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REVIEW ARTICLE

OXYGEN RADICALS, OXYGEN TOXICITY AND THE LIFE OF MICROORGANISMS

FREDERICK S. ARCHIBALD AND IRWIN FRIDOVICH

Department of Biochemistry. Duke University Medical Center. Durham, North Carolina, U.S.A.

SUMMARY

The electronic structure of dioxygen in the ground state dictates that its reduction occur most easily by a univalent pathway which involves the dangerously reactive intermediates O_2^-, H_2O_2 and OH. The oxygenation of earth's biosphere 3.5×10^9 years ago provided both opportunity and threat. The opportunity to exploit oxygen for energy-yielding and biosynthetically useful oxidations has been seized, as evidenced by the abundant and predominantly aerobic flora and fauna of this planet. At the same time the threat has been largely neutralized by a variety of defensive strategies. The superoxide dismutases are an important part of this defense against oxygen toxicity. Much of the research on superoxide dismutase has been done with microorganisms, due to their flexibility as laboratory tools and because there appears to be close similarity between oxygen toxicity threat and defense in microorganisms and in higher organisms. Data resulting from this work appear to have application in understanding a number of basic biological and medical phenomena, in particular the mechanisms of hyperbaric oxygen toxicity, anoxic tissue damage, anaerobiosis, oxidative damage in aging and mutagenesis, inflammation, phagocytosis, and the pharmacology of certain antitumor drugs and antibiotics. In each of these areas, whether the role of O_2^- and the other oxygen species ultimately proves to be major or minor, beneficial or harmful, application of the concept of oxygen toxicity to experimental design is resulting in a substantial improvement in our understanding of the mechanisms involved.

RESUMO

Os radicais oxigenados, a toxicidade do oxigénio e a vida dos microorganismos

A estrutura electrónica do dioxigénio implica que a sua redução se faça geralmente por meio da via univalente que envolve os intermediários nocivos O_2^-, H_2O_2 e OH. A oxigenação da biosfera terrestre há $3{,}5 \times 10^9$ anos permitiu por um lado a utilização do oxigénio para as oxidações biosintéticas por parte da flora e fauna aeróbica predominante e conduziu, por outro lado, à evolução de determinados mecanismos de defesa nos microorganismos. As dismutases de superoxido revelaram possuir um importante papel na defesa contra a toxicidade do oxigénio. Os dados provenientes deste estudo ajudam à compreensão de um certo número de fenómenos biológicos e médicos em especial os mecanismos da toxicidade do oxigénio hiperbárico, a lesão por anóxia dos tecidos, a anaerobiose, a lesão oxidativa que faz parte do envelhecimento e da mutagenese, a inflamação, a fagocitose e a farmacologia de certas drogas antitumorais e antibióticas. A aplicação do conceito da toxicidade do oxigénio ao modelo experimental tem contribuido para um melhor esclarecimento dos mecanismos envolvidos no papel desempenhado pelo O_2^- e por outros radicais oxigenados.

In recent years there has been a great increase in our understanding of the fundamental mechanisms of oxygen toxicity. Work with microorganisms has provided much of this information.

1. Free oxygen and the origins of life

The geological record provides evidence that for about half of its 4.5 billion years existence as a solid sphere, earth was an anaerobic planet, bathed in an atmosphere containing methane, ammonia, water vapor, nitrogen, and carbon dioxide. Under these conditions life arose and under them it evolved for at least a billion years.[1] Two of the most significant advances made by organisms on this primitive anaerobic world were photosystems I and II. The appearance of photosystem I, now known to have been present 3.5×10^9 years ago, enabled cells to utilize and store solar energy by converting it into chemical energy. The more advanced photosystem II, which was present at least 2.6×10^9 years ago, catalyzed the photolysis of water to provide abundant reducing power for biosynthetic reactions. It also produced the waste gas molecular oxygen, which accumulated in the atmosphere.[1-3] This true, water-splitting photosynthesis gradually converted a reducing atmosphere into an oxidizing one and forced all life either to make the adaptations required for oxygen tolerance or to become restricted to the anaerobic niches which exist even on an oxygenated planet. Development of oxygen tolerance then paved the way for exploitation of this gas as a terminal electron acceptor in energy yielding metabolic pathways. This *breakthrough* allowed cells to extract substantially more useable energy from foodstuffs aerobically than could be obtained from the same materials by anaerobic transformation. For example modern organisms can produce up to 38 high energy phosphate bonds in the form of ATP from one melecule of glucose by oxidative metabolism but only 2 net high energy

