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Superoxide Radical and Superoxide Dismutase

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Molecular oxygen, in the ground state, contains two unpaired electrons and is paramagnetic. The interaction of ground-state oxygen with an exogenous electron pair is therefore faced with a spin restriction. Oxidation reactions involving oxygen in the ground state are hence likely to proceed by one-electron steps and to involve free-radical intermediates.[1]

Some involvement of superoxide radicals (O_2^-) in oxidations of biological significance and the existence of a mechanism of defense against the potential toxicity of these radicals within aerobic cells might therefore have been anticipated. It was not. These realizations were, rather, reached gradually and as the result of traveling a tortuous path in search of explanations for fortuitously observed phenomena. These historical aspects are briefly summarized.

Xanthine oxidase, which catalyzes the oxidation of xanthine to urate, was observed to initiate the oxidation of sulfite and did so only in the combined presence of an oxidizable substrate and of oxygen.[2] The aerobic oxidation of sulfite had long been known to proceed by a free-radical chain reaction and the O_2^- generated by xanthine oxidase was found to be the agent responsible for initiation of this chain reaction.[3-5] Xanthine oxidase is also capable of catalyzing the reduction of cytochrome *c* by xanthine, but this process was shown to be dependent upon the presence of oxygen,[6] and H_2O_2 was definitely excluded as the agent responsible for this oxygen-dependent reduction.[7] Here, too, O_2^- generated by xanthine oxidase was shown to be the cause of the observed phenomenon.[5] Xanthine oxidase, acting on xanthine in the presence of oxygen and of luminol or lucigenin, was known to cause chemiluminescence, and this too was shown to be due to its generation of O_2^-.[8]

The abilities of certain protein preparations specifically to inhibit those actions of xanthine oxidase which were due to O_2^-, without having any effect on the enzymatic activity of xanthine oxidase,[9,10] led to the discovery of the enzyme superoxide dismutase[5,11,12] which catalyzes the reaction

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

Superoxide dismutase was assayed in terms of its ability to inhibit processes dependent upon O_2^- and was found to be ubiquitous in aerobic cells.[13] Superoxide dismutase isolated from bovine erythrocytes and heart[14] is identical with a metalloprotein previously isolated from several sources without knowledge of its activity and variously called hemocuprein, erythrocuprein, hepatocuprein, cerebrocuprein, or cytocuprein.[15-23] The generation of O_2^- by both enzymatic and nonenzymatic oxidations of biochemical significance and the action of superoxide dismutase on this radical have also been demonstrated by other investigators.[24-28]

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The pK_a for the hydroperoxyl radical is 4.8.[29] These radicals undergo a spontaneous dismutation reaction, to yield $O_2 + H_2O_2$, which is most rapid at pH 4.8 and is slower at both lower and higher pH. An explanation for this variation of the rate of spontaneous dismutation with pH was provided by the rate constants for the reactions among the species involved. Thus, for the reaction $HO_2\cdot + HO_2\cdot$ the rate constant is 7.6×10^5 M^{-1} sec^{-1}; for the reaction $HO_2\cdot + O_2\cdot^-$ it is 8.5×10^7 M^{-1} sec^{-1}; and for the reaction $O_2\cdot^- + O_2\cdot^-$ it is less than 10^2 M^{-1} sec^{-1}.[29] The relatively slow rate of spontaneous dismutation of superoxide anions probably relates to mutual electrostatic repulsions, which hinder close approach.

The need for an enzyme to catalyze what is, in any case, a rather rapid reaction may be understood in terms of the relatively low rate constant for the dismutation of the superoxide anions and of the great reactivity of these radicals, which would render even low levels intolerable to a living cell. Superoxide dismutase seems to be the catalyst evolved to answer this need. We now proceed to a consideration of the methods used in studying this enzyme, its properties, and its manyfold uses.

Assays of Superoxide Dismutase

All of the assays so far devised for superoxide dismutase depend upon its ability to inhibit some reaction dependent upon O_2^-. An assay system must therefore contain both a generator of O_2^- and a detector of O_2^-. An amount of superoxide dismutase would then be defined in terms of its ability to inhibit the change in the detector. The sensitivity of such assays will depend upon the ability of superoxide dismutase to compete with other reactants for the available O_2^-. Since the spontaneous dismutation is a second-order reaction, O_2^- must itself be considered a competing reactant. Hence, at high steady-state levels of O_2^-, superoxide dismutase will be a less effective inhibitor of O_2^--dependent processes than at low levels of O_2^-.

