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SUPEROXIDE RADICAL: AN ENDOGENOUS TOXICANT

Irwin Fridovich

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

INTRODUCTION

The Two Faces of Oxygen

Molecular oxygen is both benign and malign. On the one hand it provides enormous advantages and on the other it imposes a universal toxicity. This toxicity is largely due to the intermediates of oxygen reduction, i.e. O_2^-, H_2O_2, and $OH\cdot$, and any organism that avails itself of the benefits of oxygen does so at the cost of maintaining an elaborate system of defenses against these intermediates. We will here concern ourselves with the superoxide dismutases which, by catalytically scavenging O_2^-, provide a defense against it and against any reactive radical species which can be derived from it.

The Logic of Superoxide Dismutation

There is compelling evidence that superoxide dismutases are essential components of the biological defense against oxygen toxicity. Yet the dismutation of O_2^- is a rapid spontaneous process, and it is not self-evident why enzymes should be needed to catalyze this reaction. At pH 7.8, in an aqueous environment, the rate constant for the spontaneous dismutation is $8 \times 10^4\ M^{-1}\ sec^{-1}$. This reaction is, however, second order in O_2^- and its first half-life is therefore an inverse function of the initial concentration of O_2^-. At 1×10^{-4} M O_2^- the first half-life, in the absence of scavengers, would be approximately 0.05 sec, whereas at 1×10^{-10} M O_2^- it would be 14 h.

The reaction between O_2^- and the superoxide dismutases, in contrast, is first order O_2^-, and the half-life would be independent of the concentration of O_2^-. Given a tissue concentration of approximately 1×10^{-5} M superoxide dismutase and a reasonable steady state concentration of O_2^- of 1×10^{-11} M, we calculate that the enzymic dismutation would be 10^6-fold faster than the spontaneous process, other things being equal, merely because the likelihood of collison of an O_2^- with the enzyme is 10^6-fold greater than the

likelihood of collision with another O_2^-. In addition, the reaction of O_2^- with the enzyme is 2×10^9 M^{-1} sec^{-1}, which is 10^4-fold faster than the spontaneous reaction. Considering both of these factors, we see that the enzymic process eliminates O_2^- 10^{10}-fold faster than would the spontaneous process. The effect of these enzymes is clearly substantial.

The Defensive Team

The dismutation of O_2^-, whether spontaneous or enzyme catalyzed, produces H_2O_2, which is also a threat to the chemical integrity of cells. There are, however, catalases and peroxidases dedicated to the elimination of H_2O_2. The defensive team is thus composed of superoxide dismutases, to lower the steady state level of O_2^-, and catalases plus peroxidases to do the same for H_2O_2. These enzymes are also mutually protective, and therefore synergistic, when both O_2^- and H_2O_2 are being made. O_2^- inactivates catalase by converting the resting ferric enzyme to the poorly active ferro-oxy form (Compound III), and also by converting the perferryl intermediate (Compound I) to the inactive ferryl state (Compound II) (1). Heme-containing peroxidases are also inhibited by conversion to Compound III. SOD protects catalase and peroxidase against this inactivation. At the same time, H_2O_2 inactivates two of the three known types of superoxide dismutases (2–4), and catalases or peroxidases prevent this. Superoxide dismutases plus catalases and peroxidases thus constitute a mutually supportive defensive team.

BIOLOGICALLY RELEVANT SOURCES OF O_2^-

Autoxidations

Because of the spin restriction (5), the univalent pathway is the most facile route for the reduction of dioxygen. Seen in this light, autoxidations should often generate O_2^-, and they do. Among the autoxidations of interest from a biologist's point of view, which have been shown to produce O_2^-, we find those of hemoglobin (6), myoglobin (7), reduced cytochrome *c* (8), reduced ferredoxins (9), leukoflavins (10), tetrahydropterins (11), catecholamines (12, 13), and polyhydric phenols (14). Such autoxidations are often chain reactions in which O_2^- can serve both as initiator and as chain propagator. As such, they are strongly inhibited by superoxide dismutase and such inhibition has been used as the basis for convenient assays for this activity (12–14).