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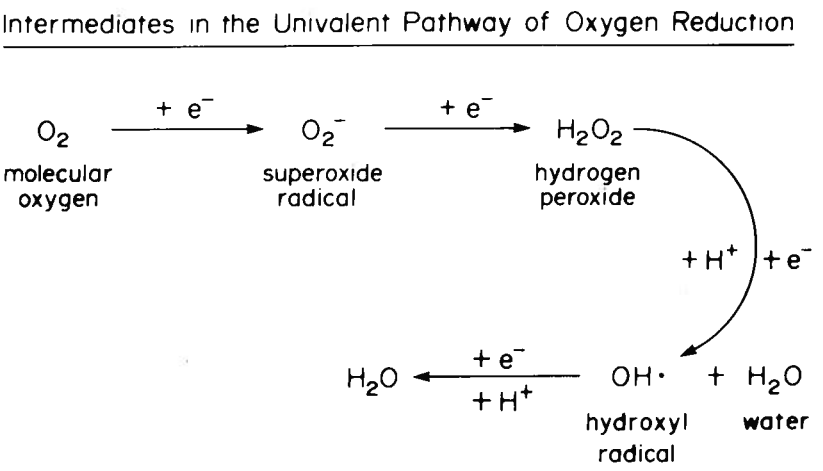

Figure 1

phosphate bonds from glucose via the substrate-level phosphorylation of anaerobic pathways.

However, while respiration remains, by all measures, a most successful adaption, organisms cannot escape the dilemma that they are formed of highly complex and relatively reduced molecules that spend their lives bathed in a sea of the oxidant O_2.

2. Oxygen in the living cell — management of a dangerous substance

The oxygen atom has six electrons in its outer shell and thus has a strong tendency to acquire two more to form a stable octed. Oxygen's potential reactivity is further suggested by its location in the periodic table, below the extremely active element fluorine. Viewed in this manner the question seems not to be why O_2 is toxic, but how cells avoid oxidative destruction by O_2. The explanation is dependent on the fortunate circumstance that O_2 is a diradical, i.e. it has two unpaired electrons, having the same or parallel spins, in its outermost orbitals. A fundamental tenet of chemical theory, the Pauli exclusion principle, permits only two electrons of opposite spin to occupy the same orbital. Therefore, an oxygen molecule is unable to directly accept the desired pair of electrons with opposite spins from a donor melecule. Since atmospheric O_2 does efficiently oxidize organic substances in living cells, there must be a way to avoid this *spin restriction*.[4, 5]

In fact, there are two basic ways.

1) Dioxygen can share its unpaired electrons with complimentary unpaired electrons of another atom, in particular the transition metals, such as Fe, Cu and Mn[6].

2) Dioxygen can be reduced one electron at a time, i.e. by a univalent pathway. Since four electrons are required to fully reduce O_2 to H_2O, three intermediates are formed, namely the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical. All are reactive and must be presumed to be toxic in living systems. Figure 1 illustrates the univalent pathway of oxygen reduction.

The chief devices enabling aerobic organisms to utilize O_2 as a terminal electron accepter are the heme-containing proteins, in particular the cytochrome oxidases. These complex proteins containing Fe and sometimes Cu at their active sites efficiently donate four electrons to a firmly bound O_2 molecule, so that oxygen intermediates are either nor formed, or if formed are not released.[4] The result is that the large majority of the O_2 consumed by most organisms is directly reduced to water. The small but significant remainder of the oxygen consumed is reduced by univalent pathways involving the production of one or more of the toxic species O_2^-, OH·, or H_2O_2.[4] While the rate of production of these reactive intermediates may be low and their existence fleeting, the total amounts of active oxygen species evolved in a cell over time may be very large and the resultant oxidative damage to the cell substancial.[7] Superoxide, while much less reactive than the hydroxyl radical,[4] can act as either a reductant or an oxidant, and has been shown to reduce or oxidize such diverse molecules as cytochromes[8-11] hemoglobins[11-13], sulfhydryl groups,[14, 15] pyrogallol,[6] adrenalin,[17] polyunsaturated fatty acids,[18-21] hydroxylamine,[22] sulfite,[23] and NADH.[24] Superoxide or derivatives thereof have also been shown to depolymerize hyaluronic acids,[25] inactivate a variety of enzymes,[26-28] nick and cleave nucleic acids,[29-30] kill bacteria,[31, 32] inactivate virus particles,[26] damage mammalian cells in tissue culture,[33] attack cell membranes and lyse erythrocytes.[34, 35] Reaction of a free radical with a stable substance begets another free radical. A particularly harmful reaction of this sort is the so-called Haber-Weiss reaction, which in the presence of catalutic amounts of Fe allows O_2^- plus H_2O_2 to give rise to OH· as follows: $O_2^- + H_2O_2 \xrightarrow{Fe} OH\cdot + OH^- + O_2$.[36-37] A living cell could not long tolerate a substantial flux of OH·, a species able to react rapidly with nearly any organic molecule. The adaptive response of most organisms to this threat appears to be the possession of antioxidants, such as glutathione and α-tocopherol, and of enzymes, such as catalases, peroxidases, and superoxide dismutases, which are capable of destroying H_2O_2 and O_2^-.

