SUPEROXIDE DISMUTASE, CATALASE, AND PEROXIDASE IN AMMONIUM-GROWN AND NITROGEN-FIXING *AZOSPIRILLUM BRASILENSE*

by

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Short Title

O_2-PROTECTING ENZYMES IN AZOSPIRILLUM

Richard W. Clara

ABSTRACT

M.Sc. Richard W. Clara Microbiology

SUPEROXIDE DISMUTASE, CATALASE, AND PEROXIDASE IN AMMONIUM-GROWN AND NITROGEN-FIXING AZOSPIRILLUM BRASILENSE

Superoxide dismutase (SOD), catalase (CAT), and peroxidase (PER) activities were studied in N_2-fixing and ammonium-grown batch cultures of *Azospirillum brasilense* sp7. PER activity, as measured using o-dianisidine or 3,3'-diaminobenzidine as the H-donor, was not significant in most growth conditions. SOD and CAT activities in cells grown aerobically with ammonium were comparable to those in aerobically-grown *E. coli* K12. Enzyme activities in *A. brasilense* so grown varied with the age of the culture, an effect that could not be attributed to changes in dissolved oxygen content. SOD activity was present in cells grown anaerobically with ammonium, but was higher in cells grown in the presence of oxygen and increased in response to higher oxygen tension. CAT activity was significantly higher in cells grown at lower oxygen tensions. SOD, but not CAT activity, increased in response to growth at oxygen levels higher than ambient. In N_2-fixing cells, SOD activity was slightly higher and CAT activity significantly lower than in cells grown similarly but with NH_4Cl as a source of fixed nitrogen. Cells grown anaerobically using NO_3^- as the terminal electron acceptor contained SOD and high CAT activity. Cells grown similarly but with N_2O as the electron acceptor, had significantly lower CAT activity but slightly higher SOD activity Polyacrylamide gel electrophoresis of the cell-free extracts revealed only one band of SOD activity under each physiological condition compared to three for aerobically-grown *E. coli* K12. This band proved to be iron-containing SOD (FeSOD) on the basis of inhibitor sensitivity.

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RESUME

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SUPEROXYDE DISMUTASE, CATALASE, ET PEROXYDASE CHEZ AZOSPIRILLUM BRASILENSE, BACTERIE UTILISANT L'AMMONIUM ET FIXANT L'AZOTE.

Les enzymes superoxyde dismutase (SOD), catalase (CAT) et peroxydase (PER) ont été étudiées chez des cultures d'*Azospirillum brasilense* sp 7 fixant d'azote et utilisant l'ammonium. Dans la plupart des conditions de culture utilisées, l'activité de la PER, telle que mesurée par le donneur d'hydrogène o-dianisidine ou le 3,3'-diaminobenzidine, n'était pas très importante. Chez les cellules cultivées en aérobiose avec ammonium, les activités de la SOD et de la CAT étaient très comparables à celles de *E. coli* K12 poussée dans des conditions similaires. Aussi chez les cellules cultivées de la même manière, les activitées enzymatiques variaient avec l'âge de la culture, un effet qui n'a pu être attribué aux changements en oxygène dissous. Chez les cellules cultivées en anaérobiose, l'activité de la SOD detectée était moindre que chez les cellules cultivées en présence d'oxygène et augmentait avec la tension d'oxygène. L'activité de la CAT était beaucoup plus élevée chez les cellules cultivées à basses tensions d'oxygène. La croissance à des concentrations d'oxygène supérieures à la concentration ambiante permettait une augmentation de l'activité de la SOD, mais non celle de la CAT. Chez les cellules fixant l'azote, l'activité de la SOD était légèrement plus élevée et l'activité de la CAT significativement plus basse que chez des cellules cultivées dans des conditions similaires mais utilisant le NH_4Cl comme source d'azote. Les cellules cultivées en

anaérobiose, utilisant le NO_3^- comme accepteur final d'électrons, contenaient la SOD et la CAT en quantité importante. Des cellules cultivées dans des conditions semblables mais utilisant le N_2O comme accepteur d'electrons possédaient une activité de CAT définitivement plus basse bien que leur activité en SOD était légèrement supérieure. L'electrophorèse des extraits cellulaires sur gel de polyacrylamide n'a révélé qu'une seule bande liee à l'activité enzymatique de la SOD comparativement à 3 bandes chez *E. coli* K12 poussée en aérobiose et ce pour toutes les conditions experimentales. Cette même bande s'est révélée être une SOD contenant du fer (FeSOD) tel que détecté par sa sensibilité aux inhibiteurs.

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INTRODUCTION

Nitrogen is an essential element for the growth of plants and is often the limiting factor in agriculture (Van Berkum and Bohlool, 1980). As heavier demands are placed on our limited agricultural resources by an increasing world population, the supplementation of natural fixed nitrogen sources by fertilizer nitrogen is necessary to maintain adequate production levels. Unfortunately, escalating production costs and uncertain availability of raw material have led to the realization that this practise may be untenable in the long term, and interest has now turned to replacing fertilizer nitrogen with biological sources.

A major physiological problem in biological nitrogen fixation is the oxygen sensitivity of the process. Although this problem has been much studied, it remains poorly understood and in fact, recent advances in our understanding of oxygen toxicity and protection have yet to be applied to this problem. This work studies the occurrence and certain physical characteristics of the oxygen-protecting enzymes of the free-living diazotroph *Azospirillum brasilense* sp7, an organism that has shown much promise as a means of providing fixed nitrogen to plants. It is hoped that such information, especially in view of the different physiological conditions under which this organism can grow, will provide some insight into the value of these enzymes to the organism in general and to nitrogen fixation in particular.

1. LITERATURE REVIEW

Oxygen holds a unique place in the evolutionary history of life on our planet, important in both its presence and absence. It was in its absence that organic matter developed and was able to coalesce into a primitive form of life, but it is now its presence that is essential in the processes of life that evolved from that point. The importance of oxygen to the maintenance of life is balanced by the fact that oxygen in levels only slightly higher than atmospheric is toxic. Thus, important as the utilization of oxygen is, protection against its toxic effects remains a critical factor.

The composition of the primitive atmosphere on earth is a contentious point among scientists studying evolution. There is a general agreement that this atmosphere was reducing in nature, since the abiotic synthesis of compounds of biological interest and their accumulation for development into more complex molecules does not occur under oxidizing conditions (Cloud, 1974; Miller and Urey, 1959; Schopf, 1975). In the absence of oxygen and lacking an adequate screen for ultraviolet rays, evolution of life could only take place in shielded areas (eg. ten meters below the surface of the ocean) where conditions would be suitable only for the development of anaerobic prokaryotes and bacterial photosynthesis (Schwartz and Dayhoff, 1978). The advent of oxygen-evolving photosynthesis by cyanobacteria (blue-green algae) approximately 3×10^9 years ago (Schopf and Barghoovn, 1967) marked a turning point in evolution. The development of an effective UV screen (ozone) and the use of respiration (as opposed to fermentation) for the production of energy supported the development of larger, more complex organisms. However, quite apart from the benefits that oxygen provides is the fact that certain biological and non-biological reactions in its presence lead to the formation of highly reactive oxygen species that are potentially toxic to cells. Thus,

the presence of oxygen necessitated the concurrent development of oxygen protection mechanisms to alleviate its toxicity (Gilbert, 1965), a key development since failure to do so would restrict the organisms to anaerobic niches in an aerobic environment.

This sequence of development has been challenged by others who question the timing of key events (Schopf, 1975) or support a greater role for oxygen produced by the photodissociation of water (Carver, 1981; Towe, 1978). It is difficult to determine with any degree of certainty the actual events from a vantage point removed by 3-4 x 10^9 years but a common theme in these theories is the key importance of oxygen in the evolution of life.

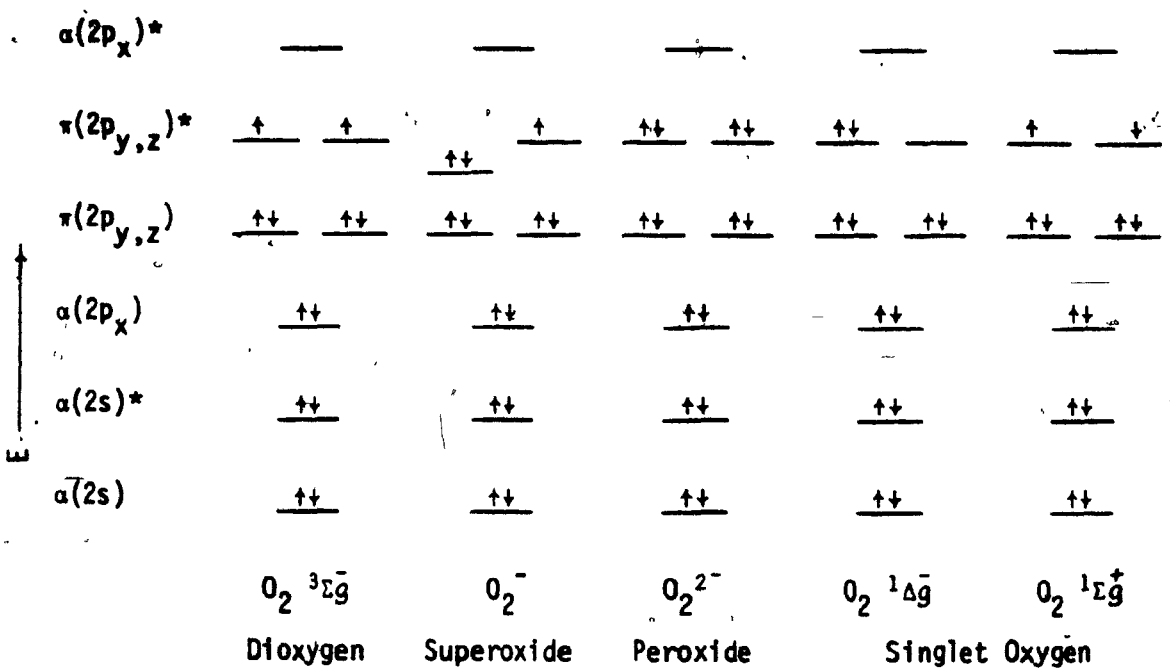
1.1 Oxygen

1.1.1 Oxygen and Related Species

The oxygen atom has eight protons with six valence electrons in its outer shell. The combination of two oxygen atoms gives rise to dioxygen (often referred to as molecular oxygen) with the electronic configuration shown in Fig. 1 (Morris, 1975). It has a triplet ground state with two unpaired electrons in the two degenerate π^* anti-bonding orbitals (Jones et al., 1979; Valentine, 1973). The presence of unpaired electrons is the source of the paramagnetism of the oxygen molecule and a barrier to reaction via a spin restriction. For an incoming pair of electrons to add to the oxygen molecule, a spin change according to the Pauli principle is required which, although possible, is a slow process (10^{-7} s) compared to the lifetime of the collisional complex (10^{-12} s) (Taube, 1965). Reactions with dioxygen must therefore avoid the spin restriction either by first combining with a transition metal (which also has unpaired electrons) or by adding electrons univalently rather than in pairs (Fridovich, 1977; Malmstrom, 1982). The latter process gives rise to active oxygen species which are

Figure 1. Schematic molecular orbital diagram for dioxygen and related species.

Orbitals indicated by horizontal lines (* indicates anti-bonding orbitals) with the vertical position corresponding to their energy level. Individual electrons are represented by arrows pointing up or down to indicate spin. Electrons are paired when two electrons of opposite spin occupy one orbital. Taken from Hill (1978) and Morris (1975).


σ(2p_x)*
π(2p_y,z)*
π(2p_y,z)
σ(2p_x)
σ(2s)*
σ(2s)
E
O2 3Σg−
O2−
O2 2−
O2 1Δg−
O2 1Σg+
Dioxygen
Superoxide
Peroxide
Singlet Oxygen

potentially toxic to the cell (Fridovich, 1975a; 1976; 1977; 1978).

In spite of its lack of reactivity, oxygen is a key molecule to biochemical processes in the cell. It is a powerful oxidizing agent (E'_o = +0.82 V) and is used in enzymatic oxidation processes, most notably as the terminal electron acceptor in respiration via cytochrome oxidase (Haddock and Jones, 1977; Wilshire and Sawyer, 1979). It is involved in biosynthetic reactions catalyzed by monooxygenases and dioxygenases (Malmström, 1982) and in other reactions such as the production of light catalyzed by luciferase in photoluminescent bacteria. It is also responsible for the regulation of synthesis of many proteins in the cell, especially those enzymes involved in aerobic and anaerobic growth.

Singlet Oxygen

Molecular oxygen is the low energy form of oxygen while the excited state (singlet oxygen) results when the ground state is energized (Fig. 1) (Hill, 1978). The outer shell electrons are of opposite spin (paired) in this case and the resulting orbital configuration determines the excitation energy. The $^1\Delta\bar{g}$ form is 23.4 kcal per mole above the ground state and has its electrons in the same orbital while the electrons in the $^1\Sigma\bar{g}^+$ form are in separate orbitals resulting in a higher energy state (37.5 kcal per mole). Generation of singlet oxygen generally involves dye-sensitized photooxidations (Fig. 2) (Foote, 1976; Krinsky, 1979) and is often the species implicated in light-dependent cellular damage.

The singlet oxygen molecule is highly unstable and has a tendency to release energy by direct relaxation to ground state (triplet) oxygen with the emission of light (fluorescence and/or chemiluminescence) (Morris, 1975). This reaction is quite rapid for the $^1\Sigma\bar{g}^+$ form (10^{-11} s, Foote, 1976) while the $^1\Delta\bar{g}$ form is longer lived and hence potentially more dangerous in biological

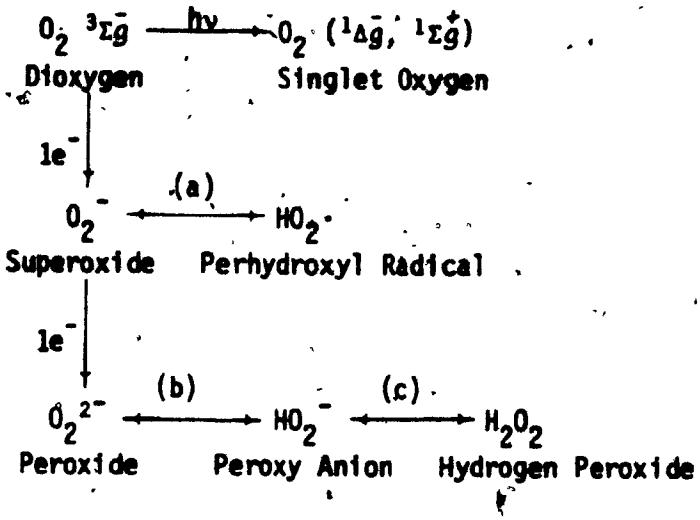
Figure 2. Redox and acid/base characteristics between dioxygen and related species.

Changes in electron content indicated vertically while that for proton content indicated horizontally (except for singlet oxygen formation). Taken from Fee and Valentine (1977) and Hill (1978).

(a) pK_a 4.8

(b) pK_a >14

(c) pK_a 11.8


$O_2\ ^3\Sigma_g^-$
hν
$O_2\ (^1\Delta_g,\ ^1\Sigma_g^+)$
Dioxygen
Singlet Oxygen
$1e^-$
(a)
O_2^-
$HO_2\cdot$
Superoxide
Perhydroxyl Radical
$1e^-$
(b)
(c)
O_2^{2-}
HO_2^-
H_2O_2
Peroxide
Peroxy Anion
Hydrogen Peroxide

systems. Since the spin restriction is no longer a barrier to reactivity (Fridovich, 1979), singlet oxygen is particularly toxic to cells but its biological significance is far from clear.

There are reports, based on luminescence studies, of the involvement of singlet oxygen in prostaglandin biosynthesis (Panganamala et al., 1976), microsomal lipid oxidase reactions (Chen and Tu, 1976) and xanthine oxidase reactions (Pederson and Aust, 1973) while others maintain that the toxicity of free singlet oxygen makes it an unlikely intermediate in biological reactions (Foote, 1976). The production of singlet oxygen by the spontaneous dismutation of superoxide (Bus et al., 1974; Khan, 1970; 1981) and its activity as a bactericidal agent produced by polymorphonuclear leukocytes (Allen et al., 1972; Rosen and Klebanoff, 1977) has also been questioned (Foote, 1979; Foote et al., 1980; Harrison et al., 1978). Singlet oxygen generation in the base-catalyzed disproportionation of H_2O_2 has also been demonstrated in vitro (Smith and Kulig, 1975), but its intracellular significance is unclear.

Superoxide

On the addition of electrons univalently to dioxygen, the first product is the superoxide anion (O_2^-, Fig. 2). The conjugate acid, the perhydroxyl radical ($HO_2\cdot$), has a pK_a of 4.8, hence the unprotonated form dominates at physiological pH. It retains one unpaired electron (Fig. 1) and is therefore paramagnetic (Fee and Valentine, 1977; Sawyer and Valentine, 1981).

The chemistry of superoxide has been studied and reviewed extensively in recent years in the light of its suggested involvement in oxygen toxicity (Fee and Valentine, 1977; Sawyer et al., 1980; Sawyer and Valentine, 1981; Singh, 1982). In aqueous media, the principal reaction is that of spontaneous dismutation (Bielski and Allen, 1977; Hill, 1978):

$$HO_2\cdot + HO_2\cdot \rightarrow H_2O_2 + O_2 \quad K_1 = 7.6 \times 10^5 \ Mol^{-1}s^{-1}$$

$$HO_2\cdot + O_2^{-} \rightarrow HO_2^{-} + O_2 \quad K_2 = 8.9 \times 10^7 \ Mol^{-1}s^{-1}$$

$$O_2^{-} + O_2^{-} \rightarrow O_2^{2-} + O_2 \quad K_3 = <0.3 \ Mol^{-1}s^{-1}$$

Though the rate is dependent on pH and is fastest when $pH=pK_a$, it is very rapid at physiological pH (Sawyer et al., 1980) and is complete even at pH 14 (Sawyer et al., 1978). In its ability to abstract protons for the dismutation from solvent or weakly acidic organic molecules, superoxide demonstrates the properties of a strong Brönsted base (Nanni et al., 1980). As well, in aqueous solution, superoxide can act as a moderate one-electron reductant to organic molecules such as tetranitromethane (Rabani et al., 1965), cytochrome c (McCord and Fridovich, 1968) and others (Hill, 1978) or to transition metal complexes (Halliwell, 1975; Singh, 1982). Though it does show the ability to oxidize certain compounds (Lee-Ruff, 1977; Misra and Fridovich, 1972b) its ability is thought to be weak at best (Sawyer et al., 1978) and may in fact be mediated by the dismutation products rather than O_2^{-} itself.