Assays Based on Xanthine Oxidase. Xanthine oxidase is capable of the univalent reduction of oxygen, and the O_2^- generated by its action can be detected in terms of the reduction of cytochrome *c*. Under standardized conditions[12] one unit of superoxide dismutase, *i.e.*, that amount which caused 50% inhibition of the rate of reduction of cytochrome *c*, was 0.10 μg/ml at pH 7.8. The reduction of tetranitromethane to nitroform and of nitro blue tetrazolium to the corresponding formazan can also be used to intercept the O_2^- generated by xanthine oxidase, and superoxide dismutase can be quantitated on the basis of its ability to inhibit these reductions.[12,30] O_2^- is an effective oxidant of epinephrine, and its conversion to adrenochrome has been used as a measure of O_2^- generated by xanthine oxidase. Superoxide dismutase inhibits this oxidation and can be quantitated on this basis.[12]

Assays Based on Autoxidations. The aerobic oxidation of sulfite to sulfate is a free-radical chain reaction which can be initiated by transition metal cations. In the presence of low levels of EDTA the mechanism of this initiation involves O_2^- and superoxide dismutase acts as a potent inhibitor. In the absence of EDTA O_2^- appears not to be involved in initiating sulfite autoxidation and superoxide dismutase did not inhibit. This system could be used to detect superoxide dismutase at a level of 0.001 μg/ml.[31]

At elevated pH (10.2), epinephrine spontaneously oxidizes to adrenochrome. This oxidation is probably catalyzed by traces of transition metal cations, present as impurities. In any case, this oxidation process involves O_2^-, and superoxide dismutase, which acts as a potent inhibitor, can readily be assayed on the basis of this reaction.[32]

Assays Based on Photosensitized Oxidation. A number of dyes, including isoalloxazine derivatives, are readily reduced by illumination in the presence of oxidizable compounds. The photoreduced dyes then react with O_2 to generate O_2^-,[26] which can be detected by its ability to reduce nitro blue tetrazolium to the blue formazan.[33,34] Superoxide dismutase interrupts this chain of events at the level of O_2^- and thus inhibits the production of the formazan. This has been used as the basis of an assay for superoxide dismutase. Because of the insolubility of the blue formazan this assay is readily applied to the detection of superoxide dismutase on polyacrylamide electrophoretograms. A band of this enzyme containing as little as 0.016 μg will signal its presence as an achromatic zone on uniformly blue gels.[30]

Properties of the Mammalian Superoxide Dismutase

Superoxide dismutase was isolated from bovine erythrocytes.[12] The purification procedure contained one very peculiar step which must indicate an unusual distribution of hydrophobic groups in this enzyme. Thus, after treatment of a red cell lysate with chloroform plus ethanol, to denature and precipitate hemoglobin (Tsuchihashi procedure), dibasic potassium phosphate was added until an organic phase was salted out. All of the superoxide dismutase activity was present in this supernatant organic phase and could be recovered therefrom by precipitation with acetone.

The purified enzyme was blue-green in color and exhibited an unusual absorption spectrum in the ultraviolet. Its spectra are shown in Figure 1. The ultraviolet spectrum suggested that this protein was devoid of tryptophan. Amino acid analysis verified this conclusion. Assays for tryptophan, by the method of Spies and Chambers,[35] also indicated no tryptophan

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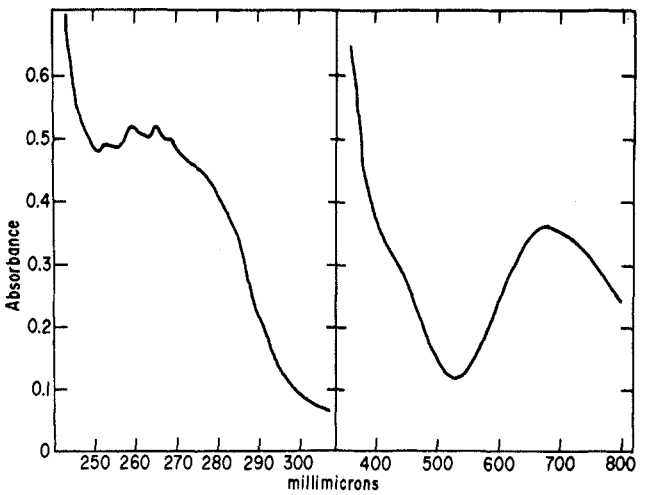