This view of the relationship of free O_2 to life is surprisingly recent. Until the 1940's there were not even data clearly showing that mammalian tissues are particularly sensitive to hyperbaric oxygen.[38] In another field, it had been known since the early part of this century that the effects of ionozing radiation are substantially enhanced by the presence of dissolved O_2 in the cells.[39, 40] Despite this, and although microbiologists had known since the last century of the toxicity of H_2O_2 and the detoxifying role of catalase, respiration physiology, radiation chemistry and microbiology went their separete ways. Few people believed that the hydroxyl and superoxide radicals actually occurred in living cells, despite a prophetic article entitled *Oxygen Poisoning and X-Irradiation: A Mechanism in Common* published in 1954.[39]

In 1968, two important findings were reported. First, it was discovered that the flavin- and heme-containing enzyme xanthine oxidase evolves substantial amounts of O_2^- and H_2O_2 in oxidizing hypoxanthine or xanthine to uric acid, the first clear demonstration of O_2^- derived from an enzymatic can evolve O_2^-, including aldehyde oxidase,[43, 44] indoleamine dioxygenase,[45-47] leukocyte superoxide synthetase,[48, 41] di-hydro-orotate oxidase,[49, 50] cysteamine oxygenase,[51] dopamine-β-hydroxylase,[52] a microbial hydrogenase,[28] and a diaphorase.[53]

Second, a copper-containing protein first isolated from bovine erythrocytes in 1939[54] was shown to be a highly efficient scavenger of O_2^-.[42] This discovery demonstrated that cell actively attemps to minimize the intracellular O_2^- concentration and also provided an invaluable O_2^- specific reagent.

3. Microorganisms as tools

Molecular biology has shown that the basic chemistry of life differs surprisingly little between organisms as diverse as microorganisms and mammals, and in general, the more fundamental the metabolic function, the more evolutionary conservatism it shows. Because of the relative ease with which microorganisms can be grown and manipulated *in vitro*, the facultatively aerobic nature mani strains, and the basic nature of the problem of active oxygen toxicity, much of the research done on O_2^- and SOD has been performed using microbial systems. So far, the evidance indicates that these organisms are susceptible to and deal with oxygen toxicity very much as higher organisms do, suggesting that this experimental approach is a useful one.

4. Superoxide dismutases

One of the first predictions made about SOD proteins was that if they really were essential defenses against a universal threat, then they should be found in all living organisms, or at least in all those exposed to oxygen.[55] A wide variety of microorganisms including hundreds of strains in nearly all major taxa have been examined for superoxide dismutase activity and, with a very few exceptions, all contained one or more SOD proteins. Surveys of a wide variety of protists, plants, and animals have likewise shown SOD to be ubiquitous. A comparison of the SOD proteins present in the various kingdoms and phyla shows both some interesting diferences and some remarkable similarities. All known SODs are stable hydrophilic Cu, Fe, or Mn-cofactored homodimers or tetramers. Subunit molecular weights are about 16,000 d for the Cu and 23,000 d for the Mn and Fe-containing enzymes. All known SODs catalyze the dismutation of superoxide (equation1) with a rate constant of about 10^9 molar^{-1} sec^{-1},

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

which is close to the diffusion limit and probably the highest rate of activity of any known enzyme.[2] The metal cofactors are essential for enzymatic activity, although the Zn in Cu-ZnSOD has been shown to play a non-catalytic role and be replaceable by Co or a variety of other metals.[56]

The Cu, Fe, and Mn each appear to function at the enzymes' active site by alternating between two formal valence states, i.e. FeII $\leftrightarrow$ FeIII, CuI $\leftrightarrow$ CuII, and MnII $\leftrightarrow$ MnIII as shown in equations 2 and 3.

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

These enzymes are very stable, usually remaining catalytically active through harsh extraction and resisting relatively high temperatures.[42] The Mn-cofactored SOD is resistant to 5 mM H_2O_2.[57] The distribution of the Cu-Zn, Fe and Mn classes of SOD in nature is instructive. Almost all eucaryote contain Cu-Zn containing SODs while Mn-SODs are found in virtually all mitochondria and in many procaryotes.[58, 59] FeSODs are found almost exclusively in procaryote cytosols, either alone or together with a MnSOD.[58, 59]