Enzymic Oxidations

A number of enzymes, including xanthine oxidase, aldehyde oxidase, dihydro-orotic dehydrogenase, and a variety of flavin dehydrogenases, all produce some O_2^- during their catalytic cycles. The most thoroughly studied of these is the milk xanthine oxidase (15), in which the partition of electron

outflow between the univalent and divalent routes of dioxygen reduction depends upon pH, pO_2 and substrate concentration (16). This behavior has been explained in terms of the extent of reduction of the enzyme during the catalytic steady state (17). The respiratory burst exhibited by granulocytes, when suitably activated, seems to be due to a membrane-associated NADPH-oxidase that reduces dioxygen exclusively to O_2^- (18).

Subcellular Organelles

Organelles such as mitochondria, chloroplasts, microsomes, and nuclei have been shown to generate O_2^-. Most often this is due to autoxidation of reduced components of electron transport assemblies and is easily demonstrated after the endogenous superoxide dismutase has been washed away. Particles prepared from mitochondria (19–21) and from chloroplasts (22) have been shown to produce O_2^-. Some of the O_2^- made by intact mitochondria can be detected in the suspending medium (23). Liver microsomes, when fortified with NADPH, have been shown to produce O_2^-. This was accomplished by trapping the O_2^- with lactoperoxidase (24), by reduction of succinylated cytochrome *c* (25), by spin trapping and EPR detection (26, 27). Isolated hepatic nuclei have also been shown to release O_2^- (28).

Intact Cells

O_2^- made within cells should not ordinarily be detectable in the suspending medium, because of scavenging by intracellular SOD. When O_2^- production by suspensions of intact cells is measured (29), one must suspect that it was actually made in the medium by autoxidation of reducing agents escaping from the cells. Thus, paraquat augments extracellular O_2^- production by suspensions of *Escherichia coli* and this was shown to be due to release of reduced paraquat, followed by its autoxidation in the medium (30). This caution does not apply to phagocytic cells such as human neutrophils (31), which secrete large amounts of O_2^-, when activated, due to the action of a membrane-associated NADPH oxidase.

It is clear from the foregoing that there are numerous potential sources of O_2^- in any given aerobic cell. However, granulocytes excepted, we know neither the specific major source of O_2^- in any cell type, nor the quantitative aspects of that O_2^- production. The ubiquity of SOD within aerobic cells hinders such measurements. In an oblique approach to this question an inhibitory antibody was used to suppress the SOD activity in extracts of *Streptococcus faecalis*. It was then possible to show that in such extracts, when fortified with NADH, 17% of the total electron flow to dioxygen was associated with O_2^- production (32). The rate of production in mammalian liver has been estimated to be 24 nmole O_2^- min^{-1} gm^{-1}, and the intramitochondrial steady state concentration of this radical to be $\sim 1 \times 10^{-11}$ M (33).

DELETERIOUS EFFECTS OF O_2^-

Direct Reactions of O_2^-

O_2^- is not indiscriminately reactive. Indeed, its failure to react rapidly with a few amino acids and citric cycle intermediates (34) has led a small number of investigators to the opinion that it is innocuous (35, 36). This is a curious conclusion, in view of the many reports of cell death due to increased production of O_2^- and preventable by elevated levels of SOD. Ignorance of mechanism is not a basis for denial of repeatable observations! Information concerning the possible chemical basis of the observed toxic effects of O_2^- is, moreover, beginning to accumulate.

NADH in free solution does not react rapidly with O_2^-, but, when NADH is bound to lactic dehydrogenase, it reacts with O_2^- at a rate of 3.6×10^4 M^{-1} sec^{-1}. Since the oxidation of NADH by O_2^- yields an NAD · radical, which can then reduce O_2 to O_2^-, a free radical chain reaction occurs (37). Lactic dehydrogenase thus appears to catalyze the chain oxidation of NADH by O_2^-. This illustrates the profound effect of microenvironment upon the reactions of O_2^-. Other compounds that have been shown to react with O_2^- include: epinephrine (38), 6-hydroxydopamine (13), catechols (39), pamoate (40), bile pigments (41), oxyhemoglobin (42), hydroxyl amines (43), phenylhydrazine (44), lipid peroxides (45), α-tocopherol (46) and the 1,2-dihydroxyethylthiamine-pyrophosphate intermediate of the transketolase reaction (47).