Figure 1. Ultraviolet and visible absorption spectra of superoxide dismutase from bovine erythrocytes. The concentration of the enzyme was 1.5 mg/ml for the ultraviolet spectrum and 47 mg/ml for the visible spectrum.

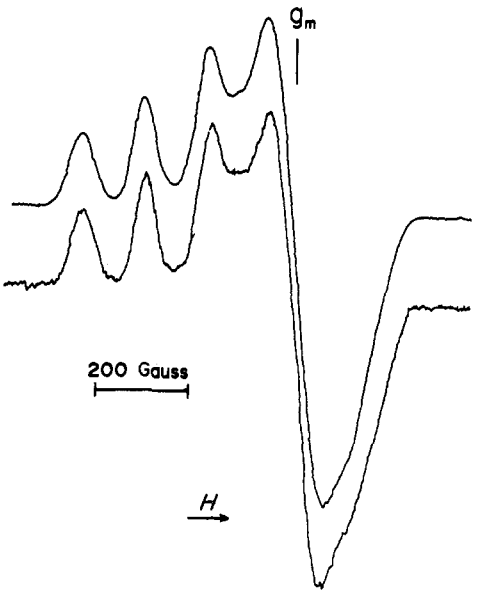

Figure 2. Electron paramagnetic resonance spectra of the superoxide dismutase from bovine erythrocytes (upper signal) is here compared with that of the enzyme isolated from bovine heart muscle (lower signal). The erythrocyte enzyme was at 47 mg/ml and the heart enzyme was at 8 mg/ml. Both were in 0.05 *M* potassium phosphate at pH 7.8. Other conditions for the erythrocyte and heart enzymes respectively were: microwave frequency, 9.082 and 9.101 GHz; microwave power, 10 and 25 mW; modulation amplitude, 4 and 3.2 G; scan rate, 500 and 125 G/min; time constant, 0.03 and 1.0 sec; receiver gain, 500 and 8000; and sample temperature, 77 K. The values of the spectral parameters are $g_m = 2.080$; $g_\parallel = 2.265$; $A_\parallel = 0.014\ cm^{-1}$.

and exposure to Koshland's reagent caused no loss of activity.[14] The blue-green color of the enzyme suggested a copper prosthetic group which was verified by the epr spectra shown in Figure 2. Double integration of this epr spectrum indicated two atoms of Cu^{2+} per molecule of superoxide dismutase. Analysis of the superhyperfine splitting of this copper signal indicated that the liganding groups contained nitrogen.[36] Chemical analyses for metal demonstrated two Cu^{2+} and two Zn^{2+} per molecule of enzyme.[14] Dialysis at low pH against EDTA caused a loss of Cu^{2+} and of color and a concomitant loss of activity. The apoenzyme was restored to full activity by Cu^{2+} alone. Thus Zn^{2+} does not appear to be part of the active site, although it may be important in stabilizing the enzyme. Cu^{2+}, *per se*, does exhibit some superoxide dismutase activity, but the activity of the enzyme is orders of magnitude greater than the activity of a comparable amount of free Cu^{2+}. Furthermore, the activity of Cu^{2+}, *per se*, is entirely suppressed by EDTA, whereas that of the enzyme is not affected by EDTA.

A good deal of the chemical characterization of superoxide dismutase had already been done by investigators working on the same proteins but without knowledge of their catalytic function. Thus Mann and Keilin[15] reported on the molecular weight and copper content and Mohamed and Greenberg[19] succeeded in reversibly removing the copper and in crystallizing the apoprotein. The ultraviolet absorption spectrum of the human erythrocuprein was reported by Markowitz, *et al.*,[16] and by Stansell and Deutsch.[20] The latter workers also reported the visible absorption spectrum, as did Nyman.[37] The discovery that zinc is also an integral part of the native protein was made by Carrico and Deutsch.[23] The epr spectrum was first reported by Malmstrom and Vanngard[38] and the fluorescence spectrum by Bannister, *et al.*[39] This is by no means an exhaustive survey of the literature on erythrocupreins.