5. Superoxide dismutases and evolution

Comparison of the amino acid compositions and especially of the amino acid sequences of homologous proteins in different species has become an important tool in determining taxonomic relationships and the course of evolution. One result of such comparisons of similarity has been increasing support for the endosymbiotic theory, which posits that some eucaryotic organelles, particularly mitochondria and chloroplasts, are descended from free-living procaryotes.[58] Since procaryotes and mitochondria both contain MnSODs, several comparisons of these enzymes have been performed. All MnSODs proved to have nearly identical size, activity, and physicochemical behavior. The N-terminal amino acid sequences of four mycobacterial MnSOD proteins showed a greater similarity to mitochondrial MnSOD than they did to other microbial MnSODs.[60] The complete amino acid sequences of chicken liver mitochondrial MnSOD and *E. coli* MnSOD are known and they show an 80% homology, that is, 80% of the amino acid sequences of the two proteins are identical.[59] In contrast, despite their similar activity in scavenging O_2^- there is apparently no relatedness between the cytosolic CuZnSOD and the mitochondrial MnSOD found within the same eucaryotic cells.[59, 61] The amino acid compositions of over 25 SOD proteins have been determined and a number have also been partially or completely sequenced.[59, 61] The data strongly suggest a high degree of similarity and evolutionary conservatism among the FeSOD and MnSOD proteins, but beyond nearly identical catalytic behavior, these proteins bear little resemblance to the CuZn proteins. Thus it seems that the CuZnSOD in eucaryote cytosols and MnSOD and/or FeSOD in mitochondria, chloroplasts, and procaryotes evolved separately.[58, 63] Fe and Mn cofactored SODs are so similar that in *E. coli*, hybrid dimers containing one subunit of MnSOD and one of FeSOD are formed.

Because of their remarlable evolutionary conservatism, the amino acid compositions and immunological cross reactivity of SOD proteins have been used to determine the relatedness of different species. The results have generally agreed very well with the taxonomic relationships determined using other proteins, 16S ribosomal RNA homology, and DNA guanine-cytosine GC) ratios.[58, 63]

Use of SOD protein relationships has also provided the first evidence for an unusual event, the natural transfer of a gene from an animal to a bacterium.[63] The tropical ponyfish (*Leiognathus splendens*) contains an organ which is luminescent due to the presence of a light-emitting symbiotic bacterium, *Photobacterium leiognathi*, in its tissues. Unlike many closely related but free-living *Photobacterium* species which contain only a single FeSOD, *P. leiognathi* also contains a CuZnSOD. Using statistical analysis of amino acid compositions, it was shown that bacterial CuZnSOD was closely related to but not identical with the CuZnSOD found in the host fish's own cells. The bacterial CuZnSOD was more distantly related to the CuZnSODs of a variety of

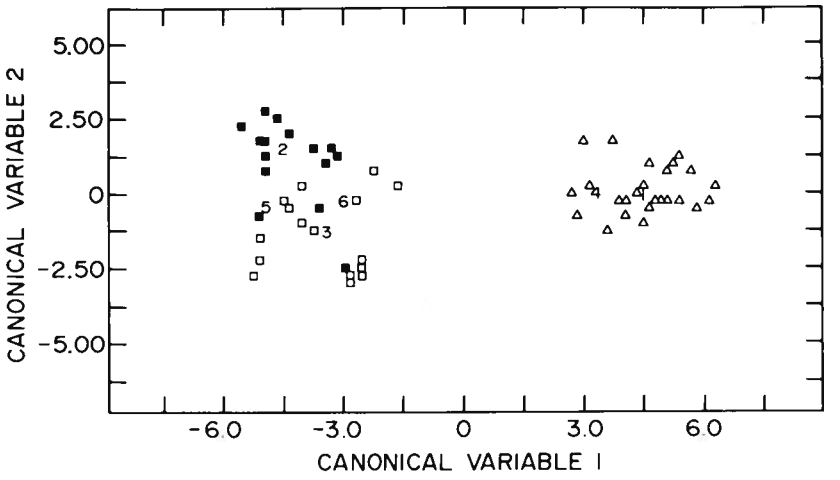

Figure 2

other organisms.[63] Figure 2 presents the results obtained when this analysis was applied to the amino acid compositions of all known superoxide dismutases. The similarities among all CuZnSODs was recognized as was the similarity among the MnSOD and the FeSOD groups. When the same analysis was applied only to the CuZnSODs, the analysis separated them into three groups, as shown in Figure 3. These groups were composed of: mammalian and bird; plant and fungal; and fish and *P. leiognathi* enzymes. The latter grouping exposes the likelihood of the ponyfish to *P. leiognathi* gene transfer.