Reactions of O_2^- Involving Conversion to $HO_2\cdot$

O_2^- is the conjugate base of the weak acid $HO_2\cdot$, whose pK_a is 4.8 in water. $HO_2\cdot$ is a much stronger oxidant than is O_2^-. Since it is a neutral acid, the ionization of $HO_2\cdot$ involves charge separation and is facilitated by high dielectric constant. Consequently, the pK_a of $HO_2\cdot$ becomes progressively higher as the dielectric constant is lowered. The movement of O_2^- from water into the low dielectric environments of lipid micelles, membranes, or the interior of globular proteins, would be accompanied by its conversion to $HO_2\cdot$. Moreover the pH adjacent to polyanionic surfaces is lower than that of the bulk solvent. The pH in the Gouy-Chapman-Stern layer adjacent to phospholipid membranes can be 3 pH units below that of the surrounding water and entry of O_2^- into this layer would result in protonation. It follows that the reactivity of $HO_2\cdot$ could contribute importantly to that of O_2^- in biological systems. $HO_2\cdot$ has been shown to react with linoleate and with arachidonate at a rate of approximately 300 M^{-1} sec^{-1} (48). A compendium of rate constants for reactions of O_2^- and $HO_2\cdot$ is available (49).

Reactions of O_2^- Due to Basicity

The dismutation of O_2^- to $H_2O_2 + O_2$ is strongly favored and it consumes protons. As a consequence, O_2^- in a nonprotic solvent behaves like a very

powerful base. It can, by proton abstraction, generate carbanions that react rapidly with O_2. Many of the oxidations in nonprotic solvents, which have attributed to O_2^-, appear to proceed by this proton abstraction pathway (50). This may have relevance to restricted low dielectric microenvironments within cells.

Reactions of O_2^- by Virtue of Conversion to OH·

O_2^- gives rise to H_2O_2 by the dismutation process, and O_2^- plus H_2O_2 can then generate OH·. This process was first noticed with an enzymic source of O_2^- plus H_2O_2 (51), and we saw the implications of the enzymic generation of OH·. We stated, "The striking conclusion that so reactive a radical as OH· can be generated in significant amounts in an aqueous enzymatic system appears to be well supported by the data presented." This process was called the Haber-Weiss reaction, because these authors had earlier proposed the reduction of H_2O_2, to $OH^- + OH\cdot$, by O_2^-. Other investigators, too numerous to list here, have since made similar observations. Much of this literature has been reviewed (52).

The importance of iron complexes as catalysts of this process, at first overlooked because iron was present as an impurity of the reagents used, was finally appreciated (53), and a biological form of iron (lactoferrin) was found to be a very effective catalyst (54). An apparently analogous process, catalyzed by bound copper, has been noted (55). Antibiotics, such as Saframycins (56) and Rifamycin SV (57), have been shown to produce OH· by this Haber-Weiss process. It has been argued that O_2^- functions as a reductant for iron in the Haber-Weiss reaction and that it would be unable to compete in this process with other reductants such as thiols. The O_2^- dependent production of OH· from H_2O_2 has, however, recently been demonstrated in the presence of thiols (58).