In aprotic media, the superoxide anion is a stable species and quite amenable to chemical analysis. It proves to be a powerful nucleophile, attacking alkyl halides with results consistent with an S_N2 mechanism (San Filippo et al., 1975). Such is not evident in aqueous solution due to the dominence of the dismutation reaction and strong solvation effects (Sawyer and Valentine, 1981; Valentine, 1979).

The occurrence of superoxide in biological systems can be a required characteristic of an enzyme or an inadvertant consequence of normal cell processes. Many of the reduced forms of cell proteins including hydroquinones (Misra and Fridovich, 1972a), reduced flavins (Ballou et al., 1969; Massey et al., 1969), thiols (Misra, 1974), ferredoxin (Misra and

Fridovich, 1971; Orme-Johnson and Beinert, 1969) and hemoglobin (Rotilio et al., 1977) will autooxidize and release superoxide. Some enzymes such as xanthine oxidase (Fridovich, 1970; Knowles et al., 1969) and aldehyde oxidase (Rajagopalan et al., 1962) will release superoxide as part of their reaction mechanism while others such as galactose oxidase (Hamilton et al., 1973) and tryptophan dioxygenase (Hirata and Hayaishi, 1971) generate it at some intermediate step in the reaction. Other intracellular sources include subcellular organelles such as chloroplasts (Asada and Kiso, 1973) and mitochondria (Cadenas et al., 1977; Loschen et al., 1973; Nohl and Hegner, 1978).

Peroxide

Peroxide (O_2^{2-}) is produced by the addition of two electrons to ground state dioxygen (Fig. 2) or via the dismutation of superoxide, and is the most stable (i.e. least reactive) of the intermediates of oxygen reduction. The protonated forms are the hydroperoxy anion (HO_2^-) and hydrogen peroxide (H_2O_2) and at physiological pH the fully protonated form predominates due to the high pK_a's (Fee and Valentine, 1977).

Hydrogen peroxide is a good oxidizing agent toward organic molecules, especially sulfhydryl compounds (Little and O'Brien, 1967; Sanner and Pihl, 1963) but these reactions are rather slow unless the concentration of H_2O_2 is high. Another important reaction is the base-catalyzed dismutation of H_2O_2 to O_2 and H_2O (Duke and Haas, 1961) which can lead to singlet oxygen (Smith and Kalig, 1975) or radical formation (O_2^- and OH·) in aprotic regions (e.g. hydrophobic areas of membranes) (Wilshire and Sawyer, 1979). A second reaction leading to the generation of radicals is the one-electron reduction catalyzed by transition metals known as the Fenton reaction (Walling, 1975):

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

This reaction generates the hydroxyl radical (OH·), a powerful oxidizing species

that forms the basis of the current theory of oxygen toxicity (below).

A number of oxidases are capable of the divalent reduction of dioxygen including d-amino acid oxidase, diamine oxidase and glycollate oxidase (Rotilio et al., 1973) while other enzymes such as xanthine oxidase and aldehyde oxidase will reduce O_2^- univalently and the resulting dismutation (with or without catalysis) will result in H_2O_2 production. Many eukaryotic sources of H_2O_2 are localized in peroxisomes (Masters and Holmes, 1977) as well as other subcellular organelles such as mitochondria (Boveris et al., 1976; Halliwell, 1971; Turrens et al., 1982), microsomes (Thurman et al., 1972) and illuminated chloroplasts via the well-known Mehler reaction (Mehler, 1951; Robinson and Gibbs, 1982).

1.1.2 Oxygen Toxicity

The toxicity of oxygen has been evident since the element was discovered and was first physiologically characterized by Paul Bert in 1865 (Haugaard, 1968). The advent of underwater diving, high altitude aircraft and, more recently, disease treatment using high pressure oxygen has spurred interest into its nature and extent. Characterization of oxygen toxicity in microorganisms and animals both in vivo and in vitro (Gottlieb, 1971; Haugaard, 1968) and its involvement in seemingly unrelated processes such as radiation damage and lyophilization (Swartz, 1973) has led to a number of theories as to its mechanism.

Hydrogen Peroxide

McLeod and Gordon (1923) put forth one of the earliest theories of oxygen toxicity with particular reference to anaerobes. They interpreted the inability of some clostridia to produce heme groups (an essential component for the production of H_2O_2-decomposing enzymes) as an indication that the toxic effects of oxygen were due to a build up of H_2O_2. To this end it

is possible to demonstrate that H_2O_2 is produced in cell-free extracts and, at high concentration, will kill most cells. However, H_2O_2 is not thought to be particularly toxic (Chance et al., 1979; Halliwell, 1974) and in fact, most organisms can tolerate low levels of H_2O_2 and some can even excrete it into the medium (Costilow and Keele, 1972). Intracellular concentrations have been measured at 10^{-9}-10^{-7} M, maintained at that level by the action of H_2O_2-scavenging enzymes. There are also examples of obligate anaerobes containing catalase (Sherman, 1926) and acatalasic aerobes (McCord et al., 1971) which argue against the importance of H_2O_2 in oxygen toxicity.

A complicating factor, however, is the reaction of H_2O_2 with transition metals (Fenton's reagent). Hydroxyl radicals produced by this reaction are highly and indiscriminately reactive, which would tend to mask the lack of reactivity of H_2O_2 itself and account for any toxic action.

-SH Oxidation

Another early proposal to explain oxygen toxicity was the inactivation of some enzymes by oxygen, especially those containing sulfhydryl groups (Barron, 1955; Dickens, 1946; Haugaard, 1946). The activity of certain key enzymes (e.g. glyceraldehyde-3-phosphate dehydrogenase) or their cofactors is maintained only when the essential thiol groups are in a reduced state and oxidation leads to irreversible inactivation (Little and O'Brien, 1969). This process is particularly applicable to anaerobic organisms but is evident in some aerobes as well (Haugaard, 1968). However, the importance of this phenomenon as a primary agent of oxygen toxicity is unclear since in at least one case (Stees and Brown, 1973), the oxidation of -SH groups proceeded rather slowly (0.2% h^{-1}) even though the level of oxygen used was immediately inhibitory,

Lipid Peroxidation

Another manifestation of oxygen toxicity is the peroxidation of polyunsaturated fatty acids contained in membranes (Halliwell, 1981). The formation of organic radicals in the presence of oxygen leads to the formation of lipid hydroperoxides (Mead, 1976):

$$RH \xrightarrow{-H\cdot} R\cdot \qquad (1)$$

$$R\cdot + O_2 \rightarrow RO_2\cdot \qquad (2)$$

$$ROO\cdot + RH \rightarrow ROOH + R\cdot \qquad (3)$$

The initial H· abstraction by previously formed peroxide radicals or hydroxyl radicals (e.g. produced via the Fenton reaction) is followed by a rapid reaction with oxygen to form a peroxide radical. Abstraction of a second H· results in hydroperoxide formation and a second organic radical, making the reaction autocatalytic. Though the normal rate of lipid peroxidation is quite slow, the presence of transition metal ions greatly accelerates the process by catalyzing the production of initiating radicals (Mead, 1976; O'Brien, 1969):

$$Me^{n+} + ROOH \rightarrow Me^{n+1} + OH^- + RO\cdot$$

In addition to damage to the structural components of membranes, lipid hydroperoxides and their degradation products are also cytotoxic, leading to further enzyme and membrane damage (Hicks and Gebicki, 1978; Putvinsky et al., 1979).

Lipid peroxidation is also a consequence of the intracellular production of singlet oxygen. This reactive species has an affinity for electron-rich areas and reacts directly with polyunsaturated fatty acids to form lipid hydroperoxides. Production of singlet oxygen can occur in the presence of light-absorbing dyes (e.g. chlorophyll) and there is some suggestion of its formation via the superoxide dismutation reaction. Much of the work in the

latter is unclear due to the lack of specificity of singlet oxygen scavengers (Foote, 1979) but recent spectroscopic evidence of such singlet oxygen formation may finally settle this contentious issue (Khan, 1981).

Oxygen Radicals

The similarity of the effects of oxygen toxicity and irradiation led Gerschman et al. (1954) to suggest a common free radical mechanism. The discovery of intracellular superoxide production by xanthine oxidase (Knowles et al., 1969) and of an enzyme (superoxide dismutase) to catalyze its removal (McCord and Fridovich, 1969) has led to its suggestion as the mediator of oxygen toxicity. In vitro studies have shown superoxide to be involved in DNA damage (Brawn and Fridovich, 1981; Lesko et al., 1980; Nishida et al., 1981), mutagenicity (Moody and Hassan, 1981), lipid peroxidation (Bus et al., 1974; Pederson and Aust, 1973; Sutherland and Gebicki, 1982; Thomas et al., 1982) including membrane damage (Goldberg and Stern, 1976; Kellogg and Fridovich, 1975; 1977; Kong and Davidson, 1981; Lynch and Fridovich, 1978), depolymerization of polysacharides (McCord, 1974), hydroxylation of aromatic compounds (Hattiwell and Ahluwalia, 1976; McCord and Day, 1978) and direct killing of bacteria and viruses (Lavelle et al., 1973; Michelson and Buckingham, 1974).

Although there is substantial biochemical evidence to suggest superoxide toxicity, it is difficult to justify on a chemical basis. Superoxide is relatively unreactive to many compounds in vitro (Bielski and Richter, 1977; Bors et al., 1980) and in fact tends to be a reducing agent in aqueous solution. The oxidizing ability is limited because the formation of O_2^{2-} is unfavourable (Fee and Valentine, 1977; Valentine, 1979) but oxidation of acidic compounds in vitro is possible via proton abstraction leading to $HO_2^{\cdot}$ (Nanni et al., 1980). Formation of O_2^{2-} can be avoided to some extent by

by reaction with the metal ion Mn^{2+}, which can lead to the oxidizing species MnO_2^+ (Bielski and Chan, 1978).

In addition to its lack of reactivity, damage is often prevented by the presence of superoxide dismutase or catalase indicating that both O_2^- and H_2O_2 are required for toxic effects (Beauchamp and Fridovich, 1970; Cohen, 1978). Based on these results, it was suggested that hydroxyl radicals were generated via the Haber-Weiss reaction (Beauchamp and Fridovich, 1970; Fridovich, 1975a):

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

and that the reactivity of these radicals could easily account for the observed oxygen toxicity. However, further study has shown that this reaction as shown is too slow to compete with the dismutation reaction of superoxide (Gibian and Ungermann, 1979; Halliwell, 1976; McClune and Fee, 1976; Melhuish and Sutton, 1978; Rigo et al., 1977). The current theory of oxygen toxicity avoids this problem by proposing a catalysis of the reaction by transition metals, in particular Fe^{2+} (Halliwell, 1981):

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

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

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

This reaction is referred to as the Fe-catalyzed Haber-Weiss reaction (or the Fe-driven Fenton reaction) and in it superoxide acts only as a reducing agent to regenerate Fe^{2+} while OH· is produced by the well-known Fenton reaction. The feasibility of this theory has been confirmed in vitro (Butler and Halliwell, 1982; Fong et al., 1973; Halliwell, 1978a; 1978b; McCord and Day, 1978) but its intracellular significance has yet to be established. There is some question as to the importance of superoxide as the reducing agent since more effective reducing agents (e.g. ascorbate) are available in

the cell (Valentine, 1979) and others have questioned the availability of normally sequestered metal ions for the reaction (Fee\ 1980). Bors et al. (1980) suggest that free OH· is in fact too reactive to be the primary cause of oxygen toxicity since much of the damage would be restricted to the site of generation and it is therefore unable to explain the selectivity and delayed onset of oxygen toxicity. They propose that the formation of organic radicals is more important in oxygen toxicity. These interesting points have raised some controversy (Fee, 1981; 1982; Fridovich, 1981; Halliwell, 1982) which will no doubt spur greater efforts into this area.

Beneficial Applications of Oxygen Radicals

In spite of their toxicity, the high reactivity of oxygen radicals has been put to good use by some organisms. For instance, it has long been known that destruction of engulfed bacteria by polymorphonuclear leukocytes is accompanied by a burst of respiratory activity (Karnovsky, 1962). This activity has now been linked to the generation of O_2^-, H_2O_2 and OH· (but not singlet oxygen, Foote, 1979) by intracellular enzymes as the destructive agents (Babior, 1978a; 1978b; Badwey and Karnovsky, 1980). Oxygen radicals released by destruction of the leukocyte can also produce damage in the host (McCord, 1974) and lead to inflammation by activating a chemotactic factor for leukocytes (McCord and Roy, 1982). Involvement of active oxygen species in other processes such as anti-tumor drugs (Bates and Winterbourne, 1982; Oberly and Buettner, 1979) and lignin degradation (Forney et al., 1982; Nakatsubo et al., 1981) indicates the diverse uses to which they have been applied by man and microorganisms.

1.1.3 Oxygen Protection

In view of the reactivity of oxygen intermediates and the essential role of oxygen in aerobic metabolism, a means to ameliorate its toxicity is necessary.

In an evolutionary sense, the initial production of free oxygen by cyanobacteria would not have been possible without a method to deal with its toxic effects. One theory suggests that the oxidation of ferrous iron external to the cell would have maintained low atmospheric oxygen levels until alternate methods of protection became established (Cloud, 1968). Others have questioned the value of extracellular oxygen protection on the oxygen produced by intracellular photosynthesis (Schopf, 1975) leading to the suggestion that oxygen produced by photodissociation may have prompted the production of oxygen-scavenging enzymes prior to the evolution of oxygen-evolving photosynthesis (Towe, 1978). Whatever the initiating event, a number of strategies have evolved to counter the threat of oxygen, each having importance in overall oxygen protection.

Avoidance

Probably the simplest method of oxygen protection is that used by the obligate anaerobes, that is, to avoid exposure to oxygen and hence its toxic intermediates. These organisms have based their metabolism on methods not involving molecular oxygen, such as fermentation (e.g. *Clostridium*), anaerobic photosynthesis (e.g. *Chromatium*) or anaerobic respiration (e.g. *Desulfovibrio*) and can inhabit environments devoid of oxygen (Morris, 1975). Exposure of an obligate anaerobe to oxygen produces growth inhibition and cell death, although oxygen tolerance among anaerobes in general is quite variable. This failure to adapt to oxygen in an evolutionary sense has restricted them to a less efficient method of energy generation but has also eliminated the problem of oxygen toxicity.

In an indirect way, aerobic bacteria also use avoidance as part of their protection against oxygen toxicity. The terminal enzyme of the respiratory chain, cytochrome oxidase (Haddock and Jones, 1977), processes the most oxygen

of all the enzymes in the cell via:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

This enzyme contains two Fe^{2+} and two Cu^{2+}, both paramagnetic transition metals, and thus is able to circumvent the spin restriction to bring about the tetravalent reduction of oxygen (Antonini et al., 1970). This is of great advantage to the cell in that the formation and release of oxygen intermediates is avoided (Fridovich, 1977). Thus, the enzyme with the greatest potential to produce harmful species is indirectly protective in nature.

Glutathione

The presence of reduced thiols in aerobic cells provides a second mechanism of protection, especially against damage due to -SH oxidation. The most intensively studied of these is glutathione (GSH), a small tripeptide containing cysteine. In the presence of oxygen, the reduced sulfhydryl group is oxidized forming the disulfide, a reaction which may be catalyzed by transition metal ions (Misra, 1974):

$$2GSH + \tfrac{1}{2}O_2 \rightarrow GSSG + H_2O$$

The enzyme glutathione reductase will reduce the disulfide at the expense of $NADPH+H^+$ maintaining a high GSH/GSSG ratio (Halliwell and Foyer, 1979). The high concentration of reduced glutathione in the cell makes it "more available" to oxygen than the -SH groups of enzymes, hence it is preferentially oxidized (Halliwell, 1981).

In animal cells, glutathione is also involved in the removal of H_2O_2 and lipid peroxides via the selenium-containing enzyme glutathione peroxidase (Chance et al., 1979; Jocelyn, 1979):

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

$$2GSH + \text{Lipid-OOH} \rightarrow \text{Lipid-OH} + GSSG + H_2O$$

This enzyme is distributed in the cytosol and mitochondria of animal cells but is not found in plants or microorganisms (Smith and Shrift, 1979).

The importance of glutathione and reduced thiols in oxygen protection and as cofactors for some cellular processes is reflected in their wide distribution in living cells (Kosower and Kosower, 1969; 1978). The distribution in prokaryotes is more complicated in that glutathione is detected in most Gram-negative cells but few Gram-positive organisms (Fahey et al., 1978) though other reduced soluble thiol compounds may replace it. In addition, mutants lacking GSH are able to grow normally in air (Fuchs and Warner, 1975) indicating that it may not be essential for aerobic organisms.

Antioxidants

The presence of antioxidant substances can also be a means of oxygen protection. By their structure, these compounds are able to dissipate the energy of active oxygen species (mainly singlet oxygen) rendering them innocuous to the cell. For instance, the presence of α-tocopherol (vitamin E) in chloroplasts can prevent lipid peroxidation by scavenging singlet oxygen and lipid radicals (Foote, 1976; Halliwell, 1981):

$$\text{Lipid-O}_2\cdot + \text{Donor-H} \rightarrow \text{Lipid-OOH} + \text{Donor}\cdot$$

This will terminate the radical chain reaction, minimizing the damage to membranes. The donor radical formed can be regenerated via reduction by ascorbic acid (Packer et al., 1979).

A similar role is played by carotenoids (e.g. β-carotene) which are present in animals, plants (chloroplasts in particular) as well as some bacteria and fungi (Krinsky, 1979). In both photosynthetic and non-photosynthetic organisms, the protective effect of carotenoids against damage due to light or singlet oxygen has been shown using mutants lacking them (Krinsky, 1978; 1979; Shimizu et al., 1979).

Catalase

The enzyme catalase brings about the catalytic decomposition of H_2O_2 and is unique in that it does so by two routes (Chance et al., 1979):

$$\text{Catalatic} \quad H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$$

$$\text{Peroxidatic} \quad H_2O_2 + AH_2 \rightarrow 2H_2O + A$$

The pathway used will depend on the concentration of H_2O_2 but in general, the catalatic reaction dominates at high levels of H_2O_2 (above 10^{-4} M) while the peroxidatic activity dominates at low levels (in the presence of a suitable H-donor) (Deisseroth and Dounce, 1970).