6. An Anomaly

It has been stated that almost all those aerobic organisms examined contained one or more SOD enzymes. However, there are exceptions, and while the lack of SOD in an anaerobe can be explained, how can O_2 be viewed as universally toxic if SOD is absent from even one O_2-consuming aerotolerant organism? In an early survey, *Lactobacillus plantarum* was found to grow well in air, yet to lack detectable SOD.[55] This organism, when in log phase growth on a glucose based medium, was found to have only about 1% of the oxygen consumption rate of a comparable *E. coli* B culture and thus it was initially thought that avoidance of respiration obviated its need for SOD. However, later work showed that on other substrates and in other phases of growth *L. plantarum* respires substantially, and furthermore is remarkably resistant to hyperbaric O_2, H_2O_2, and an internal flux of O_2^-.[64] In fact, the resistance of *L. plantarum* to hyperbaric O_2 exceeds that of several organisms containing substantial levels of SOD activity.[64] The explanation of this apparent contradiction of the oxygen toxicity theory began to appear when it was observed that *L. plantarum* and related species required and accumulated extraordinarily high levels of manganese. While most microorganisms require $< 10^{-7}$ Molar Mn in the culture medium for optimar growth, *L. plantarum* requires several hundred times this amount and accumulates $> 25 \times 10^{-3}$ Molar Mn intracellularly.[64] Free Mn^{+2} ions have been shown to scavenge O_2^- but with a rate constant 2-3 orders of magnitude lower than SOD.[64-66] However, since the lactobacillus cells contain millimolar Mn, their total O_2^- scavenging ability is roughly equivalent to that of more conventional organisms which contain micromolar SOD. The O_2^- scavenging ability of the Mn had been missed in earlier work, both because cell extracts were routinely dialyzed before assay, and because the addition of ethylene diamine tetraacetate (EDTA) to the assay to stabilize the xanthine oxidase O_2^- source, chelated the Mn in a relatively inactive form. Thus, the apparent contradiction posed by the lack of SOD in *L. plantarum* merely showed that there can be more than one solution to a common problem.

7. Induction and modulation of the superoxide dismutases

Even relatively simple organisms have multiple feedback mechanisms to maintain constant intracellular conditions and high metabolic efficiency in the face of a changing environment. One of the more prominent of these mechanisms, especially in procaryotes is the induction os specific proteins in response to specific needs. For instance, *E. coli* synthesizes the β-galactosidase enzyme and the galactoside transporter proteins required for the uptake and utilization of lactose but does so only when lactose is the best available carbon and energy source. Therefore, one of the earliest experiments was to see if microorganisms that can grow either in the presence or absence of oxygen (facultative anaerobes) altered their SOD levels in response to changes in pO_2. *E. coli* is a facultative anaerobe, and in the absense of O_2 contains only a relatively low level of a FeSOD. However, when exposed to oxygen it synthesizes a MnSOD as well.[67-70] This effect is seen in Figure 4. Elevating the pO_2 by vigorously aerating the culture causes a substantial rise in the level of the MnSOD but has no effect on the FeSOD activity.[1] These cells can thus increase their total SOD activity from 4-6 SOD units per mg protein in anaerobic cells to greater than 50 SOD units/mg protein in cells grown under hyperbaric O_2, a good example of the defense being adjusted to the threat. Similar inductions of SOD proteins in response to increasing pO_2 have been seen in streptococci,[68] *Bacteroides fragilis*,[71] *Propionobacterium shermanii*,[72] *Photobacterium leiognathi*,[73] *Vibrio cholerae* (el tor),[74] *Oscillatoria limnetica*,[75] and nearly all other microorganisms examined for this ability. It has also been seen in plants and in animal cells, such as potato slices,[76] leukocytes,[77-79] rat mammary carcinoma,[80] neonatal rat lung,[81, 82] alveolar macrophages,[77-79, 83] and yeast.[84] In higher organisms, the SOD response varies