It is clear that OH· can be generated from O_2^- plus H_2O_2, but we are left wondering about the biological relevance of this process. There are indications that it may indeed occur within living systems. Chloroplast lamellae, when illuminated, appear to produce OH· from O_2^- plus H_2O_2 (59). Alloxan, by reduction to dialuric acid followed by reoxidation, can increase intracellular production of O_2^- and H_2O_2. The diabetogenic action of alloxan is lessened by scavengers of OH· (60) and of O_2^- (61). The toxic effects of alloxan on isolated Islets of Langerhans (62, 63) and on cultured fibroblasts (64) are similarly affected. A source of oxygen radicals might be expected to initiate lipid peroxidation. Alloxan has this effect on vitamin E-deficient rats. Moreover, mannitol, which is often used as a scavenger of OH· in enzymic systems, protected (65). Liver microsomes exhibit a nonspecific alcohol-oxidizing activity that appears to be due to OH·, generated from O_2^- plus H_2O_2 (66, 67). Suspensions of neutrophils, which produce large amounts of O_2^- when activated, have also been shown to generate

OH· (68, 69). The observations enumerated above lead to the view that O_2^- plus H_2O_2 can generate OH·, or something very much like OH·, in vivo, as well as in vitro.

Evidence for Direct O_2^--Toxicity in a Bacterium

Streptococcus sanguis was chosen for study because it can grow without iron and is facultative. When grown aerobically, it accumulates mM levels of H_2O_2 in the medium. It makes a single SOD, based upon manganese, and contains 50- to 100-fold more of this enzyme when grown aerobically than when grown anaerobically. Extracts of *S. sanguis* contain an NADH-quinone reductase that mediates O_2^- and H_2O_2 production from quinones, such as plumbagin. Plumbagin increased the oxygen consumption by *S. sanguis* and imposed an oxygen-dependent toxicity, which was diminished by high intracellular levels of SOD (70).

Dimethylsulfoxide (DMSO), which freely permeates these cells, was used as an indicating scavenger for OH·. Intact aerobic cells of *S. sanguis* gave evidence of OH· production, in the presence of plumbagin, but it could be completely inhibited by SOD or by catalase added to the suspending medium. This OH· production was therefore extracellular. Moreover, 0.5 M DMSO did not protect against the oxygen-dependent toxicity of plumbagin (70). It follows that in *S. sanguis,* increased rates of O_2^- production, caused by plumbagin, exert a toxicity that is not mediated by OH·. We conclude that, although O_2^- can generate OH· from H_2O_2 it need not do so in order to exert a toxic effect.

SUPEROXIDE DISMUTASES

Varieties and Distribution

We know of three distinct types of SODs, all of which catalyze the same reaction with comparable efficiency, and which fall into two families. Given the common selection pressure of oxygenation, imposed by photosynthesis upon a varied anaerobic biota, it is not surprising that parallel evolution of SODs occurred. The iron-containing (FeSOD) and the manganese-containing (MnSOD) enzymes are characteristic of prokaryotes and are closely related, as shown by amino acid sequence homology. The copper and zinc-containing (CuZnSOD) enzymes are usually found in eukaryotes and appear to have been independently evolved (71). The distribution of these enzymes must encode a fascinating story of evolutionary events, but it is a tangled skein and difficult to unravel.

FeSOD and MnSOD are found in bacteria and a survey indicated some species with MnSOD, some with FeSOD and others which contained both (32). Eukaryotes contain CuZnSOD in the cytosol and MnSOD in the matrix of mitochondria. This is the case in yeast (72), plants (73), chicken

liver (74), and rat liver (75). The similarity between the mitochondrial and the prokaryotic MnSODs supports the endosymbiotic origin of these organelles as does their amino acid sequence homology (76). Yet, in human and in baboon liver the MnSOD is found in the cytosol as well as in the mitochondria (77).

There are anomalies in this overall pattern of distribution. Thus, the CuZnSOD is certainly characteristically found in the cytosol of eukaryotes. Yet it does occur in a bacterium (78), which lives as a symbiont in the light organ of the ponyfish and which appears to have obtained this enzyme by gene transfer from the host fish (79). Not so neatly explained is the CuZnSOD found in the free-living bacterium *Caulobacter crescentus* (80). This too may have been the result of a gene transfer, but if so it was a long time ago, the donor is unknown, and *C. crescentus* may not have been the original recipient. FeSODs, usually found only in prokaryotes, has been seen to occur in a few plants, specifically water lilies, mustards, and ginkgo trees (81). Since only these, among over forty families of plants examined, contained FeSOD, gene transfers may again have been the root cause.