The typical catalase is an oligomeric protein with a molecular weight of approximately 240,000. It consists of four identical subunits (MW 60,000), each of a single polypeptide chain and containing the prothetic group Fe^{3+}-protoporphyrin IX. The subunits are associated by non-covalent interactions and can function independently of one another (Deisseroth and Dounce, 1970; Schonbaum and Chance, 1976). In general, biosynthesis occurs by a stepwise process via synthesis of apoprotein subunits, addition of the heme and tetramerization (Ruis, 1979).

The occurrence of catalase is often associated with the possession of cytochrome systems (Deisseroth and Dounce, 1970) and it is found in most aerobic organisms. Some exceptions include *Acetobacter peroxydans*, *Bacillus popillae* (Costilow and Keele, 1972), the blue-green bacterium *Gloeocapsa* sp. LB795 (Tözüm and Gallon, 1979) and a number of aerotolerant anaerobes (McCord et al., 1971). Most anaerobes do not contain catalase, but again there are exceptions such as *Propionibacterium shermanii* (Sherman, 1926). In most plant and animal tissues, catalase is localized in peroxisomes, which also contain many of the H_2O_2-producing enzymes (Masters and Holmes, 1977). Catalase has not been detected in mitochondria or chloroplasts (Allen, 1977; Day et al., 1979)

though there may be other enzymes present (e.g. glutathione peroxidase) which scavenge H_2O_2.

It is evident, in view of the mechanism and localization in peroxisomes, that a primary function of catalase is the removal of intracellular H_2O_2 (Fridovich, 1976; Halliwell, 1981; Ruis, 1979). However, it has been suggested that it may also participate in the metabolism of certain hydrogen donors via the peroxidatic reaction (De Duve and Baudhuin, 1966). Measurements have put the intracellular concentration of H_2O_2 at 10^{-8} M which may indicate that the peroxidatic reaction is dominant (Deisseroth and Dounce, 1970).

Peroxidase

A second group of hemoproteins that will catalyze the reduction of H_2O_2 using H-donors are the peroxidases (Saunders et al., 1964).

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

In general, peroxidases have a high specificity for the peroxide but are relatively non-specific for the H-donor (though there are some with restricted H-donors such as glutathione peroxidase) (Putter, 1974). In addition to this typical reaction, there have also been reports of peroxidases acting as oxidases (Kenten, 1955) and monooxygenases (Buhler and Mason, 1961).

Though peroxidases are widely distributed, studies have centered on the easily isolated peroxidase of horseradish (HRP) as a representative example. This enzyme has a molecular weight of 40,000 and consists of a single polypeptide chain of 287 amino acid residues, a prosthetic group and some carbohydrate, probably neutral and amino sugars (Saunders et al., 1964). The prothetic group is Fe^{3+}-protoporphyrin IX (similar to catalase) and is easily removed from plant peroxidases but tightly bound in animal forms (Yamazaki, 1974). Glutathione peroxidase is a unique case in that even though selenium replaces the heme group as the active metal, it shares a similar

kinetic mechanism (Chance et al., 1979):

In the many years since it was discovered, peroxidase has been found to be widely distributed (for a list see Saunders et al., 1964 and Putter, 1974). Of the many plants in which it is found, fig tree sap and horseradish root are particularly good sources, the latter having been studied quite intensively. It has also been detected in a variety of animal tissues as well as some invertebrates and fungi. Peroxidase activity has been found in a number of bacteria but in many cases the enzyme involved is not a true (heme) peroxidase (Saunders et al., 1964). A non-specific peroxidase has been detected in *Escherichia coli* (Hassan and Fridovich, 1978).

While there has been much study of the enzymology of peroxidase, few have examined its physiological role in detail. It is generally thought that the oxidation of donor molecules is of primary importance (Yamazaki, 1974; Saunders et al., 1964) but little is known of the significance to the cell of H_2O_2 removal by this enzyme (Fridovich, 1976). In at least one case (glutathione peroxidase), the scavenging of H_2O_2 and lipid hydroperoxides is a very important function but since the intracellular substrates for a non-specific peroxidase are unknown, it is difficult to assess the magnitude of H_2O_2 removal by this means (Halliwell, 1981).

Superoxide Dismutase

The most recently identified enzyme involved in oxygen protection was known for many years as a copper-containing protein of unknown function. Originally isolated from blood, it was labelled "hemocuprein" alluding to its source and copper content (Mann and Keilin, 1938). Similar proteins were isolated from other sources (e.g. hepatocuprein, cerebrocuprein, etc.) and were well-characterized without identification of any catalytic activity. In 1969, in a totally independent line of study, an enzyme was discovered that was capable of the dismutation of superoxide and which proved to be identical to

the cupreins isolated many years previously (McCord and Fridovich, 1969). Thus, an unanticipated biological activity was uncovered which had immediate implications for the theory of oxygen toxicity.

Superoxide dismutase (SOD), as the enzyme is now known, will catalyze the dismutation of superoxide radicals with high efficiency at physiological pH (Halliwell, 1982):

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

Though this dismutation can take place spontaneously, the enzyme-catalyzed rate is 10^4 times faster (Fridovich, 1978). Hydrogen peroxide produced by the reaction is rapidly removed by H_2O_2-scavenging enzymes (Fridovich and Hassan, 1979) and the oxygen is the innocuous triplet (ground) state (Halliwell, 1981). Some early reports that SOD was able to quench singlet oxygen (Finazzi-Agrò et al., 1972; Paschen and Weser, 1973) have now been found to be in error (Schaap et al., 1974) and superoxide dismutation is the only known reaction (Hill, 1978).

Three forms of the enzyme have been identified according to their metal content. The first, isolated originally as a "cuprein", has a molecular weight of 32,000 and is composed of two identical subunits, each containing one Cu^{2+} and one Zn^{2+} (Fridovich, 1975b). The others also have two subunits and contain one Mn^{3+} or one Fe^{3+} per subunit as the active metal (MW 40,000 in both cases). The structural characteristics of the enzymes and their active sites have been well-studied (Fee, 1977; Fridovich, 1976; Lieberman et al., 1982; Walker et al., 1980).

Much study has gone into the biology of superoxide dismutase, only an outline of which is appropriate here. The CuZnSOD occurs in certain algae and in all higher eukaryotes almost exclusively (Asada et al., 1980). One of the first prokaryotic sources uncovered, the symbiotic organism *Photobacterium leiognathi* (Puget and Michelson, 1974) is thought to have acquired the enzyme

via gene transfer from its host fish (Martin and Fridovich, 1981). A CuZnSOD-like activity in *Paracoccus denitrificans* was found on characterization to be structurally distinct from eukaryotic CuZnSOD (Vignais et al., 1982). However, the discovery of true CuZnSOD in the free-living prokaryote *Caulobacter crescentus* (Steinman, 1982) raises some interesting questions regarding its origin. The similarity of CuZnSOD among the higher eukaryotes, even to primitive invertebrates, indicates that the enzyme is quite stable in an evolutionary sense (Fridovich, 1976).

The MnSOD and FeSOD are found predominantly in prokaryotes as well as some eukaryotic algae and protozoa (Asada et al., 1980). The MnSOD is also found in the mitochondrial matrix (Henry et al., 1980; Weisiger and Fridovich, 1973a; 1973b) and bound to the thylakoids of chloroplasts (Asada et al., 1980; Kanematsu and Asada, 1979) and so is often detected in higher eukaryotes. Recent evidence of FeSOD in certain higher plants (Bridges and Salin, 1981; Salin and Bridges, 1980; 1982) again raises several questions since it was previously thought to be strictly prokaryotic. Among prokaryotes, there is a general trend that MnSOD-containing organisms tend to be Gram-positive while those containing FeSOD or both FeSOD and MnSOD are Gram-negative but there are several exceptions (Britton et al., 1978).

Asada et al. (1980) have made some interesting suggestions concerning the phylogenic distribution of the three types of SOD. They find that the occurrence of FeSOD is marked in organisms having low intracellular oxygen concentration (anaerobes and aerobic diazotrophs). They believe that this form gave rise to MnSOD in response to increased atmospheric oxygen such that aerobic and facultative prokaryotes, cyanobacteria and some lower eukaryotes contain either or both of these enzymes. The CuZnSOD arose independently in primitive eukaryotes later in the evolutionary scale, hence it is not found in prokaryotes. These concepts are supported in part by

with some success. A mutant of *E. coli* was isolated that was unable to grow aerobically at high temperature due to a temperature-sensitive defect in SOD production (Fridovich, 1975a). Unfortunately, catalase and peroxidase levels were not examined so the significance of these results to oxygen toxicity in general is unclear (Halliwell, 1981). Other mutants found intolerant to oxygen were found to lack MnSOD, catalase and peroxidase. Revertants regained all of the enzymatic activities or had lost the ability to respire (Hassan, 1976).

Induction of SOD in response to oxygen has been found in *E. coli*, *Streptococcus faecalis* and *Saccharomyces cerevisiae* (Gregory and Fridovich, 1973a; Gregory et al., 1974) alluding to a possible role in oxygen protection. Higher than normal intracellular SOD activity (achieved by growth at high oxygen tension) has been correlated to increased resistance to the toxicity of hyperbaric oxygen and O_2^- generated enzymatically by streptonigrin (Gregory and Fridovich, 1973b; Gregory et al., 1973). In *Bacillus subtilis*, catalase but not SOD is induced by oxygen but no corresponding increase in resistance to toxic hyperbaric oxygen is found, which implies that SOD is more important in oxygen protection (Gregory and Fridovich, 1973b). Higher SOD levels have also been correlated directly to intracellular levels of O_2^- produced by respiration (Hassan and Fridovich, 1977a) or artificially via methyl viologen (Hassan and Fridovich, 1977b; 1977c). Induction of SOD (primarily pulmonary) in rats in response to oxygen, with a corresponding increase in resistance to oxygen toxicity, has been found (Crapo and Tierney, 1974) though results in such a complex system are difficult to interpret (Crapo and McCord, 1976).

Despite this evidence, there are those who question the importance of superoxide dismutase in oxygen toxicity and even its role in superoxide dismutation. They interpret the ability of simple metal complexes (especially those containing Cu^{2+}) to catalyze superoxide dismutation (Brigelius et al.,

1974; Rabani et al., 1973) as an indication that this activity in SOD is simply a consequence of the presence of copper and maintain that its true activity has not yet been found (Fee, 1982). Others are quick to point out that other copper proteins do not react with O_2^- as efficiently as SOD and that FeSOD and MnSOD are much more effective than their corresponding free salts (Halliwell, 1981; 1982).

1.2 Nitrogen Fixation

Nitrogen is an important element in the growth of all organisms (micro and macro) as a major component of proteins and nucleic acids. Diatomic N_2 (dinitrogen), is the most abundant form constituting approximately 78% of the earth's atmosphere. It is an inert molecule due to a high bond energy (946 kJ per mole) and an electronic configuration unfavourable to oxidation or reduction (Olivé and Olivé, 1979). Since this is unsuitable for assimilation into cells, uptake of nitrogen in its combined form, ammonia (NH_3) or nitrate (NO_3^-), is the principal means by which the biological nitrogen requirement is satisfied. In soil, removal or loss of these nutrients by uptake (e.g. plants), leaching or volatilization (denitrification) can lead to levels insufficient to support further growth. Replacement most commonly involves addition in the form of ammonia produced from dinitrogen (referred to as fixed) by biological or non-biological means. The latter is very important for the production of ammonia fertilizer for use in agriculture and is known as the Haber-Bosch process:

$$N_2 + 3H_2 \rightarrow 2NH_3$$

It uses an iron oxide catalyst with high temperatures (approximately 500°C) and high pressures to overcome the lack of reactivity of N_2 (Ozaki and Aika, 1979; Bridger et al., 1979). These extreme conditions contrast to the biological process (nitrogen fixation) in which the reaction is enzyme-

catalyzed using physiological reductants at ambient temperatures. It is a strictly prokaryotic process carried out in many and varied locations, most notably, in soil. Symbiotic nitrogen fixers do so only in association with a host plant, a classic example of which is the *Rhizobium*/legume symbiosis (though there are others, including non-legumes). These organisms invade the root of the host legume and become established as bacteroids within a root nodule (Alexander, 1977; Dart, 1977; Vincent, 1977). In return for an energy source (photosynthate), they fix nitrogen to the benefit of the host plant (Pate, 1977). A second group of nitrogen fixers are free-living and fix nitrogen non-symbiotically. They include anaerobes (e.g. *Clostridium*, *Desulfovibrio*), facultative anaerobes (e.g. *Bacillus*, *Klebsiella*) and aerobic organisms (e.g. *Azotobacter*, *Derxia*) using N_2 as their sole source of nitrogen (Alexander, 1977). A number of unicellular (e.g. *Gloeocapsa*) and filamentous (e.g. *Anabaena*, *Plectonema*) cyanobacteria also fix nitrogen (Stewart, 1980).

1.2.1 Nitrogenase

Biological nitrogen fixation is one of the most complex of enzymatic catalyses requiring six individual components. The enzyme is a complex of two proteins with different functions, both of which are essential for enzymatic activity. The true nitrogenase (directly responsible for substrate binding and reduction) is a large, tetrameric metalloprotein (MW approximately 220,000) referred to as dinitrogenase, component I or simply MoFe-protein. It consists of two dissimilar subunits (MW 50,000 and 60,000) and contains 2 molybdenum, 24-32 iron and approximately 28 acid-labile sulfur atoms (S^{2-}) per molecule (Eady and Smith, 1979: Mortenson and Thorneley, 1979). A major structural feature is an extractable "cofactor" containing $1Mo:8Fe:6S^{2-}$ (labelled FeMo-co), two of which form the active sites in the native molecule (Shah and Brill, 1977).

The second component, referred to as dinitrogenase reductase, component 2 or Fe-protein, is also a metalloprotein but is not involved in substrate binding. It has a molecular weight of approximately 60,000 and contains 4 iron and 4 acid-labile sulfur atoms. It associates with the MoFe-protein to form a tight complex and is responsible for the binding of Mg^{2+} and ATP required for the catalytic reaction (Eady and Smith, 1979; Mortenson and Thorneley, 1979). The similarity of the respective enzyme components in various organisms is illustrated by the fact that in many cases the Fe-protein and MoFe-protein from different sources will combine to form functional (albeit less active) nitrogenase in vitro (Burris, 1971; Eady and Postgate, 1974; Hardy and Burns, 1973).

A key factor that affects all aspects of nitrogen fixation is the oxygen sensitivity of the process. Most diazotrophs fix nitrogen optimally under reduced oxygen tension and all are adversely affected by elevated oxygen levels. Oxygen is an uncompetitive inhibitor of nitrogen fixation (Wong and Burris, 1972) and at higher levels causes irreversible inactivation of nitrogenase irrespective of its source (Burris, 1979). Of the purified components, the Fe-protein proves to be the most sensitive in vitro, with a half-life in air of approximately 45 seconds (Eady et al., 1972; Yates and Planqué, 1975). This sensitivity is augmented in the presence of ATP (Yates, 1972) probably due to a conformation change that occurs on binding. The MoFe-protein is somewhat less sensitive to oxygen, with a half-life of 8-10 minutes (Eady et al., 1972; Shah and Brill, 1973; Yates and Planqué, 1975) although the extractable FeMo-co separately proves to be even more oxygen sensitive than the Fe-protein (Shah and Brill, 1977). Other components required for nitrogen fixation (e.g. ferredoxin) are also oxygen sensitive (Petering et al., 1971).

Several methods have been used in an attempt to determine the mechanism of this inactivation. One proposal suggests that oxygen interacts with the

reduced state of the enzyme (e.g. reduced sulfur moieties) disrupting its structural integrity. That this is feasible is demonstrated by the denaturation of other iron-sulfur proteins (e.g. ferredoxin) by oxidation of labile sulfide to S^0 (Petering et al., 1971) and enzyme inactivation by the oxidation of sulfhydryl groups (Little and O'Brien, 1969). Air inactivation of *C. pasteurianum* Fe-protein in vitro is accompanied by the oxidation of one S^{2-} and two sulfhydryl groups (Moustafa and Mortenson, 1976). The importance of labile sulfide in the structure of Fe_4S_4 centers and in the postulated structure of the MoFe-protein active site (Cramer et al., 1978; Mortenson and Thorneley, 1979) illustrates that substantial damage can be produced by such oxidation.

The titration of the MoFe-protein through several oxidation states while retaining activity is possible (Palmer et al., 1972; Smith et al., 1972), but inactivation results on prolonged exposure to O_2. Spectral changes (EPR, Mössbauer, circular dichroism) indicate that changes occur in the redox state of the Fe atoms and in α-helical content on exposure to O_2 (Eady and Smith, 1979). In some cases, exposure leads to loss of the metal (Huang et al., 1979) which may account for the loss of activity. Using X-ray absorption spectroscopy, it is possible to detect Mo=O on exposure to air but not in its absence (Cramer et al., 1978), which may be another manifestation of oxygen damage to the enzyme.

The inactivation of the Fe-protein of *Klebsiella pneumoniae* is suggested to proceed initially through a conformation incapable of accepting electrons for the reaction (Smith and Lang, 1974) rather than direct destruction of the FeS center. Oxygen-damaged Fe-protein loses substantial α-helical content and has a Mössbauer spectrum similar to that of reduced ferredoxin but no EPR activity (Eady and Smith, 1979). It retains the ability to bind ATP but forms an inactive complex with active MoFe-protein (Biggins and Kelly, 1970)

The intracellular generation of powerful oxygen radicals and their involvement in oxygen toxicity has led to the suggestion that they may also play a role in the inhibition of nitrogen fixation (Robson and Postgate, 1980). Superoxide generated exogenously has been found to inhibit nitrogenase activity in whole cells (Buchanan, 1977) and the presence of superoxide dismutase plus catalase will prevent the air inactivation of the Fe-protein in vitro to a certain extent (Mortenson et al., 1976). Inhibition of acetylene reduction in *Gloeocapsa* sp. by H_2O_2 has also been demonstrated (Tözün and Gallon, 1979). However, little work has been done regarding the generation of these species during nitrogen fixation or their significance to the process. Misra and Fridovich (1971) demonstrated the generation of superoxide by the autooxidation of reduced ferredoxin in vitro but did not relate this to nitrogen-fixing conditions in vivo, where oxygen levels are low or negligible due to the oxygen sensitivity of the process. Radical generation may result in attack on the nitrogenase directly or interuption of the flow of ATP and/or reducing power by the disruption of cellular metabolism and membranes.