The enzyme is remarkably stable to urea and retains a large fraction of its activity in 9.0 *M* urea at neutral pH. Guanidinium chloride (6.0 *M*) does, however, cause inactivation. This denatured enzyme could be restored to an active form by dilution into solutions containing Cu^{2+}. When native bovine superoxide dismutase was exposed to 75° in 0.10 *M* potassium phosphate–1×10^{-4} *M* EDTA at pH 7.0, it underwent a first-order inactivation characterized by a half-life of 11 min.[31] Electrophoresis in acrylamide gels in the presence of sodium dodecyl sulfate ± mercaptoethanol appeared to demonstrate (Figure 3) that the enzyme was composed of two subunits of equal size (mol wt 16,300) held together by one or more disulfide bridges.[14] It has now been found that 1% SDS fails to denature this enzyme in the absence of mercaptoethanol but does so in its presence. Apo superoxide dismutase does dissociate in 1% SDS in the absence of mercaptans. It is thus clear that the bovine superoxide dismutase contains subunits which are not covalently linked.

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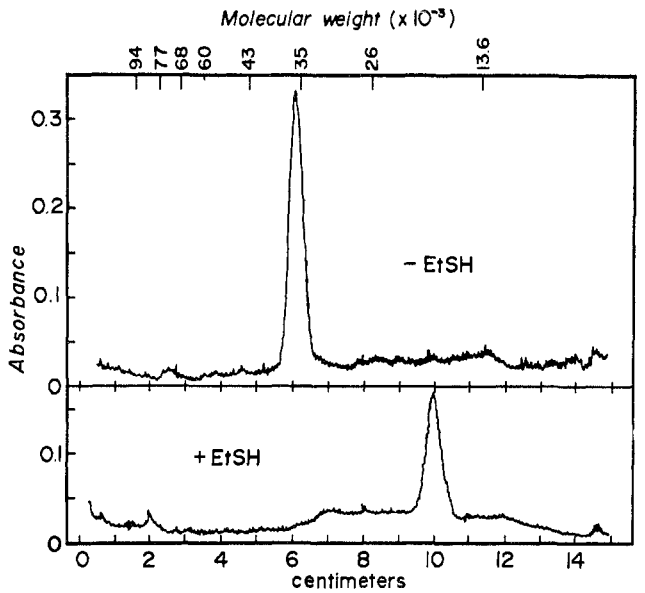

Figure 3. Determination of subunit molecular weight. Bovine erythrocyte superoxide dismutase was subjected to electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate ± mercaptoethanol. The gels were calibrated in terms of molecular weight by using, as standards, phosphorylase *a* (94,000), human transferrin (77,000), bovine serum albumin (68,000), catalase (60,000), ovalbumin (43,000), pepsin (35,000), chymotrypsinogen A (26,000), and ribonuclease (13,600). The position of the stained protein bands on the gels was recorded with a Gilford Model 2000 spectrophotometer equipped with a gel scanner. It is apparent that sodium dodecyl sulfate was able to cause dissociation of this enzyme only in the presence of β-mercaptoethanol.

Studies of end groups and of tryptic digests of this enzyme indicate that these subunits are identical.[40]

The Superoxide Dismutases of Diverse Organisms

Superoxide dismutase has been purified from a variety of sources, including bovine and human erythrocytes, bovine heart, yeast, *Escherichia coli*,[41] *Streptococcus mutans*,[42] wheat germ, summer squash, garden peas,[43] *Neurospora crassa*,[44] and chicken liver. The enzymes obtained from eucaryotic sources were uniformly blue-green in color and contained copper and zinc. In contrast the enzymes from the procaryotes were reddish in color and contained two atoms of manganese per molecule. This manganese was epr-silent until the protein was denatured, at which time the spectrum of Mn^{2+} appeared (Figure 4).[41] The bacterial superoxide dismutase had a molecular weight of 40,000 and was composed of two subunits of identical size held together by noncovalent forces.[41]

Isozymes

Electrophoresis of superoxide dismutase on polyacrylamide gels has revealed that most sources contain multiple active forms of this enzyme. Thus, *E. coli* contains two isozymes demonstrable in the crude sonicates and present in approximately equal amounts, while wheat germ extracts show three forms of superoxide dismutase and chicken liver appears to contain five distinct forms. Although considerable progress has been made in separating these isozymes by chromatography on carboxymethylcellulose, it is not yet known whether they represent distinct gene products or single gene products which have been differently modified by amidation, acetylation, or attachment of carbohydrate groups. This is obviously a question of considerable interest and is being further pursued.