Anomalies of Omission

One would expect to find SOD in all oxygen-tolerant organisms, yet a few exceptions have been noted. Thus, *Lactobacillus plantarum* (82), *Mycoplasma pneumoniae* (83), and a disseminating strain of *Neisseria* (84) have been found to lack this enzyme. Only in the case of *L. plantarum* is an explanation for this apparent anomaly in hand. These bacteria grow best in Mn-rich media and accumulate this metal to an intracellular concentration of approximately 25 mM. Mn(II) can catalyze the dismutation of O_2^-. It is a much less efficient catalyst than SOD, but given the high concentration of Mn(II) available in these cells, it suffices. When intracellular Mn(II) is depressed, by growth in manganese-deficient medium, the cells lose oxygen tolerance and become very sensitive towards the oxygen-dependent toxicity of quinones. Surveys of numerous related Lactobacilli revealed some with SOD and some with high Mn(II), but none with both. Mn(II) thus serves as a functional replacement for SOD and, in organisms that ordinarily live in Mn-rich decaying plant material, this is a sensible substitution (85–87).

Mechanism

O_2^- is unstable with respect to O_2 plus H_2O_2 and spontaneously goes over to these products by dismutation. Since O_2^- is the conjugate base of $HO_2\cdot$, whose pK_a is 4.8, we need to consider three dismutation reactions (88). These are:

$$HO_2\cdot + HO_2\cdot \rightarrow H_2O_2 + O_2 \quad \{ \quad k_2 = 7.6 \times 10^5\ M^{-1}\ sec^{-1} \qquad 1.$$

$$HO_2\cdot + O_2^- + H^+ \rightarrow H_2O_2 + O_2 \quad \{ \quad k_2 = 8.8 \times 10^7\ M^{-1}\ sec^{-1} \qquad 2.$$

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \; \{ \; k_2 < 0.3 \; M^{-1} \; 0.3 \; M^{-1} \; sec^{-1} \qquad 3.$$

It follows that the spontaneous dismutation will be most rapid at pH 4.8 and that the rate will diminish by a factor of ten for each unit increase in pH above 4.8. Reaction 3 is so slow because of electrostatic repulsion between the reactants, and because the production of $O_2^=$ would be a very unfavorable event owing to repulsion of the two negative charges during the lifetime of the collisional complex. Finally, the very high pK_a for $O_2^=$ makes its production an energetically unfavorable event at ordinary pH. The simplest mechanism for catalysis would utilize a metal cation, which could mediate the electron transfer and in the second half reaction yield a peroxo complex which could protonate as the complex dissociated.

In all of the SODs the active site metal undergoes cycles of reduction, followed by oxidation, as it mediates electron transfer between the reactants. Thus:

$$E - Me^n + O_2^- \rightarrow E - Me^{n-1} + O_2 \qquad 4.$$

$$E - Me^{n-1} + O_2^- \rightarrow E - Me^n - O_2^= \qquad 5.$$

$$E - Me^n - O_2^= \xrightarrow{H^+} E - Me^n + HO_2^- \xrightarrow{H^+} H_2O_2 \qquad 6.$$

The SODs are very efficient catalysts and operate at a rate of $\sim 2 \times 10^9$ M^{-1} sec^{-1} at 25°C, which is close to the diffusion limit. The energy of activation is 26.9 KJ/M, which is 1.5 times greater than that expected for a purely diffusion-controlled reaction (89).