1.2.2 Oxygen Protection

Since many components of nitrogen-fixing systems are oxygen sensitive, the process itself is seen as being anaerobic in nature (Yates, 1977; Yates and Jones, 1974). Yet, the capacity for nitrogen fixation exists not only in anaerobes but is found in aerobic and even oxygen-evolving photosynthetic organisms as well. This apparent paradox is resolved by the presence of protective mechanisms that ensure that adequately low intracellular O_2 tensions for nitrogenase are maintained. Since the efficiency and distribution of these mechanisms vary, nitrogen-fixing organisms cover a wide range of oxygen tolerance.

Avoidance

In avoiding oxygen to maintain their metabolism, many nitrogen-fixing obligate anaerobes (e.g. *C. pasteurianum*) indirectly produce conditions favourable for nitrogenase. In fact, some facultative anaerobes such as *Klebsiella pneumoniae* (Mahl and Wilson, 1968) and *Bacillus polymyxa* (Grau and Wilson, 1963) will fix nitrogen only under anaerobic conditions, even though they can grow aerobically.

Physical Barrier

There is a number of examples in which low oxygen tension is maintained by a barrier to reduce oxygen diffusion. One of the best known is the heterocyst of filamentous, nitrogen-fixing cyanobacteria. These specialized cells have a thick envelope acting as an O_2 barrier which, in combination with other methods (below), provide a protected environment for nitrogenase (Stewart, 1980). Nitrogen-fixing mutants whose heterocysts lack the proper envelope glycolipids are more sensitive to oxygen (Haury and Wolk, 1978).

Nodules resulting from the symbiosis between *Rhizobium* and leguminous plants are another method by which oxygen diffusion is restricted. Their impermeability (Tjepkema and Yocum, 1973; 1974) and the high respiration rates of the bacteroids conspire to keep the oxygen level low in the nodule tissue (Bergersen, 1979). Oxygen for ATP production is transported to the bacteroids by leghemoglobin, a soluble, hemoglobin-like pigment which can reversibly bind oxygen (Bergersen et al., 1973; Bergersen and Turner, 1975a; 1975b; Imamura et al., 1972; Wittenberg et al., 1972). Its high affinity for oxygen (Dilworth and Appleby, 1979) ensures that the low internal level of free oxygen is maintained (Wittenberg et al., 1972).

There is some suggestion that the production of extracellular polysaccharide (slime) by diazotrophs may impede the diffusion of oxygen thus protecting the nitrogenase (Hill, 1971). However, mutant diazotrophs not

capable of slime production are available that are no more sensitive to oxygen than the wild type (Bush and Wilson, 1959; Yates and Planqué, 1975). As well, no correlation has been found between slime production and oxygen tolerance in nitrogen-fixing *K. pneumoniae* (Wilcockson, 1977) or *Gloeocapsa* sp. LB795 (Tözüm and Gallon, 1977). These examples have raised questions regarding the significance of slime production in oxygen protection.

Removal of Oxygen

Since many metabolic processes consume oxygen, they are often used to maintain oxygen concentrations optimal for nitrogenase. For example, *Azotobacter* spp. have one of the highest known respiration rates (Williams and Wilson, 1954). Using a branched respiratory chain (Haddock and Jones, 1977) this organism is able to increase its respiratory rate to maintain low intracellular oxygen levels while fixing nitrogen under aerobic conditions (Dalton and Postgate, 1969a; 1969b). Respiration for the purpose of scavenging oxygen is poorly coupled to ATP production resulting in an energy wastage at high oxygen tensions (Jones et al., 1973). The net effect is oxygen protection at the expense of growth and nitrogen fixation efficiency.

Limited respiratory protection is found in most non-anaerobic diazotrophs, though without the unique adaptation found in *Azotobacter*. The normal metabolism of these organisms has a small but not insignificant capacity to scavenge oxygen (Biggins and Postgate, 1969; 1971). The relatively low upper limit at which it is able to maintain sufficiently low oxygen tension for functional nitrogenase restricts these organisms to microaerobic conditions when fixing nitrogen (Robson and Postgate, 1980).

This type of protection is augmented in many diazotrophs by the presence of an uptake hydrogenase:

$$H_2 + \tfrac{1}{2}O_2 \rightarrow 2H_2O$$

Aside from its value in recovering energy lost by the ATP-dependent H_2 evolution of nitrogenase, oxygen is used in the reaction with the net effect of reducing its intracellular level (Bothe et al., 1977; Ruiz-Argueso et al., 1979; Yates et al., 1981). This method has been found to be particularly important under carbon-limited conditions where oxygen uptake by normal respiration may be insufficient (Gallon, 1981; Yates et al., 1981).

Conformational Protection

Nitrogen fixation in *Azotobacter* will cease when the respiratory protection mechanism is overwhelmed by high oxygen but will resume without de novo nitrogenase synthesis if the exposure is sufficiently brief (Drozd and Postgate, 1970a; 1970b). Referred to as conformational protection, exposure to oxygen causes the nitrogenase to assume a relatively oxygen-insensitive state that is unable to fix nitrogen (Hill et al., 1972). Reactivation of this nitrogenase when the oxygen level is again suitable is possible, providing the exposure is not long enough to produce structural damage to the enzyme (Drozd and Postgate, 1970b). This protection is now attributed to the association with nitrogenase of a small, 2Fe:2S protein which is capable of conferring oxygen stability to the purified enzyme in vitro (Haaker and Veger, 1977; Robson, 1979).

Cyanobacteria

The problem of nitrogenase inactivation is compounded in cyanobacteria by the oxygen evolved by photosynthesis. Some have only simple methods of oxygen protection and are able to fix nitrogen only under anaerobic (e.g. *Synechococcus*) or microaerobic (e.g. *Plectonema*) conditions, while others possess methods effective enough to handle aerobic conditions. One strategy is to locate the nitrogenase in heterocysts (Fleming and Haselkorn, 1973; Stewart et al., 1969; Tel-Or and Stewart, 1977) which lack the

oxygen-evolving photosystem II of vegetative cells (Thomas, 1970) and have thick walls to bar atmospheric oxygen (Haury and Wolk, 1978). Under these conditions, metabolic activity (including an uptake hydrogenase) is able to maintain low internal oxygen levels (Stewart, 1980). Other cyanobacteria are capable of aerobic nitrogen fixation but lack heterocysts to separate the two processes, so they are present in the same cell (Gallon et al., 1974). Some of these organisms (e.g. *Gloeothece*) prevent nitrogenase inactivation by temporal separation in which the majority of nitrogen fixation takes place in the dark when photosynthetic oxygen evolution is at a minimum (Gallon et al., 1981). Others (e.g. *Trichodesmium*) seem to be able to localize nitrogen fixation in the interior of cell colonies where oxygen evolution does not occur (Carpenter and Price, 1976).

Superoxide Dismutase, Catalase and Peroxidase

Only a few diazotrophs have actually been examined to determine their content of O_2-scavenging enzymes and in spite of their acknowledged importance in oxygen protection, little is known of their direct significance to nitrogen fixation. Members of the genus *Azotobacter* contain FeSOD and catalase but their contribution to the protection of nitrogenase is unclear (Asada et al., 1980; Buchanan, 1977; Buchanan and Lees, 1980). Inhibition of nitrogen fixation in whole cells by exogenous superoxide is prevented by exogenous superoxide dismutase (Buchanan, 1977) which may indicate that the intracellular enzyme may also have some protection value.

A survey of *Rhizobium* spp. found that all those examined contained FeSOD and differences could be detected in the enzyme from fast- and slow-growing strains (Stowers and Elkan, 1981). The FeSOD of *R. japonicum* was inducible by increased aeration or an O_2^- flux generated intracellularly, but its importance to nitrogen fixation directly was not examined (Stowers and Elkan, 1981). The presence of CuZnSOD and catalase in the nodule itself is suggested to be of

some importance in the prevention of irreversible inactivation of leghemoglobin by H_2O_2 (Puppo et al., 1982).

Some cyanobacteria have been examined specifically to determine the significance of O_2-scavenging enzymes to the nitrogenase. The levels of catalase and superoxide dismutase were lower in the heterocysts of *Anabaena cylindrica* than the vegetative cells, possibly due to lower O_2 levels in the absence of oxygen-evolving photosynthesis (Henry et al., 1978). However, they were unable to correlate the ability of these enzymes to limit oxygen damage to nitrogenase protection.

Inhibition of acetylene reduction in non-heterocystous *Gloeocapsa* sp. LB795 by added methyl viologen, has been attributed to the generation of H_2O_2 (via O_2^-) (Tözüm and Gallon, 1979). While catalase was not detected, the fact that the levels of other H_2O_2-scavenging activities (based on ascorbate and glutathione) and superoxide dismutase were maximal during the maximum acetylene-reducing activity may indicate that they play a role in protecting the nitrogenase. However, no one mechanism was found that was able to account entirely for oxygen protection.

In short, though the removal of active oxygen species is undoubtably important for nitrogen fixation, little concrete information is available concerning the true significance of O_2-scavenging enzymes for the process.

1.3 *Azospirillum* spp.

Beijerinck (1922) first described a spirillum capable of nitrogen fixation from enrichment cultures of soil and named it *Azotobacter spirillum*, later changing it to *Spirillum lipoferum*. It generated little interest until 1974 when it was identified as the major organism responsible for nitrogen fixation in the roots of the tropical grass *Digitaria decumbens* (Day and Döbereiner, 1976; Döbereiner and Day, 1976). The group has since been renamed

Azospirillum and has been found to be a common soil and root inhabitant of the tropics and subtropics (Albrecht and Okon, 1980; Döbereiner et al., 1976; Neyra and Döbereiner, 1977). Interest in the organism has burgeoned in recent years due to its potential for supplying fixed nitrogen to agriculturally-important nonleguminous plants (Cohen et al., 1980; Kapulnik et al., 1981; Nur et al., 1980a; 1980b; Rai and Gaur, 1982; Reynders and Vlassak, 1982; Tilak et al., 1982; Van Berkum and Bohlool, 1980).

The organism is a short, vibroid, Gram-negative rod often containing refractile poly-β-hydroxybutyrate granules (Krieg, 1976; Krieg and Hylemon, 1976; Neyra and Döbereiner, 1977; Tarrand et al., 1978). It is highly motile by a polar flagellum (Hegazi and Vlassak, 1979) and the colonies on nutrient agar develop a light-pink pigment (Okon et al., 1976a) thought to be a b- or c-type cytochrome (Eskew et al., 1977) though this has been questioned (Nur et al., 1981). Its metabolism is generally considered to be respiratory in nature, with organic acids (malate, lactate, succinate, pyruvate) providing the best carbon and energy source for growth and nitrogen fixation (Okon et al., 1976a; 1976b). Certain strains have a limited ability to ferment carbohydrates, though growth is weak when using them anaerobically (Tarrand et al., 1978). Two species have been identified based on DNA homology studies and differences in metabolism (Tarrand et al., 1978): A. *brasilense*, which has no fermentative ability (does not use glucose as sole carbon and energy source) and no vitamin requirement, and A. *lipoferum*, which is capable of using glucose to support growth and nitrogen fixation but has a requirement for biotin.

A notable feature of *Azospirillum* spp. is the ability to perform all aspects of the nitrogen cycle, except nitrification (Bothe et al., 1981). Under aerobic conditions, NH_4^+ or NO_3^- (via assimilatory nitrate reductase) can be used as the nitrogen source (Okon et al., 1976a; 1976b; Neyra and Van Berkum, 1977) with optimal growth rates being obtained with NH_4^+. In the

absence of a combined nitrogen source, they are able to fix nitrogen, but due to the lack of adequate oxygen protection for the nitrogenase (below), will only do so under microaerobic conditions (Day and Döbereiner, 1976; Nelson and Knowles, 1978; Okon et al., 1976a; 1977). Under anaerobic conditions, growth is possible using nitrate as terminal electron acceptor (anaerobic respiration). Most strains are able to reduce nitrate to nitrite, while some reduce it further to N_2O and N_2 (denitrification) resulting in nitrogen losses by volatilization (Eskew et al., 1977; Nelson and Knowles, 1978; Neyra et al., 1977; Neyra and Van Berkum, 1977). There is some evidence that the energy generated by this process can support nitrogen fixation (Bothe et al., 1981; Neyra and Van Berkum, 1977; Scott et al., 1979) but its occurrence and value have been questioned (Nelson and Knowles, 1978) since assimilatory nitrate reduction is possible under anaerobic conditions (Bothe et al., 1981; Nelson and Knowles, 1978).

The respiratory metabolism of *Azospirillum* spp. makes oxygen a requirement for energy generation but when fixing nitrogen its tolerance is limited by the lack of adequate oxygen protection for the O_2-labile nitrogenase. Nelson and Knowles (1978) found only a limited ability to increase oxygen uptake in A. *brasilense*, indicating that it is incapable of the specialized respiratory protection found in *Azotobacter* and thus is restricted to oxygen tensions that are adequately scavenged by normal respiratory processes (0.003-0.007 atm). This is illustrated in N-free semi-solid medium where a pellicle of growth is formed 2-4 mm below the surface, apparently at the point where O_2 diffusion is balanced by O_2 uptake, creating optimal conditions for nitrogenase (Day and Döbereiner, 1976; Okon et al., 1976a). Uptake hydrogenase activity is also suggested to play a role in this metabolic removal of O_2 (Tibelius and Knowles, 1983; Volpon et al., 1981) though in some cases it proves to be too O_2 sensitive itself to be of value (Pedrosa et al., 1982).

Other methods of oxygen protection have also been examined. Regeneration of air-inactivated nitrogenase is not possible without new protein synthesis (Okon et al., 1976a) indicating that there is no conformational protection. A limited ability to use "reversible inactivation" for oxygen protection has been found (Berlier and Lespinat, 1980) but its value to nitrogen fixation is not known. There is some suggestion that capsules formed by A. *brasilense* isolated from plant tissue cultures may be a method of regulating O_2 flow at high oxygen tension (Berg et al., 1980). Capsules were found only when the cells were fixing nitrogen aerobically on the surface of N-free nutrient agar and not under microaerobic conditions (where presumably they would not be required for O_2 protection). However, they were unable to separate encapsulated from unencapsulated cells completely, so it is not known whether the encapsulated form is capable of fixing nitrogen. Others have raised doubts concerning the efficacy of O_2 protection by slime capsules (Wilcockson, 1977). The occurrence of carotenoids in nitrogen-fixing but not NH_4^+-grown A. *brasilense* strain cd has been interpreted to indicate that they play a role in protecting the nitrogenase by scavenging singlet oxygen and possibly other oxygen radicals (Nur et al., 1981). The carotenoids are produced under aerobic (but not microaerobic) conditions and in the presence of diphenylamine, an inhibitor of carotenoid synthesis, acetylene reduction was 50% lower than in the control. However, carotenoids are not found in other strains of A. *brasilense*, so their significance to nitrogenase protection in general is not known. Superoxide dismutase and low levels of catalase have been found in A. *brasilense* strain cd, but nitrogen-fixing cells were not examined (Nur et al., 1982).

2. MATERIALS AND METHODS

2.1 Organisms

Azospirillum brasilense sp7 (ATCC 29145), formerly known as *Spirillum lipoferum*, was received in the lyophilized state and revived by 12-18 h incubation (30°C, 300 rpm) in 10 mL of the medium of Nelson and Knowles (1978) containing ammonium (details below) in a 50-mL Erlenmeyer flask. Working cultures were prepared by inoculating nutrient agar mini-slants which, after 12 h incubation (30°C), were stored at -80°C. Purity was checked on nutrient agar plates. A fresh working culture was used for each experiment.

Escherichia coli K12 (lyophilized) was acquired from Dr. J. Wood (Department of Biochemistry, University of Guelph) via M.T. O'Reilly. Working cultures were prepared by growth of a stock culture on 10 mL TSY medium (below) in a 50-mL Erlenmeyer flask (12 h, 37°C, 250 rpm) and subsequent inoculation to nutrient agar slants in screw cap tubes. After 12-18 h incubation (37°C), the slants were stored at 4°C and transferred monthly. Purity was checked on nutrient agar plates. Each experiment used a fresh working culture.

2.2 Growth Conditions

The nitrogen-free medium for *A. brasilense* was similar to that of Nelson and Knowles (1978) and contained in g per L glass distilled water: K_2HPO_4 3.0, KH_2PO_4 2.0, NaCl 0.1, $MnSO_4 \cdot H_2O$ 0.01, $NaMoO_4 \cdot 2H_2O$ 0.002, $MgSO_4 \cdot 7H_2O$ 0.2, $CaCl_2 \cdot 2H_2O$ 0.02, Sequestrene NaFe (13% Fe) (Ciba-Geigy Corp., Greensboro, NC, USA, 27400) 0.006, L-malic acid 1.0 (neutralized with NaOH prior to addition). $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$ were autoclaved separately and added aseptically to sterile medium after cooling to prevent precipitation. The pH of the medium was adjusted to 6.9 with HCl prior to autoclaving (121°C, 20 min). Ammonium chloride (1.0 g L^{-1}) was added as a combined nitrogen source when required. Yeast extract (0.02 g L^{-1}) was also added for the

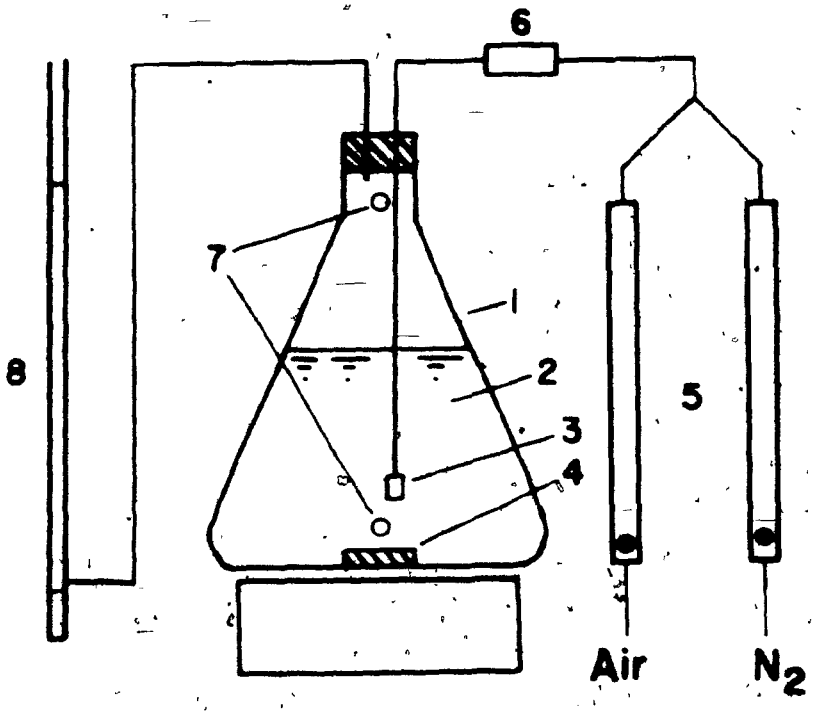
growth of inoculum cultures. For growth under anaerobic conditions, the medium with a combined nitrogen source was supplemented with 25 ug NO_3^--N mL^{-1} (1 ug NO_3^--N mL^{-1} for N_2O grown cultures) added as KNO_3.