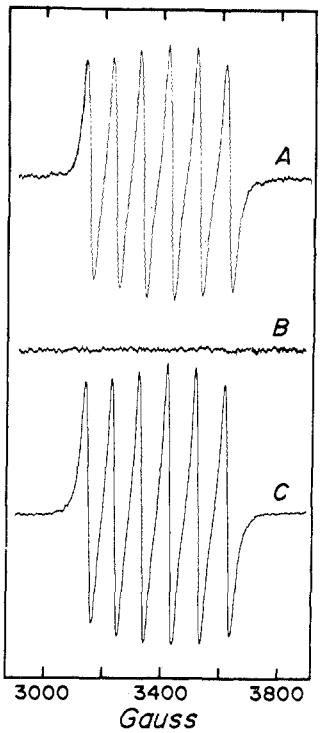

Figure 4. Detection of manganese in *E. coli* superoxide dismutase by epr spectroscopy. Curve A was obtained by mixing 0.45 ml of enzyme (1.5 mg/ml) in water with 0.05 ml of 1.0 *M* HCl and then heating to 100° for 3 min. This acid solution of denatured enzyme was examined for epr signals in a flat cell assembly under the following conditions: microwave frequency, 9.552 GHz; microwave power, 24 mW; modulation amplitude, 4 G; scan rate, 250 G/min; time constant, 1.0 sec; receiver gain, 5000; and temperature, 23°. Curve B was obtained when the native enzyme was examined at a concentration of 4.5 mg/ml in water under otherwise identical conditions. Curve C was similarly obtained from 0.1 m*M* $MnCl_2$ dissolved in 0.1 *m* HCl.

The Biological Significance of Superoxide Dismutase

If we take the position that respiration leads to some univalent reduction of oxygen and that superoxide dismutase exists to protect cells against the deleterious actions of this reactive radical, then we may expect that obligate anaerobes, which never grow exposed to oxygen, have no need for this enzyme. A number of microorganisms were surveyed for their content of superoxide dismutase and of catalase. These organisms were selected so that some were aerobes, some microaerotolerant anaerobes, and some obligate anaerobes.

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Aerobes are defined as organisms which can grow in air and which when growing aerobically develop electron-transport chains which use oxygen as the terminal electron acceptor. Microaerotolerant anaerobes are organisms which can tolerate exposure to air and can grow under aerobic conditions but which never utilize oxygen as their major electron sink. Obligate anaerobes, in contrast, cannot grow when exposed to air. The results of this survey which are presented in Table I demonstrate that aerobes contained high and fairly uniform levels of superoxide dismutase whereas microaerotolerant anaerobes contained less and obligate anaerobes contained no superoxide dismutase.[13] The one apparent exception to these generalizations was *Lactobacillus plantarum*, a microaerotolerant anaerobe which contained no superoxide dismutase. As organism which does not utilize oxygen at all will not be subject to an internal flux of O_2^-. It was reasoned that *L. plantarium* might escape the need for superoxide dismutase, even when growing in air, because it did not reduce oxygen. Suspensions of this organism were found, in fact, not to consume detectable amounts of oxygen. Two additional obligate anaerobes, kindly supplied by Dr. Dave C. White of Lexington, Kentucky, *i.e.*, *Bacterioides melaninogenicus* and *Bacterioides ruminicola*, have also been found to be devoid of superoxide dismutase. These results are consistent with the proposition that superoxide dismutase is essential only to organisms which metabolize oxygen.

