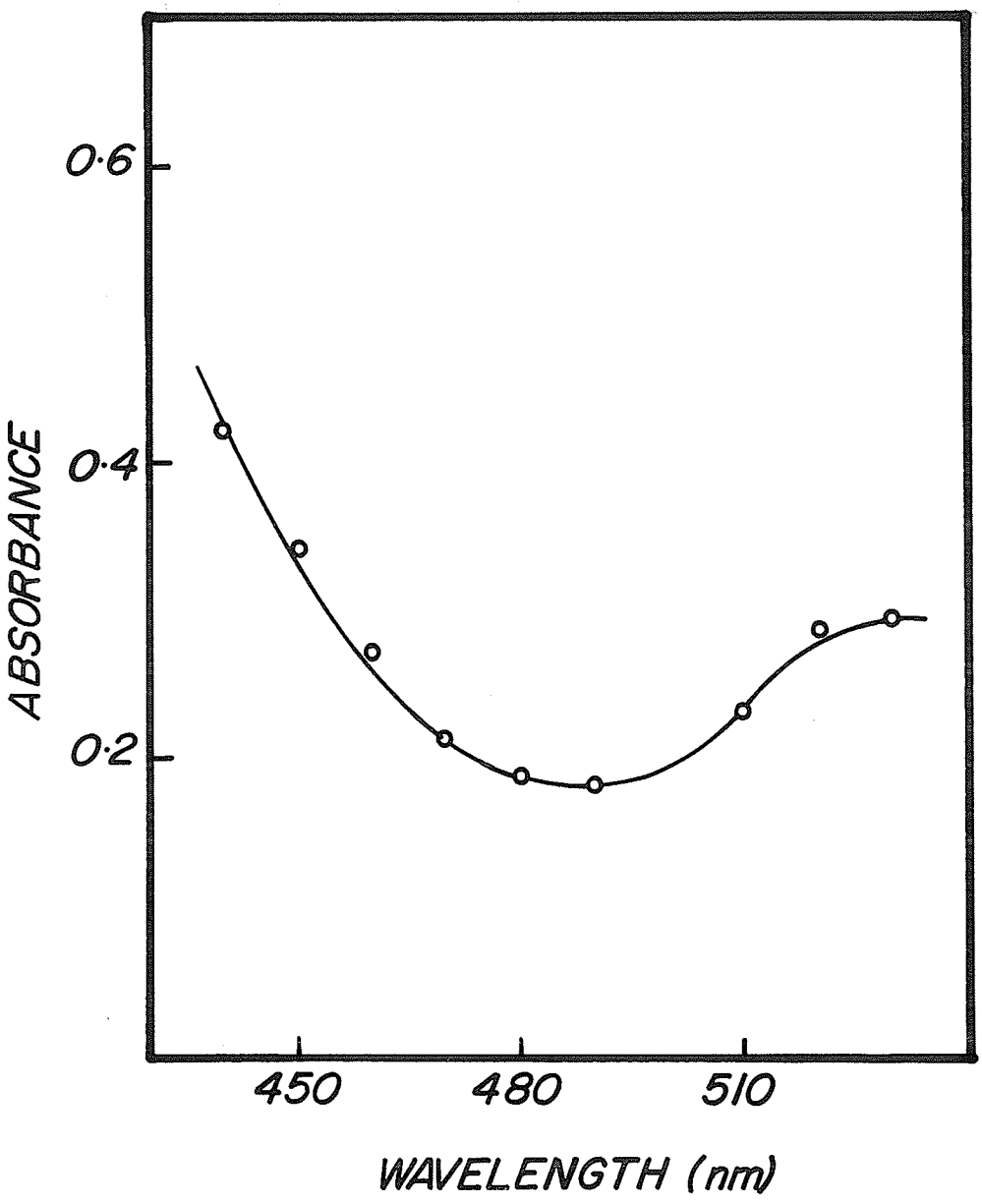
Figure 44 Characterisation of SOD from A. chroococcum: visible spectrum.

0.6 - 1 mg/ml. protein soluton was scanned as indicated using a Beckmann Acta II.


0·6
0·4
0·2
ABSORBANCE
450
480
510
WAVELENGTH (nm)

SUMMARY AND GENERAL CONCLUSIONS

SUMMARY AND GENERAL CONCLUSIONS

A.

The nitrogen fixing aerobe, Azotobacter chroococccum, was grown in continuous culture with different oxygen concentrations supplied to the organisms. The adaptive responses of the organism to oxygen were examined. The examination was restricted largely to the effects of oxygen on whole cell nitrogenase activity, and to changes in the levels of selected enzyme systems. The enzymes examined presumably played some role in the oxygen scavenging and protective mechanisms assosciated with the response of A.chroococcum to oxygen. The following enzymes were examined:-

1.Superoxide dismutase

2.Catalase

3.NADH oxidase

4.NADPH oxidase

5.Cytochrome oxidase

1.Superoxide dismutase.