An isolated colony from a nutrient agar plate (previously streaked from a thawed mini-slant) was used to inoculate 10 mL of inoculum medium in a 50-mL Erlenmeyer flask. After aerobic incubation on a gyratory shaker (12-18 h, 30°C, 300 rpm), 0.5 mL of this culture was used to inoculate 50 mL of the same medium in a 125-mL flask and incubated 12 h under similar conditions. These cells were harvested by centrifugation (8,000 x g, 4°C, 10 min) and washed twice with 30 mL portions of the sterile medium into which they were to be inoculated. The final pellet was resuspended in 10 mL of sterile medium and approximately 4.5 mL used to inoculate 900 mL (unless otherwise indicated) of the desired medium in a 1-L batch culture flask (Fig. 3). These cultures were maintained at 30°C, continuously stirred with a teflon-coated magnetic bar and sparged (at 450 mL min^{-1} unless otherwise indicated) with the appropriate gas mixture. Cultures at ambient oxygen levels were sparged with air, while for anaerobic cultures 100% N_2 was used (10% N_2O in N_2 for N_2O-grown cultures). For levels of oxygen lower than ambient (including 0.75% oxygen for N_2-fixing cultures) air and N_2 were mixed in the correct proportions using gas flowmeters. Oxygen levels higher than ambient were obtained by substituting 100% O_2 for air and mixing with N_2 accordingly. A bubble flowmeter attached to the exit line was used to monitor the gas flow-rate. Two ports, sealed with silicon-rubber reinforced Suba-seals (William R. Freeman and Co. Ltd., Barnsley, England), were used to sample the culture and gas phase.

The medium for growth of *E. coli* was the TSY medium of Hassan and Fridovich (1977d) consisting of 3.0% (w/v) Trypticase soy broth (Difco) and 0.5% (w/v) yeast extract (Difco) and sterilized by autoclaving at 121°C (15 min). An

Figure 3. Growth vessel and sparging apparatus for batch cultures of *A. brasilense*.

1. 1-L Erlenmeyer flask 2. medium (900 mL unless otherwise indicated) 3. fritted glass sparger 4. teflon-coated magnetic bar 5. gas flowmeters 6. sterile cotton filter 7. sampling ports 8. bubble flowmeter.


6
7
1
8
2
5
3
4
Air
N_2

isolated colony from a nutrient agar plate was used to inoculate 10 mL TSY medium in a 50-mL Erlenmeyer flask. After 12 h of incubation aerobically on a shaker (37°C, 250 rpm), 0.5 mL of this culture was used to inoculate a 100 mL batch culture of the same medium in a 500-mL flask and incubated similarly.

2.3 Preparation of Cell-Free Extract

Cultures in late-log phase (unless otherwise indicated), were divided into two (or more) samples of equal size and the cells harvested by centrifugation (10,000 x g, 4°C, 10 min). After washing three times with 50 mM phosphate buffer (pH 7.8) containing 10^{-4} M ethylenediaminetetraacetic acid (PB/EDTA), the final pellet was resuspended in 7.0 mL of buffer and the cells disrupted by two runs through a French press (American Instrument Co., Silver Spring, MD, USA) at 12,000-16,000 psi (83,000-110,000 kPa). Cell debris was removed by centrifugation (60,000 x g, 4°C, 60 min) and the supernatant (cell-free extract) retained for enzyme analysis. Where indicated, the cell-free extract was dialyzed overnight in several changes of PB/EDTA (pH 7.8). The pellet, when required, was resuspended in 2.0 mL PB/EDTA using a homogenizer.

2.4 Enzyme Analysis

Enzyme assays were performed spectrophotometrically using a Gilford 240 spectrophotometer equipped with a temperature-controlled cell compartment at 25°C.

2.4.1 Catalase

Catalase was measured by the spectrophotometric method of Beers and Sizer (1952). An appropriate amount of cell-free extract (sufficient to produce a significant rate) was suspended in approximately 2.0 mL of 50 mM phosphate buffer (pH 7.0). The volume of stock H_2O_2 (0.4 mL 30% H_2O_2 in 50 mL phosphate buffer) required to produce a final concentration of 17 mM was

determined immediately prior to the assay using the extinction coefficient, thus eliminating problems due to H_2O_2 decomposition during storage. The exact amount of phosphate buffer used to suspend the cell-free extract was adjusted so as to result in a final volume of 3.0 mL upon addition of H_2O_2. The rate of decomposition was determined by following the decrease in absorbance at 240 nm over time. One unit of activity is expressed as 1 umol H_2O_2 decomposed per minute at 25°C, calculated using the extinction coefficient for H_2O_2 of 43.6 $M^{-1}cm^{-1}$. Only the initial (linear) portion of the trace was used to determine the rate and the mean of a minimum of three runs (per sample) was used in the calculation. Specific activity is expressed as units (U) per milligram cell-free extract protein. Data are the mean ± SE of two or more cultures each assayed in duplicate.

2.4.2 Peroxidase

The activity of o-dianisidine peroxidase was determined according to the Worthington Enzyme Manual (Worthington Biochemical Corp., 1972) except that o-dianisidine-HCl was used. To 6.0 mL of substrate (10 uL of 30% H_2O_2 in 100 mL of 10 mM phosphate buffer pH 6.0), 0.05 mL of a solution of o-dianisidine-HCl (1% w/v) in phosphate buffer was added. A 2.9 mL aliquot of this mixture was transferred to the cuvette and the changes in absorbance at 460 nm were followed upon addition of 0.10 mL of the cell-free extract. One unit of activity is expressed as 1 umol H_2O_2 decomposed per minute at 25°C based on an extinction coefficient for H_2O_2 of 1.13×10^4 $M^{-1}cm^{-1}$ at 460 nm. The rate used in the calculation is the mean of duplicate assays for each sample. Specific activity is expressed as units (U) per 100 milligrams of cell-free extract protein. Data are the mean ± SE of two or more cultures each assayed in duplicate. Controls for non-specific dye oxidation were obtained by omitting H_2O_2 from the reaction mixture.

A second peroxidase assay with 3,3'-diaminobenzidine (DAB) as the

H-donor, was kindly provided by Dr. F. Archibald. The assay mixture contained 0.5 mM DAB and 2.0 mM H_2O_2 in 50 mM phosphate buffer (pH 7.2). The reaction was started by the addition of 0.10 mL cell-free extract to 2.9 mL of the reaction mixture and the subsequent changes in absorbance at 482 nm were followed. The activities are expressed as the change in absorbance per minute per milligram of cell-free extract protein and are the mean ± SE of duplicate cultures each assayed in duplicate.

2.4.2 Superoxide Dismutase

Superoxide dismutase (SOD) activity was determined by the method of McCord and Fridovich (1969) as described by Crapo et al. (1978). Reaction mixtures contained 50 uM xanthine and 10 uM oxidized cytochrome c (as determined using the extinction coefficient of Massey (1959) of 21,000 $M^{-1}cm^{-1}$ at 550 nm) in 3.0 mL PB/EDTA (pH 7.8). The amount of xanthine oxidase required to produce a rate of reduction of cytochrome c of 0.025 absorbance units (AU) per min at 550 nm was determined prior to the assay. Various amounts of cell-free extract were added to the reaction mixture (replacing an equal amount of buffer) to determine the amount necessary to inhibit this rate of reduction by 50%. This amount is defined as one unit and was used to calculate the specific activity, expressed as units (U) per milligram of cell-free extract protein. Data are the mean ± SE of two or more cultures each assayed in duplicate. The effect of added cell-free extract on the xanthine/xanthine oxidase reaction was determined by monitoring its effect on product formation (urate) which absorbs at 295 nm.

2.5 Visualization of SOD Activity by Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis was performed on 10% polyacrylamide gels prepared according to the method of Davis (1964) as described by Shuster (1971). The separating gels were prepared in 0.375 M Tris-HCl buffer (pH 8.9) and

contained 10% (w/v) acrylamide, 0.03% (w/v) N,N-methylene-bis-acrylamide, 0.03% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED) and 0.04% (w/v) ammonium persulfate. This mixture was deoxygenated under vacuum and cast into 8 cm lengths in 10-cm acid-washed glass tubes (6 mm ID). The spacer gel consisted of 3.5% (w/v) acrylamide, 0.06% (w/v) bis-acrylamide, 0.06% (v/v) TEMED and 0.005% (w/v) riboflavin in 0.062 M Tris-HCl (pH 6.8). After polymerization of the separating gel, 0.20 mL of the spacer gel mixture (deoxygenated) was layered on top and photopolymerized under fluorescent light. Completed gels were placed in a Biorad model 150 disc electrophoresis apparatus maintained at 4°C with 0.025 M Tris-glycine buffer (pH 8.3) as the electrode buffer. Gels were pre-run at 4 mA/gel for 30 minutes prior to the addition of sample.

Samples were prepared in spacer buffer (0.062 M Tris-HCl, pH 6.8) and contained 100 ug cell-free extract protein, 5 mg bromophenol blue and 20% (v/v) glycerol in a total volume of 100 uL. Samples were layered carefully on the top of the gel and run at 2 mA/gel (1 mA/gel for the initial 30 minutes) until the bromophenol blue marker had traversed to very near the end of the tube. The apparatus was then disassembled and the gels removed from the glass tubes.

The stain for SOD activity was that of Beauchamp and Fridovich (1971). Gels were soaked in 2.45×10^{-3} M nitrotetrazolium blue for 20 minutes in screw cap tubes, followed by a brief rinse with distilled water. They were then soaked in a solution containing 0.028 M TEMED and 2.8×10^{-5} M riboflavin in 0.036 phosphate buffer (pH 7.8) for 20 minutes. The tubes were then drained and the gels illuminated for 5-15 minutes (depending on colour development) by a 20 W flourescent lamp in a foil-lined box. Achromatic zones in the uniformly blue gels (indicating SOD activity) were measured for their distance from the origin. Sensitivity to inhibitors was tested by soaking the

gels in 5.0 mM H_2O_2 or 1.0 mM CN^- (in PB/EDTA pH 7.8) for 60 minutes prior to staining. Control gels were soaked in phosphate buffer that did not contain either inhibitor. Gels were photographed or scanned at 560 nm using a Gilford 240 spectrophotometer with a linear transport accessory.

2.6 Analyses

Growth of the cultures was followed by changes in optical density at 430 nm (*A. brasilense*) or 600 nm (*E. coli*) using a Spectronic 20 spectrophotometer (Bausch and Lomb). Oxygen levels in the gas phase of N_2-fixing cultures (0.75% O_2 in N_2) were monitored by gas chromatography as described by Brouzes et al. (1971). A 0.5 mL sample, removed from the gas phase by syringe, was injected into a Fisher-Hamilton Model 29 gas partitioner with a 183 cm x 6 mm column of Chromosorb P coupled to a 198 cm x 5 mm molecular sieve and a thermal conductivity detector. The carrier gas was He (40 mL min^{-1}) and the column temperature was ambient. Higher oxygen levels were monitored with a Servomex paramagnetic oxygen analyzer (Servomex Controls Ltd., Crowborough, Sussex, England) previously calibrated with air and N_2. Dissolved oxygen concentrations in the culture were determined by a sterilizable galvanic dissolved oxygen probe (New Brunswick Scientific, New Brunswick, NJ, USA), calibrated by sparging the medium with air and N_2 prior to inoculation.

For determination of NO_2^- and NO_3^- levels in anaerobic cultures, samples were filtered to remove the cells (0.22 um membrane filter) and frozen until they were analyzed by the Griess-Ilosvay method (sulfanilamide reagent with and without reduction with hydrazine copper) using an autoanalyzer (Chemlab Instruments Ltd., Hornchurch, Essex, England).

Nitrous oxide production by denitrifying cultures was detected by injection of 0.2-mL gas phase samples into a Perkin-Elmer Model 3920 gas chromatograph with an electron capture detector and a 200 cm x 6 mm column

of Poropak Q. The carrier gas was 5% CH_4 in Ar (30 mL min^{-1}) and the column temperature was 65°C. The concentration of N_2O in N_2O-sparged cultures of *A. brasilense* was monitored by injection of 0.5-mL samples of the gas phase into a Hewlett-Packard Model 5750 gas chromatograph equipped with a thermal conductivity detector and a 200 cm x 6 mm column of Poropak Q. The carrier gas was He (46 mL min^{-1}) and the column temperature was 60°C.

Protein was analyzed by the method of Lowry et al. (1951) except that sodium citrate was used in the place of sodium tartrate (Eggstein and Kreutz, 1955). Bovine serum albumin was used as the standard.

2.7 Chemicals

All special gases (O_2, N_2 and 10% N_2O in N_2) for sparging gas mixtures were obtained from Liquid Carbonic, St. Laurent, Québec H4X 1X1. Xanthine, xanthine oxidase, cytochrome c (type IV), superoxide dismutase, o-dianisidine-HCl and nitroblue tetrazolium were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Horseradish peroxidase was obtained from Boeringer-Mannheim Canada (Dorval, Québec) and the 30 H_2O_2 and KCN came from Fisher Scientific Ltd. (Montréal, Québec). The H-donor 3,3'-diaminobenzidine was kindly provided by Dr. F. Archibald.

3. RESULTS

Preliminary tests were carried out to determine the suitability of *A. brasilense* cell-free extract for SOD analysis. Retention of SOD activity by the cell-free extract after overnight dialysis in PB/EDTA (pH 7.8) indicated that it is a true enzymatic activity rather than a manifestation of high levels of O_2^--scavenging metals such as Mn^{2+} (Archibald and Fridovich, 1981). To ensure that the decrease in the rate of cytochrome c absorbance was not due to an effect of the cell-free extract on O_2^- generation, the rate of product (urate) formation by the xanthine/xanthine oxidase reaction was followed at 295 nm in the presence and absence of an amount of cell-free extract equal to 1.0 U SOD. No inhibitory effects were found using cell-free extract from any of the growth conditions used in this study. Cytochrome oxidase can be a problem in some SOD assays since it can reoxidize cytochrome c reduced by O_2^-, mimicking the inhibition of reduction by SOD. The addition of 10^{-6} M CN^- to the reaction mixture (which inhibits cytochrome oxidase but has no effect on SOD) had no effect on the assay, indicating the absence of interference of this type. Low molecular weight compounds capable of chemically reducing cytochrome c were also not a problem in these assays.

3.1 Enzyme Activities in Cell-Free Extracts

3.1.1 Ammonium-grown cells

Growth curves (Fig. 4) showed that ammonium-grown and N_2-fixing *A. brasilense* had generation times of approximately 2 and 7 h, respectively, while that for *E. coli* (Fig. 5) was 30 min. In general, the stages of growth as determined by optical density or protein content (mg mL^{-1}) were very similar, indicating that optical density was a satisfactory method for monitoring culture growth.

The activities of peroxidase (PER), catalase (CAT) and superoxide dismutase

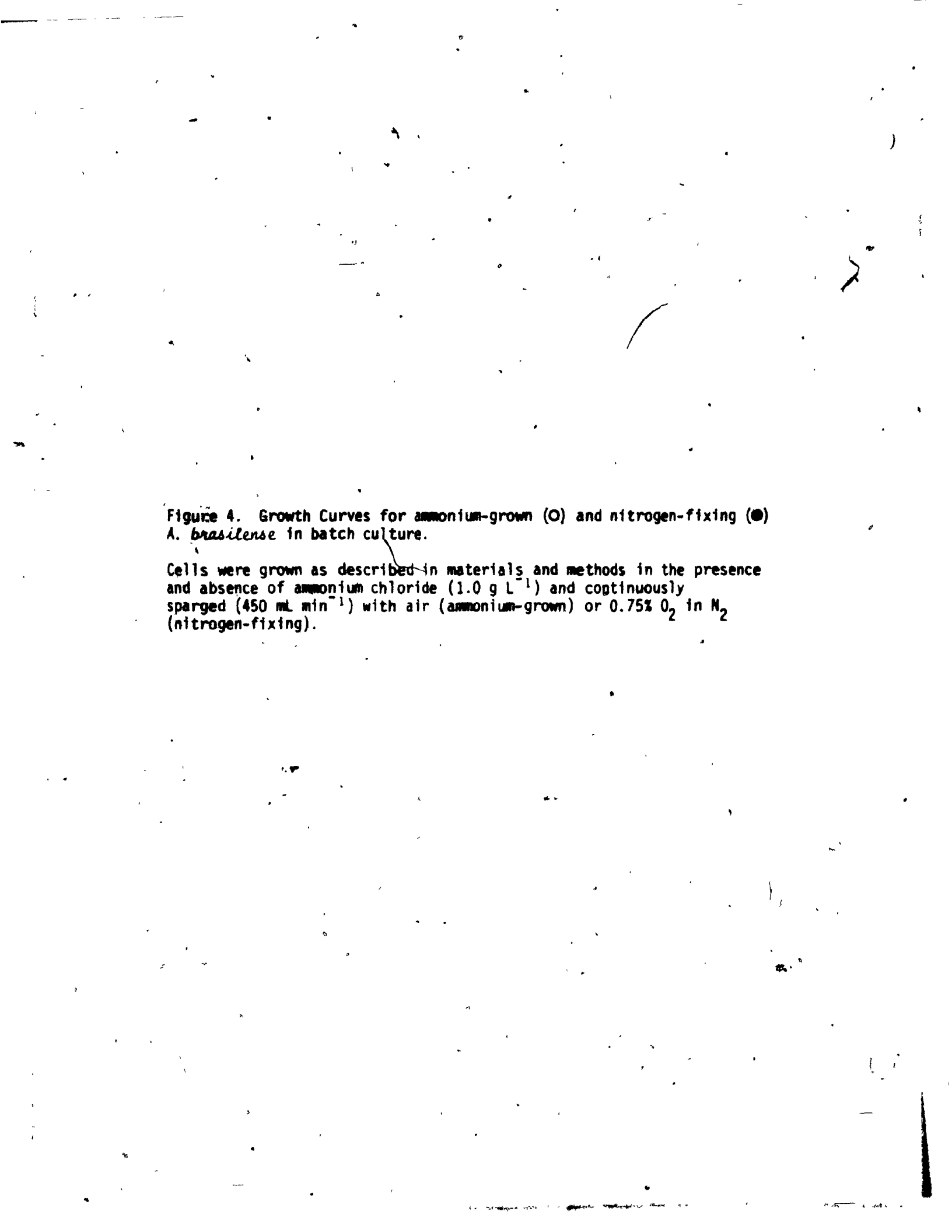


Figure 4. Growth Curves for ammonium-grown (O) and nitrogen-fixing (●) *A. brasilense* in batch culture.