Table I
Superoxide Dismutase and Catalase Contents of a Variety of Microorganisms

	Superoxide dismutase, units/mg	Catalase, units/mg
Aerobes		
Escherichia coli	1.8	6.1
Salmonella typhimurium	1.4	2.4
Halobacterium salinarium	2.1	3.4
Rhizobium japonicum	2.6	0.7
Micrococcus radiodurans	7.0	289
Saccharomyces cerevisiae	3.7	13.5
Mycobacterium sp.	2.9	2.7
Pseudomonas sp.	2.0	22.5
Strict anaerobes		
Veillonella alcalescens	0	0
Clostridium pasteurianum, sticklandii, lentoputrescens, cellobioparum, barkeri	0	0
Clostridium acetobutylicum	0	
Clostridium sp. (strain M.C.)	0	0
Butyrivibrio fibrisolvens	0	0.1
N2C3[a]	0	<0.1
Aerotolerant anaerobes		
Butyribacterium rettgeri	1.6	0
Streptococcus fecalis	0.8	0
Streptococcus mutans	0.5	0
Streptococcus bovis	0.3	0
Streptococcus mitis	0.2	0
Streptococcus lactis	1.4	0
Zymobacterium oroticum	0.6	0
Lactobacillus plantarum	0	0

[a] N2C3 is an unclassified cellulolytic Gram-negative rod isolated from the rumen of an African zebu steer and has been described by S. S. Margherita and R. E. Hungate, *J. Bacteriol.*, **86**, 855 (1963).

Applications of Superoxide Dismutase

Superoxide dismutase is a useful tool which can be used to investigate the role of O_2^- in a variety of reactions. If O_2^- is an essential intermediate in any process, then superoxide dismutase might be expected to inhibit that process. This is based upon the supposition that superoxide dismutase is specific for superoxide and is without effect on other radicals. Although not known with certainty, it seems a reasonable assumption, in view of the usually great specificity of enzymes. Superoxide dismutase has been used in probing a number of reactions, some of which will now be discussed.

Ethylene Production. The biogenesis of ethylene has been a matter of general interest because of the action of ethylene as a plant-ripening hormone.[45] It was suggested that methional, derived from methionine, was the precursor of ethylene and that $O_2\cdot^-$ or possibly OH· was the species whose attack upon methional led directly to the production of ethylene.[46] If O_2^- were the attacking radical, it was anticipated that xanthine oxidase, acting on xanthine, in air, would serve as a source of O_2^- and would therefore give rise to ethylene in the presence of methional. This was found to be so, and superoxide dismutase did inhibit ethylene production.[47] However, a lag in the rate of accumulation of ethylene suggested that some stable reaction product was also required. H_2O_2 eliminated this lag and catalase was found to inhibit the production of ethylene. These results, which indicated that H_2O_2 and O_2^- were both required for the generation of ethylene from methional, recalled the reaction proposed by Haber and Weiss,[48] *i.e.*, $O_2\cdot^- + H_2O_2 \rightarrow OH\cdot + OH^- + O_2$. If this reaction actually represented a reality, then OH· could be secondarily generated in the xanthine oxidase reaction mixture and could be the species actually reacting with methional. The ability of scavengers of OH·, such as benzoate or ethanol, to inhibit the production of ethylene lent support to this notion since benzoate and ethanol do not react with O_2^-. If a powerful oxidant such as OH· were generated as a consequence of the interaction of O_2^- with H_2O_2, then xanthine oxidase might be expected to exhibit an activity-dependent ferrocytochrome *c* peroxidase action which was inhibited by superoxide dismutase. Such an activity was observed.[47]

Cathodic Reduction of Oxygen. There has been controversy concerning the mechanism of cathodic reduction of oxygen in aqueous solutions. Workers in this area have generally concluded that O_2^-, generated at the cathode, would either be further reduced or would dismute before it could diffuse from the immediate surface of the electrode. Since electrolytically generated O_2^- has been shown capable of initiating

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sulfite oxidation[4] it appeared likely that other means of detection of O_2^- could be applied to the detection of electrolytically generated O_2^- and that superoxide dismutase could be used to intercept this O_2^- and thus both to verify its production and to demonstrate that it diffused far enough from the electrode to react with an enzyme. An oxygen-dependent, superoxide dismutase inhibitable, electrolytic conversion of epinephrine to adrenochrome has been observed. Its voltage dependency was similar to that of an oxygen reduction half-wave.[49] Superoxide dismutase has thus been used to demonstrate that the electrolytic reduction of oxygen in aqueous solutions does give rise to univalently reduced oxygen which can diffuse far enough from the electrode to react with small molecules and with an enzyme.