Since the active site metal represents less than 1% of the surface area of SOD, one wonders how it is possible to achieve rates close to the diffusion limit. Koppenol (90) has proposed a virtual electrostatic funnel based upon a net negative charge on the protein, combined with a cluster of positive charges at the active site. This would minimize unproductive collisions with the protein, while favoring collisions with the active site region. In the case of the bovine erythrocyte CuZnSOD enough structural information is in hand to know that these features are present (91, 92). Increasing ionic strength decreased the activity of the CuZnSOD and acylation of lysine residues inverted this response to ionic strength (93). These results indicate that lysine residues do provide electrostatic facilitation for the reaction of the enzyme with O_2^-. There is an arginine residue in the active site crevice, close to the copper. Modification of this arginine with α,β-diketones markedly reduces catalytic activity (94, 95), yet does not alter the response of the enzyme to ionic strength (93). It seems likely that this arginine provides for ion-pairing and hence for stabilization of the O_2^- as it leaves the

bulk solvent and enters the lower dielectric environment of the active site cleft.

PHYSIOLOGICAL FUNCTIONS OF SUPEROXIDE DISMUTASES

Specificity

Given that the SODs catalyze a reaction which occurs quite rapidly even in the absence of catalysis and that free Cu(II) can catalyze the dismutation of O_2^-, at pH 8 and below, the question of specificity is a serious one. The CuZnSOD, MnSOD, and FeSOD were obtained by isolation from diverse biological sources solely on the basis of activity. Electrophoresis on polyacrylamide gels, followed by activity staining, indicates that the materials isolated exhibit the same mobility as the active components of the crude extracts. Moreover, all of the bands of activity seen in the crude extract can be accounted for in terms of the isolated SODs. It follows that the SODs are the only significant catalysts of O_2^- dismutation in the cell types examined. Free metals, low molecular weight metal complexes, and the plethora of other metallo proteins present in cell extracts do not contribute detectably to the SOD activity of these extracts. Certain lactobacilli, discussed above (85–87), are exceptions in that they contain very high intracellular levels of Mn(II) as a functional replacement for SOD. All of this information indicates a high degree of specificity of SOD for O_2^-, and the pulse radiolysis data thus far available supports this idea (96, 97).

Induction of SOD in Bacteria

Control of the biosynthesis of enzymes by their substrates, or by products uniquely derived from these substrates, provides cells with important economies. Microorganisms, which must be able to accommodate to a variety of growth conditions, make good use of this mechanism so that they will not waste cell substance and energy producing particular enzymes, except when they are needed. SOD synthesis is controlled in this way, and induction of SOD by increased intracellular fluxes of O_2^- has been seen in numerous microorganisms including *Streptococcus faecalis* (98), *Photobacter leiognathi* (78), *Escherichia coli* (99, 100), *Listeria monocytogenes* (101), *Vibrio eltor* (102), *Bacterioides fragilis* (103), *Rhizobium japonicum* (104), *Salmonella typhimurium* (105), *Selenomonas ruminantium* (106), and *Streptococcus sanguis* (70).

In most cases these inductions were brought about by increased exposure to oxygen, but inductions can be caused at fixed pO_2 by conditions which increased the intracellular production of O_2^-. In *E. coli,* growth on a fermentable substrate, such as glucose, depresses SOD, whereas dependence upon a respiratory energy supply elevates SOD (107). When grown in a

glucose-limited chemostat, *E. coli* exhibits greater respiration and higher SOD at increased growth rates (108). Most striking were the inductions brought about by redox-active compounds, such as methyl viologen or quinones, which divert intracellular electron flow from the cytochrome pathway to an O_2^--producing pathway. Such compounds markedly increased cyanide-resistant respiration and SOD in *E. coli* (109–111).

In the case of *E. coli,* which contains both MnSOD and FeSOD, the FeSOD is constitutive and is made even under anaerobic conditions, when O_2^- production is not possible (99). In contrast, the MnSOD is under tight control and is inducible by O_2^-. Anaerobically-grown *E. coli* contain only FeSOD, whereas cells induced by growth at high pO_2 or in the presence of methyl viologen or quinones contain a high level of MnSOD, in addition to FeSOD (99). In a facultative organism this is a sensible arrangement in that the FeSOD provides a constant standby defense against oxygen toxicity, while the MnSOD allows fine tuning of the defense to match the level of the threat.