Cells were grown as described in materials and methods in the presence and absence of ammonium chloride (1.0 g L^{-1}) and continuously sparged (450 mL min^{-1}) with air (ammonium-grown) or 0.75% O_2 in N_2 (nitrogen-fixing).

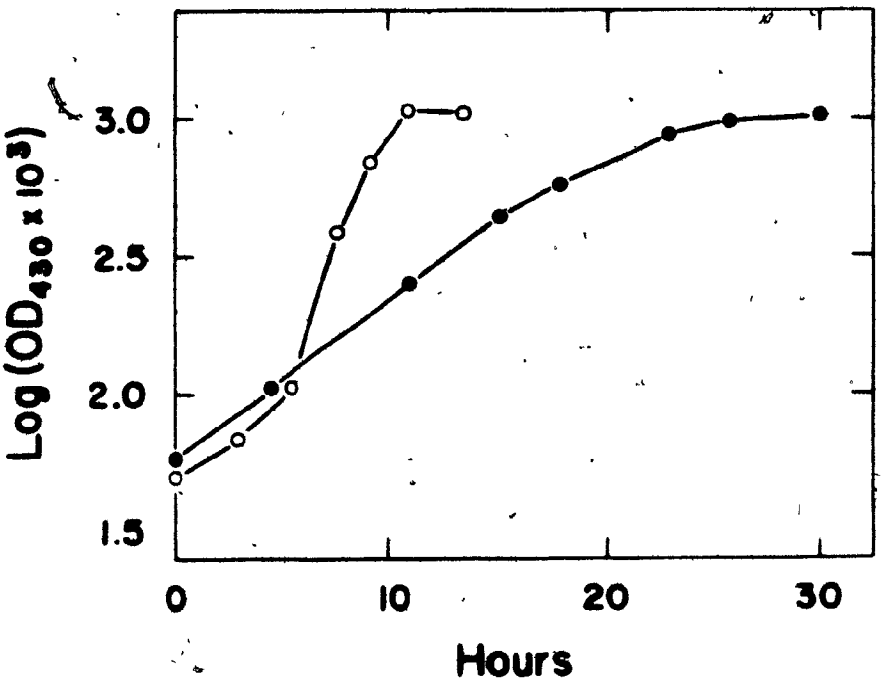
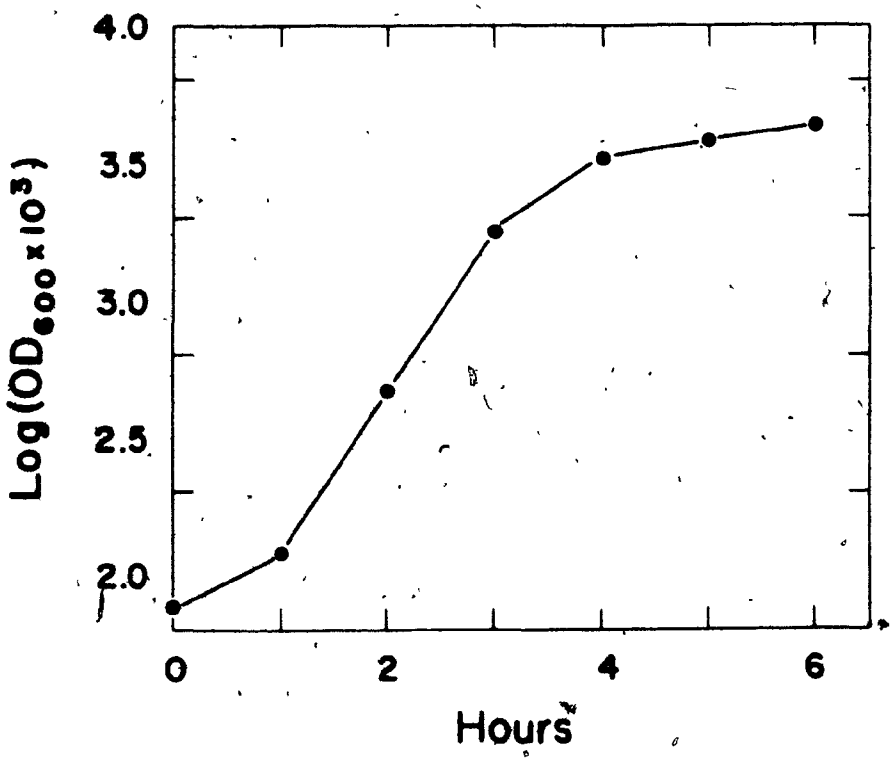

Log (OD430 x 10³)
3.0
2.5
2.0
1.5
0
10
20
30
Hours

Figure 5. Growth of *E. coli* K12 in batch culture.

Cells were grown aerobically at 37°C on a gyratory shaker (250 rpm) in 100 mL of TSY medium in a 500-mL Erlenmeyer flask. Samples with high cell density were diluted appropriately with sterile TSY medium.


4.0
3.5
3.0
2.5
2.0
Log(OD600 x 10³)
0
2
4
6
Hours

(SOD) in late-log phase A. *brasilense* grown aerobically on ammonium, are compared to those of aerobically-grown *E. coli* (as reference) in Table 1. No o-dianisidine PER activity was found in A. *brasilense*, though such activity was easily detected in *E. coli* and horseradish peroxidase. A slightly higher level of CAT was found in aerobically-grown A. *brasilense* as compared to *E. coli*, while the reverse was true for SOD activity.

The changes in CAT and SOD activity during aerobic growth of A. *brasilense* are shown in Fig. 6. CAT activity was high in early to mid-log cells but decreased steadily during growth to relatively low levels in the stationary phase. SOD levels decreased only slightly over the same period, but increased in the stationary phase. PER activity was not detected.

The possibility that the change in CAT activity during growth might be due to changes in dissolved oxygen content of the culture was examined. During growth of A. *brasilense* under the conditions in Fig. 6, the concentration of dissolved oxygen decreased from 100% air saturation in the early stages to approximately 26% in late-log phase (Fig. 7). The level returned to air-saturated conditions in the stationary phase. This substantial decrease in the dissolved oxygen content could be avoided by increasing the sparging and stirring rate of the culture to increase the efficiency of aeration (Fig. 8). In this case the concentration of dissolved oxygen decreased only slightly (approximately 10%) at the highest cell density. Cells grown under these two conditions were harvested in late-log phase (where the differences in dissolved oxygen content were greatest) and their enzyme activities compared (Table 2). CAT and SOD levels did not differ significantly while PER was not detected in either case.

The response of the enzyme activities to oxygen was examined by changing the sparging gas oxygen content (Table 3). Increasing the oxygen content to 60% resulted in a slight increase in SOD activity but no significant change in

Table 1. Peroxidase, catalase and superoxide dismutase activities in aerobically-grown, late-log phase cells of A. brasilense and E. coli K12.*

Organism	PER (100 mg)	CAT mg	SOD mg
A. brasilense	ND	18.9 ± 1.0	12.7 ± 0.7
E. coli K12	4.9 ± 1.3	13.4 ± 0.7	19.6 ± 1.0

*Cells were grown as in Fig. 4 (ammonium) or Fig. 5 and harvested in late-log phase for analysis of enzyme activities. Data are the mean ± SE of duplicate cultures. ND - not detected.

Figure 6 Changes in catalase (□) and superoxide dismutase (△) activities during aerobic growth (●) of A. brasilense

Cells were grown in 600 mL of medium containing ammonium chloride (1.0 g L^{-1}) and sparged with air (225 mL min^{-1}). At the appropriate times, the cells were harvested by centrifugation and enzyme activities determined as described in materials and methods

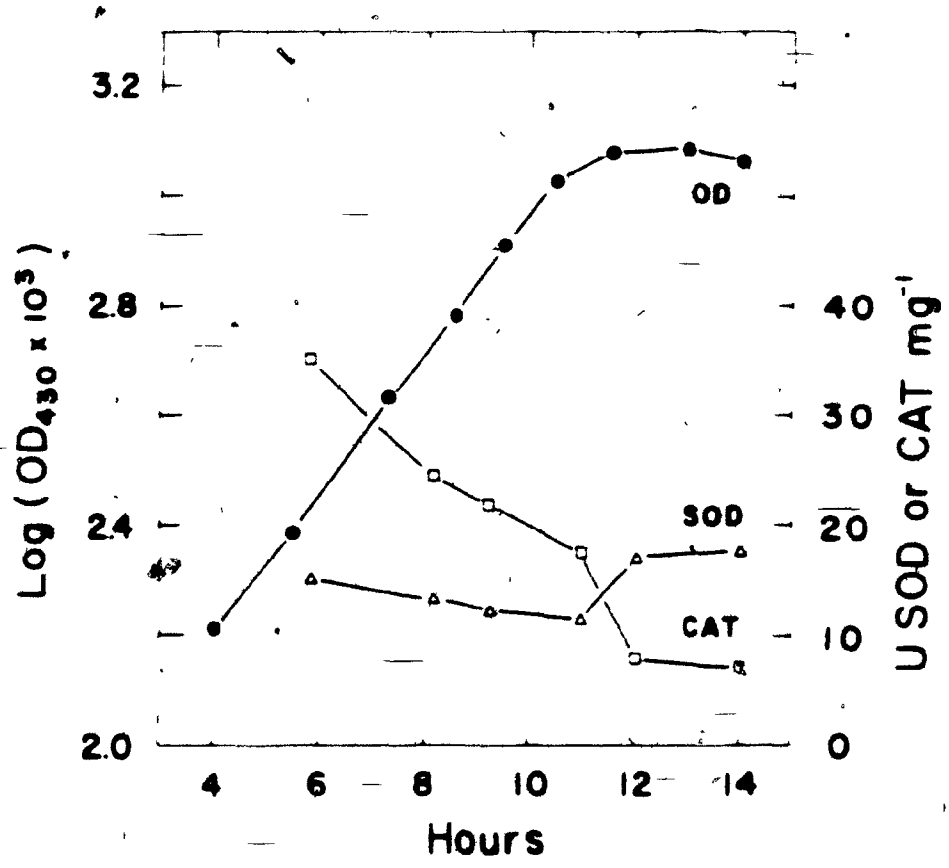

3.2
2.8
2.4
2.0
Log (OD_{430} x 10^3)
OD
SOD
CAT
40
30
20
10
0
U SOD or CAT mg^{-1}
4
6
8
10
12
14
Hours

Figure 7. Changes in dissolved oxygen content (O) during aerobic growth (●) of *A. brasilense* sparged at 225 mL min^{-1}.

Cells were grown as in Fig. 6 with a galvanic dissolved oxygen probe (New Brunswick Scientific Ltd.) for the measurement of dissolved oxygen. Duplicate cultures were examined with typical results shown.

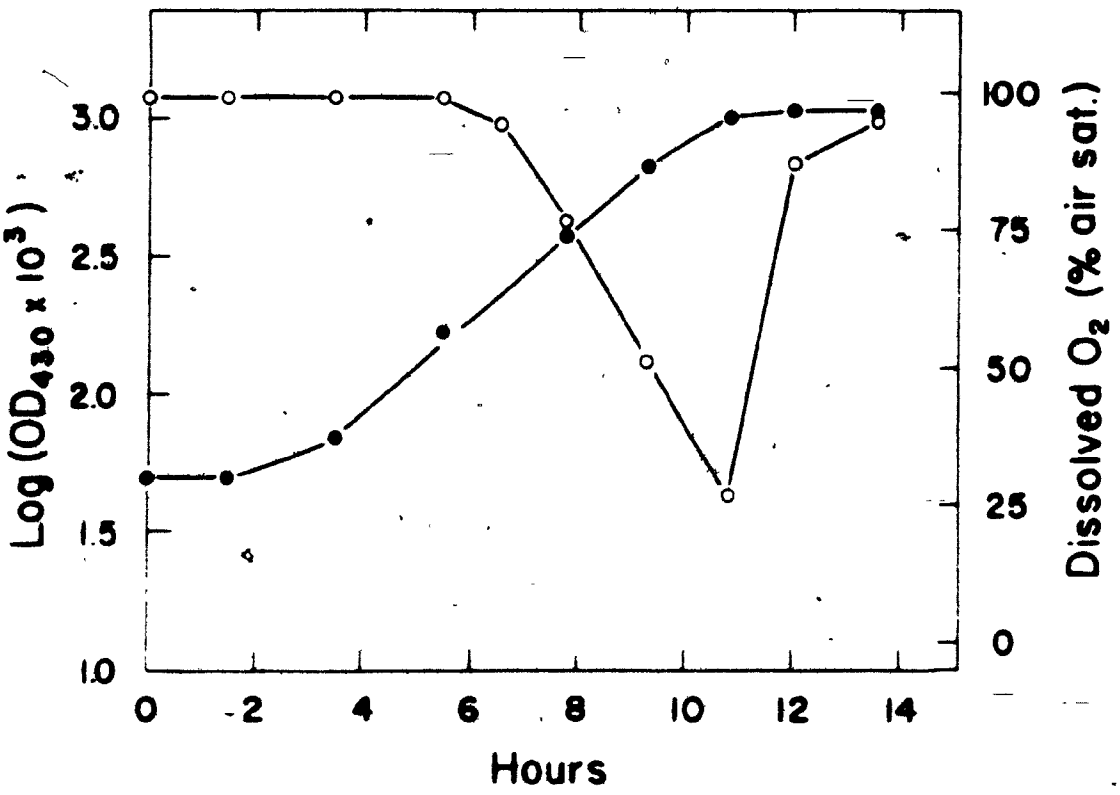

Log ($OD_{430} \times 10^3$)
3.0
2.5
2.0
1.5
1.0
0
-2
4
6
8
10
12
14
Hours
100
75
50
25
0
Dissolved O_2 (% air sat.)

Figure 8. Changes in dissolved oxygen content (○) during aerobic growth (●) of *A. brasiense* sparged at 450 mL min^{-1}.

Conditions similar to those in Fig. 7 except that the culture was sparged with air at 450 mL min^{-1} and stirred more vigourously. Duplicate cultures were examined with typical results shown.

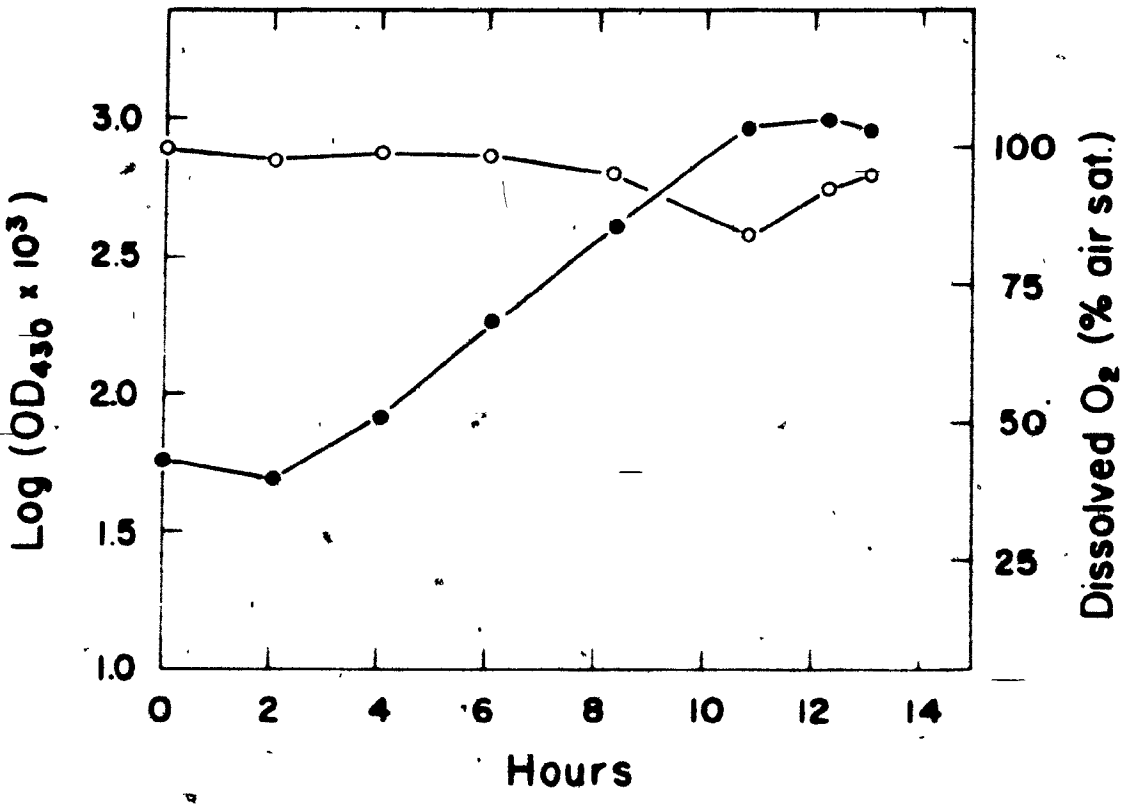

3.0
2.5
2.0
1.5
1.0
Log (OD436 x 10³)
100
75
50
25
Dissolved O2 (% air sat.)
0
2
4
6
8
10
12
14
Hours

Table 2. Effect of sparging and stirring rate on the enzyme activities in late-log phase cells of aerobically-grown *A. brasilense*.[1]

Sparging Rate mL min^{-1}	Stirring Rate	PER U $(100\ mg)^{-1}$	CAT U mg^{-1}	SOD U mg^{-1}
225	low	ND	17.7 ± 2.6	11.3 ± 0.3
450	high	ND	18.9 ± 1.0	12.7 ± 0.7

[1]Cells were grown as in Figs. 7 and 8 with the sparging and stirring rates as indicated. Late-log phase cells were harvested and enzyme activities determined as in materials and methods. Data are the mean ± SE of duplicate cultures. ND - not detected.