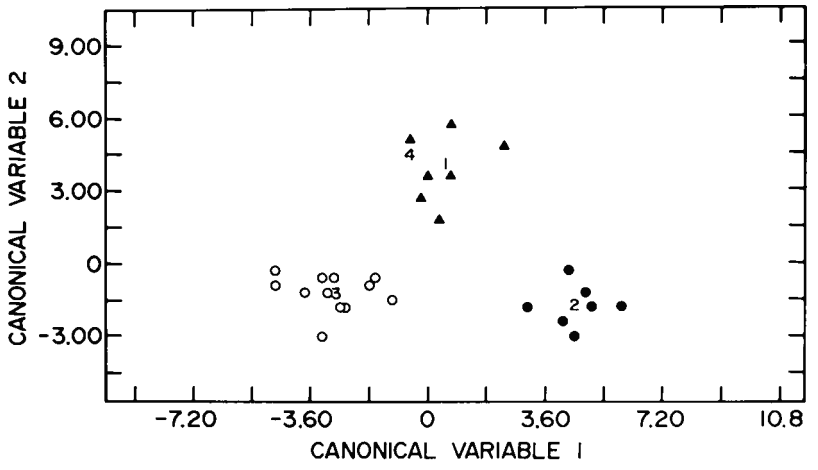

Figure 3

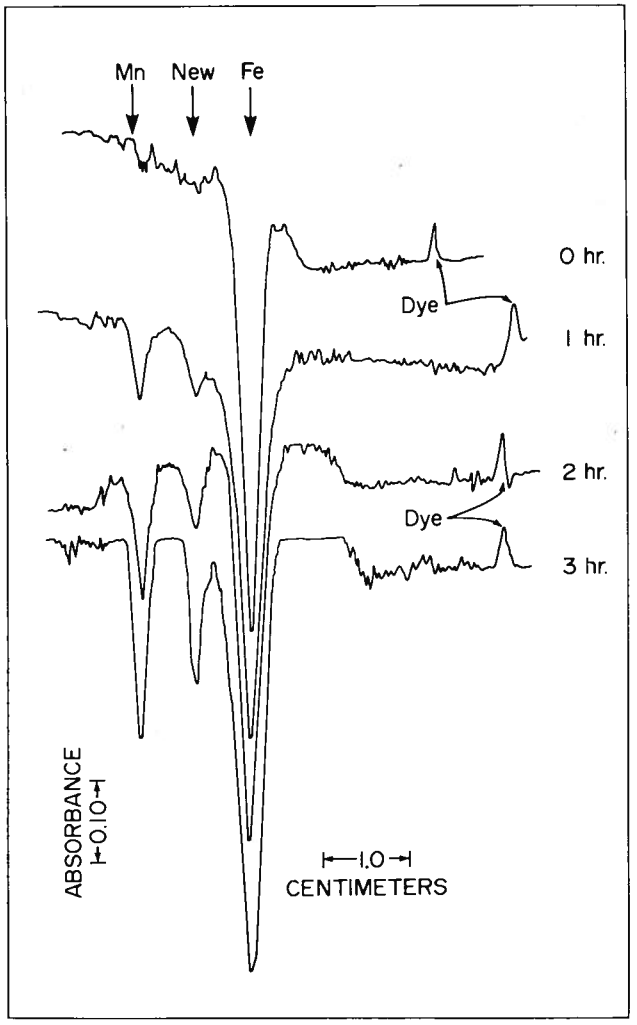

Figure 4

with the type and age of the tissue.[85] In all those organisms examined, increased SOD levels, achieved by aerobic growth rendered the cells more resistant to hyperbaric O_2.[85]

While these results are suggestive of the *in vivo* role of SOD, there is some circular reasoning involved in demonstrating increased O_2 tolerance after exposure to elevated pO_2 and it would be more convinvcing to induce SOD by means other than high pO_2 and then show the resulting cells to have increased resistance to hyperbaric O_2. To this end, some elegant experiments have been performed with *E. coli*. Since the intracellular production of O_2^- is presumably proportional to the rate of cyanide resistant O_2 uptake, any strategy increasing this O_2 consuption by the cells shoud lead to increases in their SOD content.

In one experiment, *E. coli* B was grown using either glucose or succinate as the primary carbon and energy source.[86] When grown aerobically on glucose, its preferred substrate, *E. coli* metabolizes the sugar chiefly via the glycolytic pathway despite the availability of O_2, excreting organic acids and resipring relatively little. However, when subsisting on organic or amino acids, the intermediates of the tricarboxylic acid cycle and respiratory chain are fully induced so that oxidative phosphorylation and consequently respiration rates are high.[86, 87] When cells were grown on glucose, lactate and succinic acid, under equal aeration, those grown on succinate and therefore having higher rates of respiration showed substantially higher SOD levels. In fact when *E. coli* B is grown in a medium containing both low levels of glucose and amino acids, the cells consume the glucose first and during this have low intracellular levels of SOD, but upon depletion of the glucose the cells both switch to the amino acid substrate and sharply increase their SOD content.[87]

Another strategem to increase the respiratory rate in cells of *E. coli* is to manupulate their growth rate.[86] Cells were grown under continuous culture conditions with cell density and aeration held constant and the rate of growth limited by a low glucose concentration.[86, 87] Upon addition of more glucose, there was a short lag followed by a sharply increased rate of growth. By periodically assaying the cells for their SOD content, it was found that the growth lag exactly

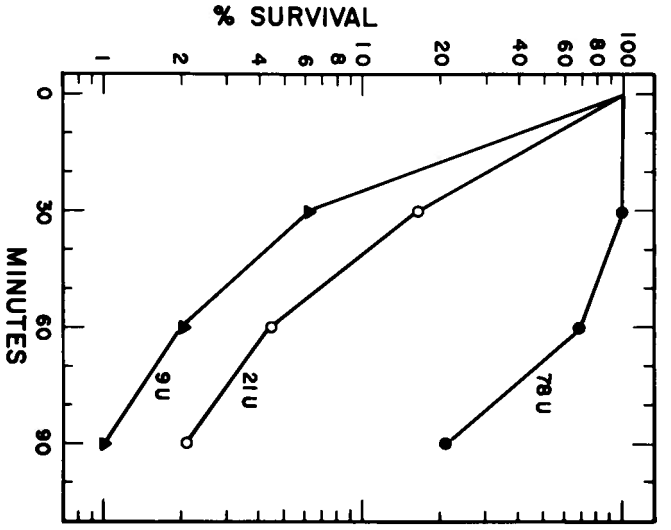

Figure 5

corresponded to the length of time required for the cells to induce a new and substantially higher level of SOD.[86, 87] Cells of *E. coli*, which by any one of the above techniues had elevated SOD levels, were markedly more resistant to hyperbaric O_2 than were uninduced control cells.[67-69, 80, 88]