A method of assay for the enzyme was developed. This method enabled the examination of crude extracts of the organism for their superoxide dismutase content. The superoxide activities did not vary greatly with the different concentrations of oxygen supplied to the cultures. Activity was evaluated on a unit dry weight basis. This result suggested that superoxide dismutase was constitutive under the conditions herein described. The daily levels of the enzyme were relatively constant.

Azotobacter chroococcum, when grown in air on agar plates, did not secrete superoxide dismutase into the growth medium. The enzyme was apparently assosciated with the cytoplasmic fraction of the cell, but some contact with the membrane was not ruled out.

Electrophoretic examination of crude extracts from the continuous cultures showed that two or three forms of the enzyme were present. One of these types was apparently sensitive to cyanide.

A partial purification of one of the superoxide dismutases present in the organism was effected. The enzyme was characterised as to its molecular weight, absorption spectra, and thermal stability. The enzyme was thermostable, and retained activity after being heated to sixty degrees for ten minutes. The spectra obtained indicated that the enzyme isolated was similar to the manganese-containing enzyme found in other bacteria. The molecular weight of the enzyme was approximately 33 000.

2.Catalase.

Catalase activity of A.chroococcum whole cells increased with increasing oxygen supply to the continuous cultures. Highest levels of activity were found in cells from cultures grown with thirty percent oxygen, and proportionally lower levels in those from twenty percent and from five percent oxygen.

The daily levels of catalase in whole cells were relatively constant. Catalase was localised to the cytoplasm by methods involving analysis of cell fractions obtained by osmotic shock of whole cells. Histochemical detection, and electron microscopic examination, indicated that the enzyme was localised to the periplasmic space, and to the membrane vesicles

assosciated with the periplasmic space. Electron microscopic examination of the cell fractions revealed that sonication of osmotic shocked cells effected fragmentation of the membrane enclosures normally present in the cell.

Catalase activity in A.chroococcum was apparently inducible by an increased supply of oxygen to the continuous cultures. This observation was presumably related to the relative significance of catalase (as compared to superoxide dismutase) in the oxygen scavenging and protective mechanisms of A.chroococcum. A peroxidase activity was not detected in the organism.

3.NADH oxidase.

Significantly larger quantities of NADH oxidase (compared to NADPH oxidase) were found in the crude extracts from the continuous cultures. Highest levels of activity were found in cells grown with thirty percent oxygen. NADH oxidase levels were higher in cells grown with five percent oxygen than in cells grown with twenty percent oxygen. The relatively higher activities at thirty percent oxygen were possibly assosciated with an uncoupling of NADH dehydrogenase activity under highly aerobic conditions, as discussed previously (Results and Discussion). NADH biosynthesis might possibly also have been stimulated by an increase in oxygen supplied to the organism.

4.NADPH oxidase.

NADPH oxidase activities were highest in extracts from cells grown with five percent oxygen. Three times as much NADPH oxidase was found in cells from thirty percent oxygen as was present in cells from twenty percent oxygen.

Much larger quantities of NADPH oxidase (compared to NADH oxidase) were found in extracts form cells grown with five percent oxygen. These differences in NADH, and NADPH, oxidase at low and high oxygen supply might reflect oxygen-prompted changes in the relative importance of the different pathways of electron transfer as influenced by a variation in the oxygen supply. The NADH-mediated pathway presumably assumes a larger share of electron transfer functions at increased levels of oxygen than does the NADPH -mediated pathway.

5.Cytochrome oxidase

Cytochrome oxidase in crude extracts varied inversely with the oxygen supplied to the continuous cultures. Crude extracts from cells grown with five percent oxygen had the highest activities, and twice as much was found in cells grown with twenty percent oxygen as was found in cells grown with thirty percent oxygen..

B.

Superoxide-mediated effects.

Much of the work desribed herein was carried out to show that the effects formerly attributed to oxygen could be mediated by the superoxide anion. The effect of exogenous O_2^- on the organism's activities was thus examined,as related to the folowing:-

1.Nitrogenase activity of whole cells

2.The effects of O_2^- on cell viability

3.Oxygen uptake in the presence of exogneous O_2^-

4.The production of O_2^- by whole cells

5.The protection conferred by exogenous superoxide dimsutase.