Table 3. Effect of sparging gas oxygen content on the enzyme activities in late-log phase cells of aerobically-grown *A. brasilense*.[1]

Oxygen Content	PER U $(100\ mg)^{-1}$	CAT U mg^{-1}	SOD U mg^{-1}
60	ND	17.8 ± 0.2	15.2 ± 0.5
21	ND	18.9 ± 1.0	12.7 ± 0.7
10	ND	23.9 ± 1.2	13.0 ± 3.6

[1]Cells were grown as described in materials and methods in the presence of ammonium chloride (1.0 g L^{-1}) and sparged with the indicated gas mixture (450 mL min^{-1}). Data are the mean ± SE of duplicate cultures. ND-not detected.

CAT activity relative to air-sparged cultures (21% O_2). Decreasing it to 10% oxygen produced an increase in CAT activity but had no effect on the SOD level. PER activity was not detected under any of these conditions.

3.1.2 Nitrogen-fixing cells

The enzyme activities in N_2-fixing A. *brasilense* is shown in Table 4 and compared to cells grown under the same conditions except with ammonium chloride (1.0 g L^{-1}). PER activity was detectable but low in both cases. CAT activity in cells grown on ammonium under these conditions was strikingly higher than that of N_2-fixing cells or cells grown similarly at ambient (or higher) oxygen tensions (Tables 1 and 3). SOD activity in both cases was lower than that found in cells grown aerobically on ammonium, though of the two, N_2-fixing cells had slightly higher activity.

3.1.3 Anaerobically-grown cells

For growth under denitrifying conditions, the medium containing NH_4Cl was supplemented with nitrate and sparged with N_2 (Fig. 9). Growth proceeded initially using NO_3^- as the terminal electron acceptor, reducing to NO_2^-, which was further reduced to N_2O. Growth stopped when the supply of electron acceptor was exhausted. The generation time for denitrifying cultures was 6-7 h. Under these conditions, N_2O was not reduced further (to N_2) since as a gas it was swept out of the culture. For growth using N_2O as the terminal electron acceptor, a continuous supply of N_2O was provided by sparging with 10% N_2O in N_2 resulting in an N_2O concentration of 1.9 mM in solution as calculated using the Ostwald coefficient (Wilhelm et al., 1977). A small amount of nitrate (which was rapidly exhausted) was apparently required to aid in the induction of the N_2O reductase. The generation time for N_2O-grown cells was approximately 4 h (Fig. 10).

The enzyme activities were examined in cells grown anaerobically as in

Table 4. Effect of nitrogen source on the enzyme activities in late-log phase cells of A. *brasilense* sparged with 0.75% O_2.[1]

Nitrogen Source	PER U $(100\ mg)^{-1}$	CAT U mg^{-1}	SOD U mg^{-1}
NH_4Cl	.07 ± .01	52.9 ± 4.1	6.2 ± 0.2
N_2	.04 ± .01	6.3 ± 0.9	8.3 ± 0.1

[1]*A. brasilense* was grown as described in materials and methods in the presence and absence of ammonium chloride and sparged with 0.75% O_2 in N_2 (450 mL min^{-1}). Data are the mean ± SE of duplicate cultures.

Figure 9. Characteristics of growth of *A. brasilense* under denitrifying conditions.

- ● Optical Density
- □ Nitrate
- ■ Nitrite
- ○ Nitrous Oxide

Cells were grown using medium supplemented with ammonium chloride (1.0 g L^{-1}) and nitrate (25 ug NO_3-N mL^{-1}) and sparged with N_2 (120 mL min^{-1}). Samples were taken at various times for analysis of nitrate and nitrite. Nitrous oxide in gas phase samples was determined by gas chromatography.

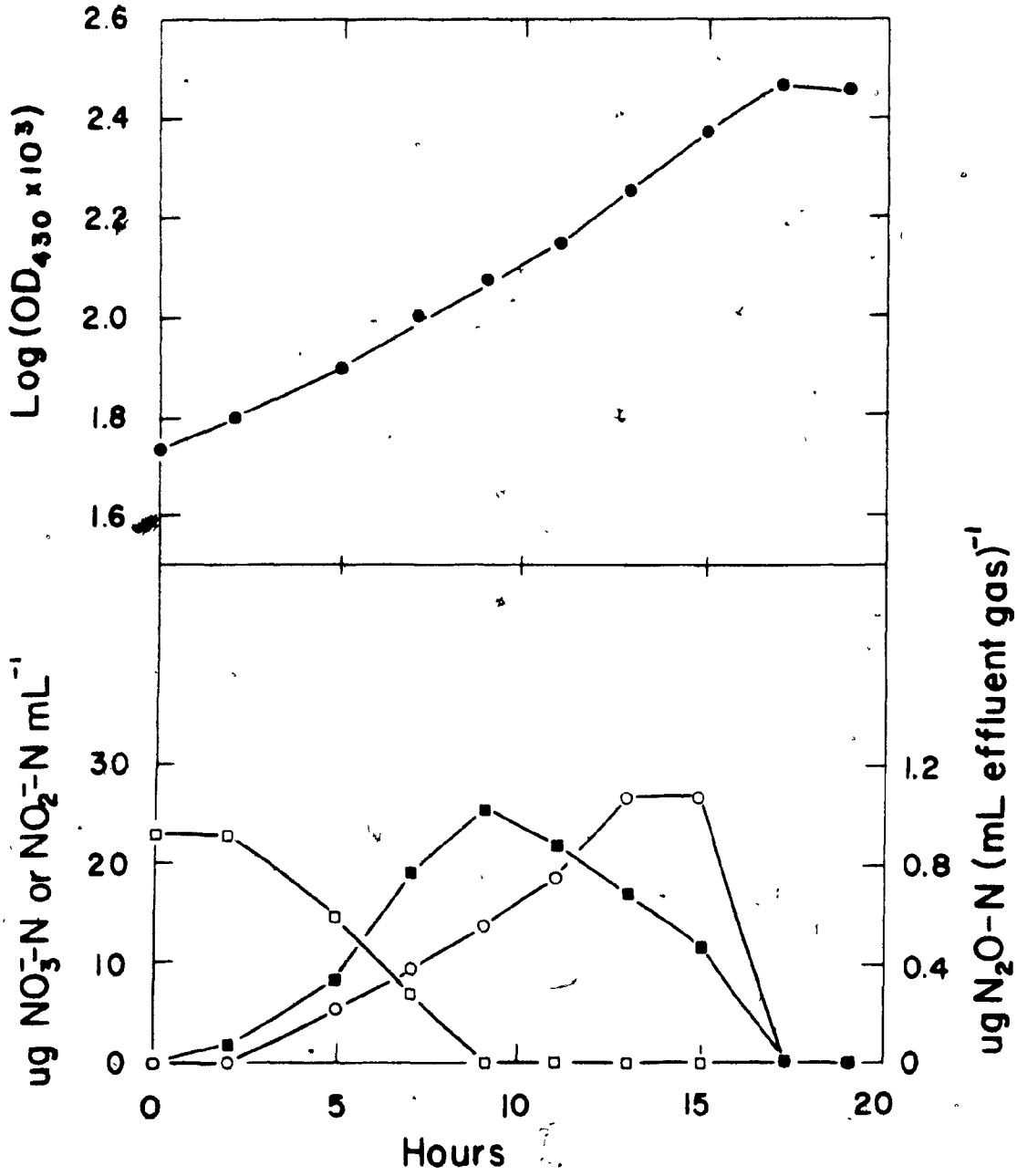
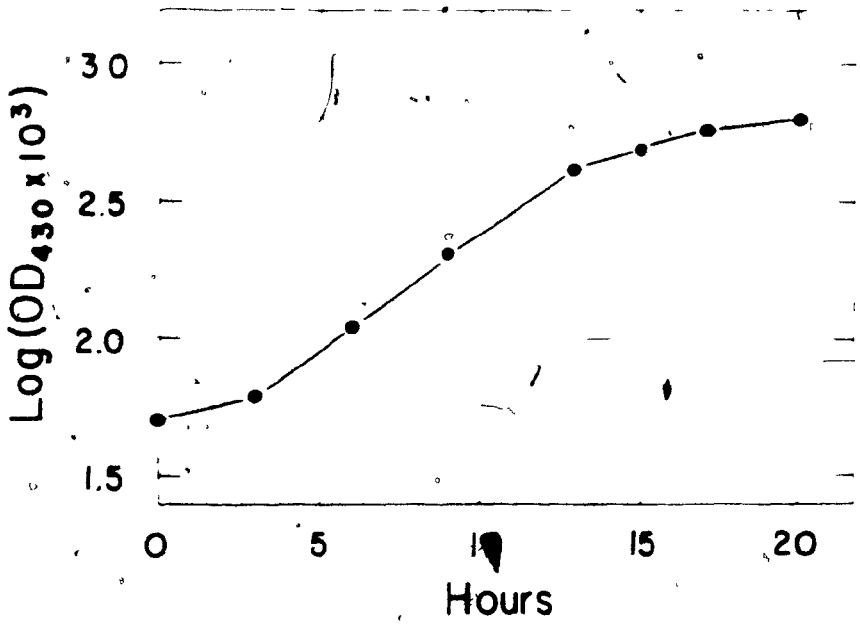

Log (OD430 x 10³)
2.6
2.4
2.2
2.0
1.8
1.6
ug NO3-N or NO2-N mL-1
30
20
10
0
ug N2O-N (mL effluent gas)-1
1.2
0.8
0.4
0
0
5
10
15
20
Hours

Figure 10. Growth of A. *brasilense* using nitrous oxide as the terminal electron acceptor.

Cells were grown as in Fig. 9 except that only 1 ug NO_3-N mL^{-1} was used and the sparging gas was 10% N_2O in N_2 (25 $mL \cdot min^{-1}$).


Log (OD430 x 10³)
3.0
2.5
2.0
1.5
0
5
15
20
Hours

Figs 9 and 10 Table 5 The activity of o-dianisidine PER was low under both conditions CAT activity in denitrifying cultures was the highest detected under any of the conditions tested, and contrasted with the low level in N_2-grown cells SOD activity was present in both conditions, although it was slightly higher in N_2O-grown cells

c 2 Detailed Study of Peroxidase Activity

Since peroxidase is a heme enzyme (Saunders et al., 19[illegible], CN^- sensitivity is often used to identify 'true' peroxidase activity (Putter, 1974 This aspect of PER activity and the possibility that activity was present in the high-speed pellet fraction were examined (Table 6 The low levels of o-dianisidine PER in cell-free extract from both ammonium-grown and N_2-fixing cells proved to be CN^- resistant The latter also had partial activity in the absence of the substrate H_2O_2, indicating the presence of non-specific dye oxidation. The cell-free extract from denitrifying cells had somewhat higher activity but much of it was also CN^- resistant. The PER activity found in the cell-free extract of aerobically-grown *E. coli* proved to be very sensitive to 1.0 mM CN^- and had no activity in the absence of H_2O_2. The pellets had evidence of "true" (CN^- sensitive) PER activity though still at low levels. The pellet from N_2-fixing cells had substantially higher activity than that of other cultures with one-third of the activity independent of H_2O_2. The pellet of *E. coli* did not exhibit any o-dianisidine PER activity.

A second PER assay, using 3,3'-diaminobenzidine (DAB) as the H-donor, was found to produce higher activities than the o-dianisidine assay with *A. brasilense* cell-free extract. To ensure that this increase in activity was not due simply to the change in buffer, the o-dianisidine PER activity in *A. brasilense* using both buffer systems was compared (Table 7). In cell-free extracts from ammonium-grown and N_2-fixing cells, no differences

Table 5. Effect of electron acceptor on the enzyme activities in late-log phase cells of anaerobically-grown *A. brasilense*.[1]

Electron Acceptor	PER U (100 mg)$^{-1}$	CAT U mg^{-1}	SOD U mg^{-1}
NO_3^-	.08 ± .01	79.8 ± 9.4	6.8 ± 0.2
N_2O	.03 ± .01	10.8 ± 0.5	9.1 ± 0.4

[1]Cells were grown as in Figs. 9 and 10 to late-log phase and enzyme activities were determined as in materials and methods. Data are the mean ± SE of two or more cultures.

Table 6. Cyanide sensitivity of o-dianisidine PER activity in cell free extracts and particulate material of *A. brasilense* and *E. coli* K12

Organism	Growth Condition	$\Delta A_{...}$ (mg protein) min^{-1}									
		Cell-Free Extract						Particulate Material			
		No change		+CN$^-$		H_2O_2		No change		+CN	
A. brasilense	Ammonium	0011	0001	0011	0002	0		0021	0003	0	
	N_2-fixing	0077	0002	0082	0006	0041	0003	0287	0016	0008	0001
	Denitrifying	0111	0023	0075	0019	0		0119	0003	0011	0011
E. coli K12	Aerobic	1805	0188	0127	0014	0		0		NT	NT

[1]The cell-free extracts and high-speed pellets (particulate material) of *A. brasilense* and *E. coli* K12 grown under the conditions indicated were assayed for o-dianisidine PER activity as described in materials and methods. Cyanide (1.0 mM) was added or H_2O_2 omitted from the reaction mixture where indicated. Data are the mean ± SE of duplicate cultures. NT not tested.

Table 7. Effect of buffer conditions on o-dianisidine PER activity in cell-free extracts of A. *brasilense*.

Growth Condition	ΔA_{460} (mg protein)$^{-1}$ min^{-1}	
	pH 6.0	pH 7.2
Ammonium	.0012 ± .0001	.0012 ± .0000
N_2-fixing	.0078 ± .0006	.0075 ± .0019

[1]Cell-free extracts of A. *brasilense* grown under the conditions indicated were assayed for o-dianisidine PER activity using 10 mM phosphate buffer (pH 6.0) or 50 mM phosphate buffer (pH 7.2) in the reaction mixture. Data are the mean ± SE of duplicate cultures.

in activity were found. These results also indicate that the low PER activities found in A. brasilense cell-free extracts were not due to the presence of a peroxidase enzyme sensitive to the slightly acid conditions of the o-dianisidine assay. The DAB peroxidase assay was used in a manner similar to that shown in Table 6 to examine PER activity (Table 8. The cell-free extract of ammonium-grown A. brasilense provides some evidence for CN^--sensitive PER activity but the activity in cell-free extracts from both denitrifying and N_2-fixing cells is entirely accounted for by CN^--resistant or H_2O_2-independent dye oxidation. Using this H-donor, PER activity in the cell-free extract from aerobically-grown E. coli was quite low, and was in fact lower than that found in A. brasilense cell-free extracts. All pellets, except that from E. coli, exhibited high rates of CN^--sensitive dye oxidation, but in all cases this activity was independent of the presence of H_2O_2.

3.3 Visualization and Inhibitor Sensitivity of Superoxide Dismutase Activity

The visualization of SOD activity in cell-free extracts using polyacrylamide gel electrophoresis is a powerful technique that can supply information regarding certain physical characteristics of the enzyme. For instance, it can resolve two or more forms of the enzyme in the cell if they are sufficiently different structurally to be separated. The type of SOD present in the cell-free extract (with respect to metal content) can be determined by taking advantage of their different sensitivity to inhibitors. Thus, CN^- inhibits CuZnSOD but not FeSOD or MnSOD and H_2O_2 inhibits FeSOD but has no effect on CuZnSOD or MnSOD (McEuen et al., 1980). The staining method is that of Beauchamp and Fridovich (1973) in which the presence of SOD is revealed by achromatic zones in the uniformly blue gels (Fig. 10). Using this technique (Table 9), it is evident that the cell-free extract of A. brasilense grown under all conditions has only one achromatic band (R_f

approximately 0.35) and that it is only sensitive to inhibition by H_2O_2. Aerobically-grown *E. coli* exhibits three bands (Rf 0.18, 0.27, 0.35), only two of which are inhibited by H_2O_2. Neither organism contains SOD activity sensitive to inhibition by CN^-.

Table 8 Assay and cyanide sensitivity of diaminobenzidine PER activity in cell-free extracts and particulate material of *A. brasilense* and *E. coli* K12[1]

Organism	Growth Condition	ΔA_{436} (mg protein)$^{-1}$ min^{-1}												
		Cell-Free Extract							Particulate Material					
		No change		+CN$^-$		$-H_2O_2$		$-H_2O_2$+CN$^-$	No change		+CN		$-H_2O_2$	$-H_2O_2$+CN
A. brasilense	Ammonium	0252	0006	0100	0009	0032	0004	NT	0305	0011	0035	0021	0290	0010	0
	N_2-fixing	0788	0046	0876	0040	0285	0009	0	0936	0036	0064	0064	0987	0101	0
	Denitrifying	0325	0002	0326	0047	0191	0007	0003	0003	1347	0656	0216	0216	1434	0720	0
E. coli K12	Aerobic	0167	0028	0075	0013	0020	0011	0010	0001	0033	0005	0010	0013	0		NT

[1]Cell-free extracts and high-speed pellets (particulate material) of *A. brasilense* and *E. coli* K12 grown under the conditions indicated were assayed for diaminobenzidine PER activity as described in materials and methods. Cyanide (1.0 mM) was added or H_2O_2 omitted from the reaction mixture as indicated. Data are the mean ± SE of duplicate cultures. NT-not tested

Figure 11. Visualization of superoxide dismutase activity of *A. brasilense* and *E. coli* K12 using polyacrylamide gel electrophoresis.