8. Intracellular O_2^-

These results indicated that not just hyperbaric O_2 but some basic component or product of respiration itself poses a toxic threat to *E. coli*. Since SOD proteins are thus far known to perform only a single function, i.e., the rapid dismutation of O_2^-, and since the other known oxygen protective enzymes, i.e., the catalases and peroxidases are induced only in some of the conditions resulting both in elevated respiration and SOD, it was suspected that O_2^- was either an essential precursor of, was itself the primary oxygen product in these experiments. There are now several reports suggesting that the classical respiratory chain in intact mitochondria and chloroplasts each evolve significant levels of O_2^-.[89-93] If the diversion of electrons from reduced respiratory chain intermediates to univalent pathways of O_2 reduction is important in oxygen toxicity, then blocking the terminal oxidase should make the intermediates more reduced and increase the univalent flux. In fact it has been shown that when the cytochrome oxidase of *E. coli* is partially blocked with low levels of CN^-, the level of SOD in the cells increases.[94] Although the near universal presence of SOD in cell extracts makes it difficult to determine which reactions of the respiratory chain evolve O_2^- is evolved at the NADH dehydrogenase and ubiquinone levels in a reduced respiratory chain.[89, 90] Nevertheless, using cyanide as a means of exacerbating O_2^- flux and increasing SOD levels has serious shortcomings due to the plethora of direct and indirect effects that this ion may exert *in vivo*.

Fortunately, another and much less equivocal method of increasing intracellular O_2^- is available. A variety of *redox--active* compounds are known; these being coumpounds which will divert electrons from the normal cytochrome--cytochrome oxidase respiratory pathway to produce O_2^-. These compounds are initially reduced at the expense of NADH or NADPH via a diaphorase, but once reduced they rapidly autoxidize by transferring an electron to O_2. The herbicide paraquat (methyl violegen), numerous antitumor antibiotics, some dyes, and natural naphthoquinones such as plumbagin and juglone are such compounds and can produce O_2^- so rapidly in cells with a compatible diaphorase that their overall cyanide resistant O_2 consumption is greatly enhanced.[67, 94-96] If oxygen-mediated cell damage is due at least in part to the production of low levels of intracellular O_2^- then an artificial flux of intracellular O_2^-, engendered by a redox-active compound, should both have detrimental effects and substantially induce SOD. This has proved to be true. Using paraquat, which passes readily into the cells of *E. coli*, cyanide-resistant respiration and SOD, catalase, and peroxidase activities were all greatly increased.[94, 95] Growth was retarded by very low concentrations of paraquat, while higher levels killed the cells.[95, 97] If this toxicity was due primarily to the intracellular production of O_2^-, then both dissolved O_2 and substantial levels of reduced coenzyme would have to be present for paraquat to be toxic. In the absence of a metabolizable substrate, the cells, although viable contained little or no reduced coenzyme and were unharmed by paraquat.[96] Likewise, if the cells were exposed anaerobically, paraquat had no effect on them.[97]

If paraquat is demaging *E. coli* through its generation of O_2^-, then the resistance of a cell to paraquat should be proportional to that cell's SOD content. This is shown in Figure 5. Cells grown anaerobically contained low total SOD and were very sensitive to aerobic paraquat, while those grown in air were more resistant.[94, 97] However if induction of SOD was prevented by an inhibitor of protein synthesis, such as puromycin, then exposure to low levels of SOD inducers neither increased cellular SOD nor the cell's resistance to paraquat.[95, 97] In short, the toxicity of paraquat is dependent on its ability to produce O_2^- *in vivo* and SOD is an essential defense against this toxicity.

In other studies *Streptococcus faecalis* was also shown to respond to redox-active compounds such as the anti-tumor antibiotic streptonigrin and to increased pO_2 by greatly increasing its SOD content, and as with *E. coli*, cells high in SOD were then much more resistant to both.[57, 67, 68, 88] If a low SOD content makes *E. coli* or *S. faecalis* more sensitice to intracellular O_2^-, then in *Lactobacillus plantarum* and related organisms[64, 98] should one not see the same effect by lowering the intracellular Mn? *L. plantarum* cells grown on a medium deficient in Mn and exposed to the redox-active

O_2^--generating naphthoquinone plumbagin show 10^4 fold greater kill by the intracellular flux O_2^- than identical controls grown in sufficient Mn.[98]

9. Superoxide dismutase in anaerobes — a useless protein?

If the hypothesis that the total SOD of *E. coli* is adjusted to that of the O_2^- threat is valid, then why does the Fe-SOD activity remain unchanged in cells grown for many generations in the complete absence of O_2? There is reason to suspect that this may be a safety device. Since it requires a substantial length of time for a cell to induce, transcribe, and translate a protein, sudden exposure of anaerobically grown cells, having no SOD activity, to O_2 would leave them completely unprotected againsts O_2^- for a critical period of time. A low constitutive level of SOD, such as is found in *E. coli* would alleviate this problem.