Inductions of SOD in Eukaryotes

Bacteria were not unique in their ability to increase SOD biosynthesis in response to increased pO_2 or to increased rates of intracellular O_2^- production. Similar responses have been seen with yeast (112), potato slices (113), endothelial cells (114), and rat lung (115, 116). The sea anemone *Anthopleura elegantissima* contains 100-fold more SOD when exposed to the oxygen generated by the symbiotic alga *Symbiodinium microadriaticum,* than when not so exposed (117). These inductions are clearly related to enhanced O_2^- production. Less obvious is the induction of SOD in poplar leaves by SO_2 (118). SO_2 hydrates and ionizes to sulfite, which can be oxidized by a free radical chain pathway initiated by O_2^-. The lethality of SO_2 could thus be exacerbated by O_2^- and therefore minimized by SOD. The induction of SOD by SO_2 could signify that O_2^- production is augmented in the presence of sulfite or that a sulfite radical, produced by interaction of O_2^- with sulfite, is a powerful inducer of SOD biosynthesis.

Protections by SOD

Elevation of intracellular SOD levels by induction in bacteria has been seen to impart resistance against the toxicity of oxygen and against the oxygen-dependent toxicities of streptonigrin, methyl viologen, plumbagin, pyocyanine, and related substances (98, 99, 103, 107, 108, 110, 111, 119, 120). This correlation held true in several organisms and when induction was achieved by several different strategies. Induction of SOD, with parallel acquisition of enhanced tolerance toward oxygen toxicity, has also been seen in yeast (112), rat lung (115, 121), and *Oscillatoria limnetica* (122).

SOD added to the medium has been seen to protect bacteria and bacteriophage against the lethality of an extracellular source of O_2^- (30, 123–125). SOD in the medium enhances the oxygen resistance of *Campylobacter fetus* (126) and protects mammalian 3T3 cells, in culture, against the damaging effect of an enzymic source of O_2^- (127). Application of an enzymic source of O_2^- to the hamster cheek pouch initiates a physiological cascade resulting in leucocyte margination and vascular leakage, and SOD prevented these deleterious effects (128). Near ultraviolet irradiation of coliphage T7 in the presence of H_2O_2 caused a loss of infectivity that could be prevented by SOD (129). There are also reports of protection, by SOD, against ionizing radiation (130–138). Chromosomal aberrations seen in autoimmune diseases (139), in some strains of inbred mice (140), and induced by phorbol ester in cultured 3T3 cells (141), are diminished by SOD.

Alloxan, a well-known diabetogenic agent, is capable of O_2^- production via cyclical reduction to dialuric acid and autoxidation back to alloxan. Enhanced production of O_2^- and H_2O_2 could lead to OH· production, and if OH· were the proximal damaging agent then a scavenger of OH·, such as ethanol, might protect. Ethanol was shown to protect against the diabetogenic action of alloxan (13). When a series of OH·-scavengers were tested in mice their abilities to mitigate the effects of alloxan paralleled their rates of reaction with OH· (60). When alloxan was applied to isolated mouse islets, SOD, catalase or OH·-scavengers were seen to protect (62), a clear indication that O_2^-, H_2O_2, and ultimately OH·, were involved in the process leading to cell damage. These results were repeated and extended by the observation that a chelating agent, capable of preventing the iron-catalyzed production of OH· from O_2^- plus H_2O_2, also prevented the damaging action of alloxan on islets (63). SOD, coupled to polyethylene glycol to extend its circulating lifetime, prevented the diabetogenic effect of alloxan on mice (61). This protective effect of SOD has been observed repeatedly (64, 142, 143).

We may conclude that O_2^- is the substrate for the superoxide dismutases, in vivo as well as in vitro, and that a wide variety of deleterious effects, which are mediated by O_2^-, can be diminished or eliminated by SOD.