A. *A. brasilense* cell-free extract - untreated (CN^- treatment produced similar results)

B. *A. brasilense* cell-free extract - treated with H_2O_2 (also appearance of control without cell-free extract)

C. *E. coli* cell-free extract - treated with H_2O_2

D. *E. coli* cell-free extract - untreated (CN^- treatment produced similar results)

Cell-free extracts of *A. brasilense* and *E. coli* prepared as indicated in materials and methods were applied to 10% polyacrylamide gels and stained for SOD activity after electrophoresis. Sensitivity to inhibitors was tested by soaking the gels in CN (1.0 mM) or H_2O_2 (5.0 mM) in PB/EDTA pH 7.8 for 60 minutes prior to staining. Control gels were soaked in PB/EDTA containing no inhibitor. Photographs illustrate typical results.

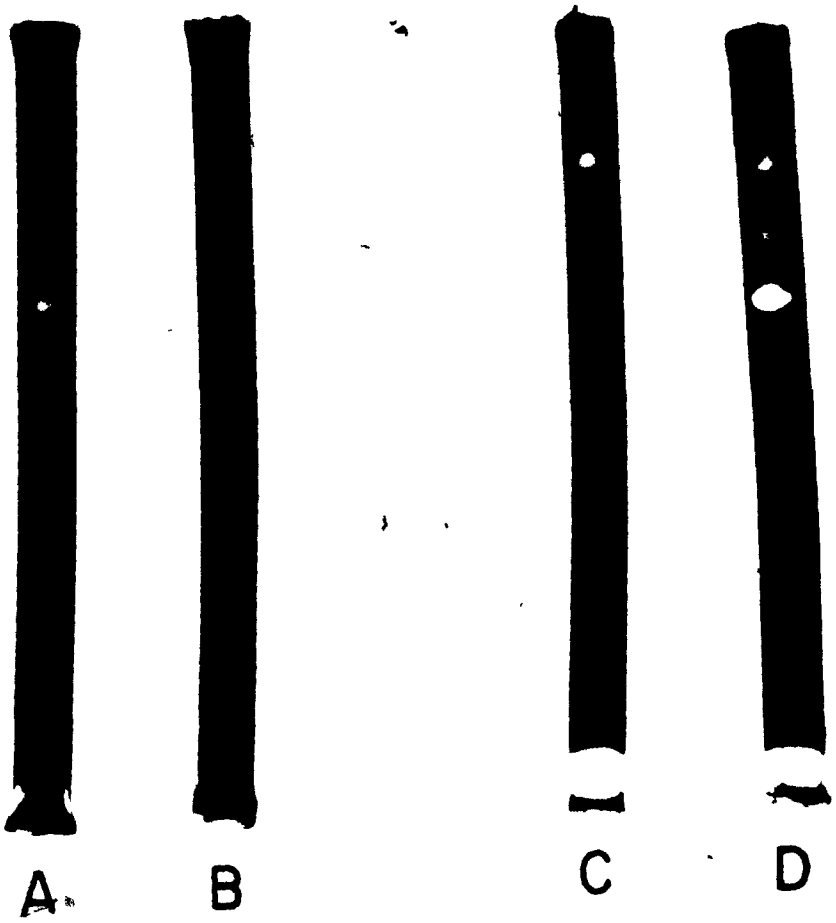

A
B
C
D

Table 9. Localization and inhibitor sensitivity of *A. brasilense* and *E. coli* K12 superoxide dismutase activity in polyacrylamide gels.[1]

Organism	Growth Condition	R_f	Inhibition by:	
			CN^-	H_2O_2
A. brasilense	ammonium	.35	-	+
	N_2-fixing	.33	-	+
	denitrifying	.35	-	+
E. coli K12	aerobic	.18	-	-
		.27	-	+
		.35	-	+

[1]The superoxide dismutase activity was visualized in 10% polyacrylamide gels as in Fig. 10. Data for R_f are the mean of results from duplicate cultures. Sensitivity to inhibitors was determined by complete or partial loss of the band in treated versus control gels using linear densitometry.

4. DISCUSSION

The intracellular production of highly reactive oxygen species and the presence of enzymes to deal with them have led to many modifications of the theories of oxygen toxicity (Halliwell, 1981; Morris, 1975). Nitrogen fixation is a well-known oxygen-sensitive process to which these concepts have only begun to be applied (Gallon, 1981; Yates, 1977). The results of this study establish the presence and certain characteristics of oxygen-protection enzymes in the free-living diazotroph A. *brasilense* sp7. Such information will be useful in the continuing investigation of their importance to nitrogen fixation and as a basis for the planning of further studies.

SOD and CAT activity were present in A. *brasilense* at levels which varied (in some cases considerably) according to the culture conditions. In cells grown aerobically on ammonium, the enzyme levels were comparable (with slight differences) to those in aerobically-grown *E. coli* K12 except for o-dianisidine PER activity, which was present only in the latter. In view of the current theories of oxygen toxicity, many organisms have been examined for their content of oxygen-protecting enzymes but there is little information available concerning factors governing their activity. One important factor is the intracellular concentration of their substrates or other substances derived from them (Hassan and Fridovich, 1977a). However, the nature and magnitude of intracellular generation of these species by different organisms, as well as the importance of external factors, is not well understood, and the enzyme content of the many organisms examined is varied and spans a wide range. Among other aerobic diazotrophs grown aerobically with a combined nitrogen source, *Azotobacter* spp. possess substantially higher SOD activity than *A. brasilense* (Asada et al., 1980) while that of *R. japonicum* is similar (Stowers and Elkan, 1981). *A. brasilense* strain cd grown in continuous culture had high SOD

activity but low CAT activity (Nur et al., 1982). However, comparisons such as these must be interpreted with caution due to differences in assay methods and the importance of external factors to enzyme levels (e.g. *R. japonicum* SOD levels vary according to the carbon source) (Stowers and Elkan, 1981).

There is no general trend that can be identified for the kinetics of synthesis of O_2-protecting enzymes and changes may simply reflect physiological changes unique to each organism. Thus, SOD activity in *R. japonicum* grown aerobically with a combined nitrogen source increased dramatically in mid- to late-log phase cells (Stowers and Elkan, 1981) while that in the obligate aerobe *Bdellovibrio stolpii* decreased during logarithmic growth and increased gradually during stationary phase (Von Stein et al., 1982). In the latter case, it was suggested that active repair mechanisms in log-phase cells obviate the need for O_2-protecting enzymes while the decreasing efficiency of such mechanisms in stationary-phase cells necessitates alternate means of oxygen protection. An increase in CAT and PER activity in *E. coli* during aerobic growth in TSY medium was attributed to the switch to respiratory metabolism when glucose for fermentation was exhausted (Hassan and Fridovich, 1978). Regulation of CAT activity in a number of organisms in the presence of glucose has been identified as catabolite repression (Hassan and Fridovich, 1978) though this has been questioned (Richter and Loewen, 1982). The variation in SOD and CAT activity during aerobic growth of *A. brasilense* in this study, could not be attributed to changes in the dissolved oxygen content of the culture and evidently results from undetermined physiological changes during growth.

Induction of SOD activity by oxygen is a major factor in support of its importance in oxygen toxicity. In *E. coli*, SOD activity increases with an increase in the oxygen tension, including levels higher than ambient (Gregory and Fridovich, 1973a; Hassan and Fridovich, 1977d). Other organisms in which SOD induction by oxygen has been demonstrated include *Streptococcus faecalis*

(Gregory and Fridovich, 1973a), *Spirochaeta aurantia* (Austin et al., 1981), the anaerobe *Bacteroides fragilis* (Privalle and Gregory, 1979), certain halophilic vibrios (Daily et al., 1978) and ammonium-grown *Azospirillum brasilense* strain cd (Nur et al., 1982). These increases are generally accompanied by a corresponding increase in resistance to the toxicity of hyperbaric oxygen or O_2^- generated by streptonigrin (Gregory and Fridovich, 1973a; 1973b; Privalle and Gregory, 1979) although not all of the above examples have been so tested. On the other hand, *Bacillus subtilis* SOD activity was not induced by increased oxygen and although CAT was induced, there was no increase in resistance to toxic hyperbaric oxygen (Gregory and Fridovich, 1973b). In the obligate aerobe *B. stolpii*, SOD is induced by higher oxygen tension only in the initial phases of growth (Von Stein et al., 1982). In this organism and in *E. coli*, constituitive SOD activity is provided by an FeSOD and oxygen induction affects only separate enzyme forms, such as MnSOD (*E. coli*) or other FeSOD isozymes. In this study, SOD activity in ammonium-grown *A. brasilense* doubled when conditions were changed from microaerobic to fully aerobic and there was also an increase in activity when oxygen levels higher than ambient were used. Induction of SOD activity by oxygen apparently involved only one FeSOD enzyme since only one band of SOD activity was found by electrophoresis of cell-free extracts from aerobically-grown cells.

The induction of CAT activity by oxygen is less clear. Increased CAT activity due to higher oxygen tension was found in *E. coli* K12 (Hassan and Fridovich, 1977d; Yosphe-Purer et al., 1977), *B. subtilis* (Gregory and Fridovich, 1973b), *Leptospira pomonas* (Rao et al., 1964), *Saccharomyces cerevisiae* (Sulebele and Rege, 1967; 1968) and two strains of *Streptococcus faecalis* (Jones et al., 1964) but not *E. coli* B (Gregory and Fridovich, 1973a) or *B. stolpii* (Von Stein et al., 1982). In *A. brasilense*, CAT activity increased as the oxygen content of the sparging gas was, in fact, lowered and was not

induced by levels higher than ambient. The stimulation of CAT activity by by this means was also found in *Neisseria meningitidis* (Archibald and DeVoe, 1978; Yu, 1980) and *Pseudomonas fluorescens* (Lenhoff and Kaplan, 1953). Although the CAT activity was quite low, a similar trend was found in *A. brasilense* strain cd (Nur et al., 1982). This implies that substrate induction is not the only controlling factor for CAT activity since H_2O_2 production would be expected to decline with a decrease in oxygen tension. The kinetics of CAT induction by O_2 in *E. coli* K12 led Yosphe-Purer et al. (1977) to suggest that it was in fact an indirect effect of a general increase in metabolic activity. Hassan and Fridovich (1978) demonstrated that the synthesis of CAT and PER in *E. coli* K12 was linked to the synthesis of electron transport components and independent of O_2 and H_2O_2. They found that mutants defective in certain respiratory proteins did not show normal synthesis of CAT until phenotypic normality was restored. The stimulation of respiratory proteins by low oxygen tension is well-documented in *E. coli* (Wimpenny et al., 1963), *N. meningitidis* (Archibald and DeVoe, 1978) and *P. fluorescens* (Lenhoff et al., 1956; Rosenburger and Kogut, 1958) and is suggested to increase the efficiency of respiration at low O_2 tension by increasing the number of target sites for oxygen (Archibald and DeVoe, 1978). Although growth conditions were slightly different than those used in this study, such an increase in *A. brasilense* sp7 cytochrome content in response to a decrease in oxygen tension has been demonstrated (Lalande and Knowles, unpublished). In view of the above arguments, this increase may account for the stimulation of CAT activity in *A. brasilense* at lower oxygen tensions.

Asada et al. (1980) did not find any difference in the SOD content of ammonium-grown or N_2-fixing *Azotobacter* spp. Evidently, higher enzyme levels were not required for the protection of nitrogenase and the high respiration rates attained during aerobic N_2 fixation did not result in O_2^- levels high

enough to induce SOD synthesis. In the non-heterocystous cyanobacterium *Gloeocapsa*, the activity of O_2-protecting enzymes (including SOD) was highest during maximum nitrogenase activity and were suggested to provide it with O_2 protection (Tozum and Gallon, 1979). In this case, the absence of alternate methods of protecting the nitrogenase may increase the importance of O_2-protecting enzymes in this function. Nitrogen-fixing cells of *A. brasilense*, with their requirement for low oxygen tension, had lower SOD activity than aerobically-grown cells but slightly higher activity than cells grown at a similar oxygen tension but with ammonium as a source of fixed nitrogen. It is tempting to speculate that this latter difference reflects the need for increased O_2 protection in cells actively fixing nitrogen but there is no direct evidence to substantiate this hypothesis. The low level of CAT in N_2-fixing *A. brasilense* as compared to cells grown similarly on ammonium highlights the physiological differences between the two states. The process of nitrogen fixation drains much of the available energy and reducing power, which is reflected in the longer generation time. The shunting of electrons to nitrogenase may result in lower H_2O_2 production and possibly lower cytochrome levels (if indeed involved) for the induction of CAT.

Under anaerobic conditions, O_2^- and H_2O_2 are not produced and the presence of their respective enzymes in organisms grown anaerobically has been a major stumbling block to the current theory of oxygen toxicity. SOD activity is found in anaerobically-grown *E. coli* (Gregory and Fridovich, 1973a); Hassan and Fridovich, 1977d) and is present at low levels in many strict anaerobes (Gregory and Dapper; 1980; Hewitt and Morris, 1975; Tally et al., 1977). The presence of SOD under these conditions indicates that induction is not entirely dependent on the presence of O_2^-. In this study, the SOD activity in anaerobically-grown cells is similar to that in cells grown microaerobically on ammonium and likely represents the level of activity

independent of the presence of O_2^-. The difference in SOD activity between the two types of anaerobic culture (NO_3^-- and N_2O-grown cells) indicates that certain physiological elements may also be important in the induction of SOD.

Production of CAT during anaerobic growth with NO_3^- as found here, was first demonstrated in *E. coli* K12 (Hassan and Fridovich, 1978). The absence of H_2O_2 production under anaerobic conditions and the respiratory nature of anaerobic growth by this organism was further evidence that the synthesis of CAT was independent of H_2O_2 and in fact regulated by the synthesis of respiratory proteins. Similar arguments may apply to A. *brasilense*, since anaerobic growth in the presence of NO_3^- in this organism is also respiratory in nature. It is interesting to note that cells so grown, which had the highest CAT activity of all the culture conditions examined, had higher cytochrome levels than aerobically-grown cells (Lalande and Knowles, unpublished). Similarly, lower CAT activity in N_2O-grown cells as compared to those grown with NO_3^- corresponds to lower cytochrome levels in these cells (Lalande and Knowles, unpublished).

It is evident on the basis of resistance to CN^- inhibition, that the low levels of o-dianisidine PER activity detected in some A. *brasilense* cell-free extracts do not represent "true" peroxidase activity. Although higher rates were obtained using DAB as the H-donor, the CN^--sensitive, H_2O_2-dependent activity was very low. The pellet fractions (except that from *E. coli*) did contain low but detectable levels of CN^--sensitive o-dianisidine PER activity, N_2-fixing cells in particular. The DAB assay proved to be unsuitable for these pellets due to the presence of a CN^--sensitive factor capable of H_2O_2-independent dye oxidation. The nature and significance of this o-dianisidine PER activity associated with the particulate material, although quite low, is not known. The fact that these assays were unable to detect significant PER activity does not preclude the possibility that such activity is present. Even in cases

where PER is present, the nature of the intracellular H-donor is often difficult to determine and may be sufficiently unique that there is no activity with the one present in the assay mixture. This is amply demonstrated in this study, in that *E. coli* cell-free extract clearly demonstrates peroxidase activity using o-dianisidine but with DAB as the H-donor its activity is much lower.

Only one achromatic band was produced by electrophoresis of the *A. brasilense* cell-free extract from any growth condition. This is strong but not conclusive evidence of a single enzyme, since in separation on the basis of size and charge it is possible for two structurally-distinct enzymes to co-migrate. Inhibition only by H_2O_2 indicates that the enzyme is an iron-containing SOD. Other diazotrophs that have been so examined, including *Azotobacter* spp. (Asada et al., 1980), *Rhizobium* spp. (Stowers and Elkan, 1981) and *Anabaena cylindrica* (Asada et al., 1980) also contain only FeSOD. Thus, results from *A. brasilense* resembles that from other diazotrophs and lends support to the suggestion of Asada et al. (1980) that FeSOD is associated with cells characterized by low intracellular O_2 concentrations such as aerobic diazotrophs. Three enzymes are resolved in *E. coli* grown aerobically, the fastest is FeSOD while the slowest is MnSOD. The middle achromatic band has been found to be a hybrid SOD containing one subunit from each of FeSOD and MnSOD (Fridovich, 1981). It is interesting to note the similarity of R_f between the FeSOD of *A. brasilense* and that of *E. coli*. Though this implies structural similarity, no such conclusions can be drawn on the basis of this technique, but it does provide rich ground for further study.

Although this study did not address the question of the importance of O_2-protecting enzymes directly to nitrogen fixation, it does provide a foundation upon which such studies can be based. Compounds capable of generating an intracellular flux of O_2^- would appear to be ideal tools (Hassan

and Fridovich, 1979). In addition to the testing of the susceptibility of nitrogenase to O_2^- in vivo, their ability to induce SOD and to a certain extent CAT and PER, offer a unique opportunity to demonstrate their contribution to the oxygen protection of nitrogenase. Assays to measure its oxygen sensitivity are available (Pedrosa et al., 1982; Tibelius and Knowles, 1983) but the presence of an appropriate diaphorase (the enzyme responsible for the reduction of the redox active compound) has yet to be demonstrated in A. *brasilense*. An increase in nitrogenase oxygen tolerance due to elevated levels of oxygen-protecting enzymes would be evidence of their contribution to its oxygen protection.

Another area deserving further study is the induction of CAT by low oxygen tension and its presence under anaerobic conditions. Arguments that CAT activity in A. *brasilense* may be closely linked to the synthesis of respiratory proteins have been presented. The presence of heme as the prosthetic group in both CAT and some respiratory proteins raises the possibility that it may have a regulatory function as well. Studies using *Saccharomyces cerevisiae* have found that oxygen, glucose and heme regulate the synthesis of a number of hemoproteins, including CAT, at the level of transcription (Hortner et al., 1982; Richter et al., 1980; Woloszczuk et al., 1980). There is also evidence that heme is involved in the control of certain post-translational events in some cases (Ross and Schatz, 1976; Salzgaber-Muller and Schatz, 1978). Thus, heme may be an important factor in the co-induction of respiratory proteins and CAT in A. *brasilense*.

To summarize, the presence of SOD and CAT has been demonstrated in ammonium-grown, N_2-fixing and denitrifying A. *brasilense* sp7. CAT and SOD activities varied during growth and in response to changes in culture conditions. PER activity using o-dianisidine or 3,3'-diaminobenzidine as the H-donor was mostly low or undetectable. Only a single achromatic band of SOD activity was evident in polyacrylamide gels, which proved to be FeSOD on the basis of inhibitor sensitivity.

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