1. Nitrogenase activity of A. chroococcum whole cells

Exogenous O_2^- inhibited the nitrogenase activity of whole cells. The inhibition produced was directly related to the quantities of O_2^- generated, inhibition being more marked with larger quantities of O_2^- . The inhibition produced by a particular concentration of O_2^- could be lessened by increasing the cell density of the reaction mixtures. Free-radical scavengers, ethanol, and sodium benzoate,did not prevent the inhibition produced by exogenous O_2^-.

The superoxide anion had considerably less effect on the nitrogenase activity of cells which were cultured with a source of fixed nitrogen, on comparison to that produced in cells which were fixing nitrogen. Inhibition was complete in the nitrogen-fixing cells,whereas the same quantity of O_2^-caused approximately ninety percent inhibition of nitrogenase activity in cells which were supplied with ammonia. A similar response has been documented to occur with molecular oxygen,thus the superoxide anion can duplicate the effects of oxygen on the nitrogenase of Azotobacter chroococcum whole cells.

Hydrogen peroxide,and ethyl hydrogen peroxide,also inhibited the nitrogenase activity of whole cells. The inhibitions noted were not attributable to generally cytotoxic effects, as was reflected in the different viabilities of the organism subjected to the respective treatments.

2. The effect of O_2^- on cell viabilities.

Treatment of whole cells with exogenous O_2^- lowered the viability of the organism. Hydrogen peroxide,and ethyl hydrogen peroxide,were also toxic to A.chroococcum.

3. Oxygen uptake in the presence of O_2^-

Exogenous O_2^- inhibited the uptake of oxygen by whole cells from batch and continuous cultures. There was no apparent difference in the inhibitions produced as related to the oxygen supply with which the organisms were cultured. Oxygen uptake in batch-grown samples was somewhat less sensitive to O_2^- mediated inhibition than were samples from cells grown with higher concentrations of oxygen in continuous cultures.

4. The production of O_2^- by whole cells.

Significant quantities of O_2^- were produced by whole cells, as influenced by the oxygen supplied to these cells. Smaller quantities of O_2^- were produced by cells grown with lower oxygen concentrations than that produced by cells grown with higher concentrations of oxygen. The production of O_2^- by A.chroococcum whole cells was thus related to their respiratory rates,larger quantities of O_2^- being assosciated with higher respiratory rates.

5. The protection conferred by exogenous superoxide dismutase.

The addition of bovine SOD to whole cells before generating the O_2^- anion prevented the inhibitions produced by O_2^-. The inhibitions noted were thus produced or mediated by the superoxide anion, and not by other species which might have been present.

C.

The proposed model for the O_2^- mediated inhibition of nitrogenase.

Examination of the data available from other sources, and of the work herein discussed, prompted the formulation of a model for the O_2^- mediated inhibition of nitrogenase in A.chroococcum. This model was described as the "ON-SITE", "OFF-SITE" model. The nitrogenase complex was regarded as being functional when in the on-site position,and non-functional when off-site. The complex was thought to be on-site when in contact with the membrane. An increased rate of production of O_2^- in response to an increased supply of oxygen produced detachment of the complex from the membrane to give the off-site form of the enzyme. Conformational and other changes might accompany movement of the complex from the membrane. These conformational and other changes were regarded as being mediated or prompted by O_2^-,which is the signal produced in response to rising oxygen tension. It might be possible to account for the activity of nitrogenase which is not apparently assosciated with the membrane, by assuming that the nitrogenase complex can be obtained in a conformational state permitting activity. Some assosciation of membrane with this complex would be preserved,as was influenced by the method used to isolate the complex.

D.

The inlfuence of Ammonium, and of increased oxygen supply, on whole cell nitrogenase activity.

Growth on a source of fixed nitrogen resulted in an immediate inhibition of whole cell nitrogenase activity. The level of activity

subsequently increased after two hours,and the level attained by stable cultures was significantly lower than that with growth under nitrogen fixing conditions. Similar results were obtained by a sudden and marked increase in the oxygen supplied to the continuous cultures.

It therefore seems plausible that several of the responses to oxygen which were exhibited by Azotobacter chroococcum can be mediated by the superoxide anion. The oxygen protective and scavenging mechanisms displayed by the organism will therefore be those which deal with the effects produced by this reactive specie. The fact that A.chroococcum is still sensitive to the effects of excess oxygen implies that even though these defense mechanisms are invoked in response to oxygen, that a finite limit is imposed. The protective mechanisms are therefore not adequate to confer protection when the organism is subjected to oxygen stress. These mechanisms are apparently adequate under conditions which are assosciated with normal growth,when oxygen stress is not expected. The explanations herein presented will hopefully prompt an examination of related phenonema. More cogent support would be provided by the demonstration of similar effects of O_2^- on other aspects of Azotobacter physiology and metabolism.

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LITERATURE CITED

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