O_2^-, INFLAMMATION AND REPERFUSION INJURY

Inflammation

Injected SOD has been seen to exert an antiinflammatory effect. Much of this literature has been reviewed (114, 145). The mechanism of this effect appears to be multifactorial. Thus, the O_2^- secreted by activated neutrophils can cause tissue injury that can be prevented by SOD placed into the extracellular compartment (146–148). In addition, O_2^- acts upon a compo-

nent of normal plasma and converts it into a potent neutrophil chemotaxin (149, 150). The latter effect provides for a self amplification of the inflammatory response, which will persist as long as the neutrophils arriving at the site of inflammation continue to encounter an activating stimulus, such as opsonized bacteria. When bovine CuZnSOD is injected into a rat its circulating half-life is approximately 6 minutes and its rapid clearance from the extracellular compartment limits its antiinflammatory effect. Coupling the SOD with ficoll extends its circulatory lifetime, without inactivating this enzyme, and thus multiplies its antiinflammatory effect (151). A similar effect has been achieved by coupling SOD to a homologous serum albumin (152) and to polyethylene glycol (153).

Inflammation of the lung could be caused by inspiration of xanthine oxidase plus xanthine, which would act as a source of O_2^-. This caused acute lung damage which could be prevented by SOD, but not by catalase (154). Similar results were obtained in an inflammation caused by the reverse passive Arthus reaction (155).

Reperfusion Injury

Temporary interruption of blood flow to a tissue results in damage to that tissue. It has usually been assumed that this damage occurs during the period of hypoxia and is due to depletion of ATP. Another possibility is that the deleterious effects actually occur during reperfusion and are due to free radical generation (156). Thus, one can imagine an accumulation of reduced substances during hypoxia such that reperfusion and reoxygenation results in a burst of free radical production. Several reports now lend substance to this proposal. Injected SOD has been seen to diminish infarct size and creatine kinase depletion in rats subject to left coronary artery ligation (157, 158). Myocardial infarction has been shown to result in complement activation with the release of C5A which in turn activates neutrophils, causing them to become adherent and to release O_2^- and H_2O_2. In an in vitro model, plasma from animals suffering myocardial infarction damaged endothelial cells in culture and this was prevented by SOD and catalase (159).

The mechanism of reperfusion injury has been elegantly studied in the feline intestine and the role of O_2^- confirmed (160–162). Thus, SOD protected against the increased capillary permeability and the mucosal lesions imposed by 3 h of ischemia. Pretreatment of the animals with allopurinol, to inhibit xanthine oxidase, also protected. It thus appears that tissue hypoxia is accompanied by conversion of the xanthine dehydrogenase to xanthine oxidase, probably by limited proteolysis, and the concomitant degradation of ATP to hypoxanthine. Reoxygenation would then allow the xanthine oxidase to oxidize the accumulated hypoxanthine with production of O_2^- and H_2O_2 (16).

CONCLUDING REMARKS

Limitations of time, space, and patience have precluded an exhaustive survey of the large and rapidly growing literature of the superoxide radical and the superoxide dismutases. It is nevertheless clear, from the data cited, that O_2^- is a commonly encountered intermediate of oxygen reduction in both biotic and abiotic systems and that this free radical constitutes a threat to the chemical integrity of living cells. This threat may arise from the intrinsic and relatively selective reactivity of O_2^-, or by way of its protonation to the more strongly oxidizing $HO_2\cdot$, or it may be due to generation of the vastly reactive OH· by the iron-catalyzed Haber-Weiss process. Indeed, all of these modalities, plus others yet to be discovered, are probably germane to the deleterious actions of O_2^- in cells. Whatever the mechanism, some defense is essential and in the great majority of organisms it is provided by the superoxide dismutases, which catalytically scavenge O_2^-.

O_2^- is thus to be viewed as an endogenous toxicant and the superoxide dismutases as the endogenous antidote. It has already become apparent that O_2^- is an important factor in phagocytosis, the inflammatory process, and reperfusion injury. This information opens the way to new and beneficial interventions in pathological processes. Superoxide dismutases and covalently modified variants thereof are currently being studied as injectable antiinflammatory agents, and we may hope that such work will soon make a new and useful addition to our pharmaceutical armamentarium.

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