Oxygen Radicals Generated by Ultrasonication. The widespread use of ultrasonication in breaking cells for biochemical preparative work and in clinical diagnostic procedures makes sonochemistry a matter of great interest. Studies of the sonochemical generation of H_2O_2 suggested that O_2^- was generated when aerobic aqueous solutions were sonified.[50] A direct means of investigating this point would be to detect sonically generated O_2^- by a chemical action and to inhibit this chemical action with superoxide dismutase. Buffered solutions of ferricytochrome *c* were reduced when sonified in the presence of oxygen, and superoxide dismutase inhibited this process. Since both cytochrome *c* and the superoxide dismutase are restricted to the solution phase, we must conclude that $O_2\cdot^-$, generated in the cavities, diffuses into the bulk solution. To the extent that OH· was also generated in the cavities it would decrease the detectable $O_2\cdot^-$ by the reaction $OH\cdot + O_2^- \rightarrow OH^- + O_2$ which would occur in the cavities. Adding formate as a scavenger of OH· should therefore have had the effect of increasing the rate of superoxide dismutase inhibitable reduction of cytochrome *c*. It did so.

Other Applications. Superoxide dismutase has also been used to expose the involvement of $O_2\cdot^-$ in the following: (1) air oxidation of reduced ferredoxins;[28,51] (2) air oxidation of reduced quinones and flavins;[26,27,52–54] (3) oxygen-dependent hydroxylations;[55–57] (4) mechanism of tryptophan pyrrolase;[58,59] (5) mechanism of bacteriocidal action of streptonigrin;[60] (6) autoxidation of epinephrine.[32]

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Summary and Possibilities

Discovery of a new phenomenon is generally followed, in short order, by its recognition in diverse situations, which had previously been only imperfectly understood. In this perspective, there are several areas of great interest in which we may hope to have gained a foothold, because of the discoveries of the biological production of O_2^- and of the existence of superoxide dismutase. We will close this discussion by briefly considering some of these possibilities.

Radiation Damage. The lethality of ionizing radiation to living cells is enhanced by the presence of oxygen. The passage of ionizing radiation through water would generate H· and ·OH, and in the presence of oxygen the H· would rapidly transfer its electron to O_2, yielding O_2^-. Of these radicals the only one with sufficient stability to diffuse appreciably from the ionization spur would be O_2^-. We may therefore anticipate that the enhancement of the lethality of ionizing radiation by oxygen is due to the formation of O_2^- and that superoxide dismutase limits the extent of this oxygen enhancement.

Oxygen Toxicity. The desire to utilize hyperoxia as a means of controlling infections by anaerobes and as an adjunct to surgical procedures which involve temporary blocks to blood circulation has led to the observation that exposure to hyperoxic atmospheres is damaging and can be lethal. It appears likely that one facet of the toxicity of oxygen is the production of O_2^- in amounts greater than normal, so that the capacity of the defensive system, *i.e.*, superoxide dismutase, is exceeded. In this case any strategy which could raise the level of superoxide dismutase could enhance tolerance for hyperoxia. We presume that the great sensitivity of obligate anaerobes to oxygen is due to their complete lack of superoxide dismutase.

Senescence. It appears likely that senescence may be due to an accumulation of irreparable damage to the macromolecules of the organism. Organisms evidently differ greatly in their capabilities for repair, as evidenced by the species variability of average lifespans. The chemical insults which the organism must attempt to counter may well be due to the reactivities of oxygen radicals. In this case exposure to increased concentrations of oxygen should lead to more rapid senescence, whereas any increase which could be effected in the levels of superoxide dismutase or of other radical scavengers should retard the onset of age deterioriation.

I have been extremely fortunate in my choice of graduate students and postdoctoral associates, and it is a pleasure to comment on their contributions to the work on the superoxide dismutase. In chronological order of their involvement with this problem are J. M. McCord, C. Beauchamp, B. B. Keele, Jr., S. Goscin, H. P. Misra, H. Forman, R. Weisiger, M. Gregory, and B. Lippett. Special recognition must be given to J. M. McCord because it was his insight which first drew back the blinds.

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