Such standby protection be a particularly useful adaptation in rumen and intestinal organisms that live and multiply anaerobically, but which must survive sudden exposure to oxygen during transmission to a new host. This may explain why a number of obligately anaerobic bacteria have been shown to have SOD activity.[55, 99-102] *Bacteroides fragilis*, unable to grow in the presence of O_2, maintains a low constitutive level of SOD ans will substantially increase this level when exposed to low levels of O_2. At least some of the most oxygen intolerant organisms known, the methanogens, also contain SOD.[103] These organisms require highly reducing conditions (an ϵ_h <-300 mv) to grow but clearly must survive at least brief exposure to O_2 to initially colonize the rumen. The methanogen *Methanospirillum hungateii* provides a specific example of why the presence of SOD activity may be required in obligate anaerobes. This organism contains an NADH-dependent flavin diaphorase which can directly reduce O_2 to O_2^-, so that exposure of metabolically active cells to O_2 ensures that a substantial flux of O_2^- will be generated intracellularly.[53] Presumably not coincidentally, this species a substantial level of SOD (T. Kirby personal communication). There is a similar case among the SOD-free, Mn containing lactobacilli. The rumen anaerobe *Lactobacillus ruminis* contains high Mn levels and while unable to grow aerobically will remain viable in air for long periods and will survive substantial intracellular fluxes of O_2^-.[98] In contrast, strains of *L. acidophilus* and *L. bulgaricus* cantaining neither high Mn levels nor SOD are extremely sensitive to O_2^- and lose viability upon exposure to air.[98] *Lactobacillus ruminis* also provides a clear demonstration that inability to grow in air may arise from causes other than the presence of toxic oxygen species.

10. Extracellular superoxide

It has been known since 1933[104] that phagocytes are *activated* by exposure to any of a variety of substances, including microbial cells or extracts, certain short peptides, phorbol myristate acetate, fluoride, the ionophore A23187, and zymosan. Activation involves a large increase in O_2 consumption, hexose monophosphate shunt activity, lacate production, increased cell motility and phagocytosis and the production of large amounts of H_2O_2. It is now known that most or all of the respiratory burst can be accounted for by a membrane-bound NADPH oxidase or superoxide synthetase whose primary product is O_2^-, and that the observed accumulation of H_2O_2 is due largely to the dismutation of the O_2^-.[47] Patients with chronic granulomatous disease (CGD) are characterized by having neutrophils that can phagocytose but not kill microorganisms, and are thus dangerously prone to microbial infections.[48] The PMNs of these people appear normal but lack detectable superoxide synthetase activity and do not exhibit the respiratory burst or O_2^- and H_2O_2 production. There is direct evidence that these oxygen species are important in the microbicidal activity of phagocytes. The myeloperoxidase system shown to efficiently kill bacteria by halogenating their cell walls and membranes requires the presence of H_2O_2 and primary amines to function. When ferrated, the iron chelating protein lactoferrin, released by PMNs catalyzes a Haber-Weiss type production of OH· from O_2^- with 5,000 times the rate of Fe-EDTA.[105] Superoxide has also been shown to produce a specific fatty acid derived product which is a potent neutrophil chemotactic factor.[18] It is therefore nor surprising that SOD has been found to be an effective anti-inflammatory agent.[106-108] Thus the ability of a microorganism to resist exogenous O_2^- may be important to its ability to resist phagocytic kill, although direct evidence for this is still regrettably scanty.

A number of different approaches to determining the effects of extracellular O_2^- on bacteria have been tried. When paraquat is reduced to its monocation radical by the diaphorase of *E. coli*, it can readily pass through the envelope of *E. coli* although O_2^- cannot.[96] Since the rate of paraquat radical oxidation and hence of O_2^- formation is limited by the availability of O_2, the lower the pO_2, the more reduced paraquat diffuses out of the bacterial cells to autoxidize and form O_2^- in the extracellular medium. It has been shown that only when the intracellular pO_2 is sufficiently low to permit substantial egress of reduced paraquat do extracellular SOD and catalase protect the cells.[96] Less elaborate methods of producing exogenous O_2^-, i.e., via the xanthine oxidase mediated generation of O_2^-[42] or by a photochemical source of O_2^- (illuminated riboflavin and methionine), wee equally toxic to *E. coli* B[31, 69] and extracellular SOD afforded a large measure of protection.[96] Likewise, *L. plantarum* was killed by exogenous O_2^- and protected by exogenous SOD and catalase.[31] However another study found that while both *E. coli* and *Staphylococcus epidermidis* were killed by exogenous O_2^-, and *S. epidermidis* was protected by exogenous SOD and catalase only exogenous catalase benefited the *E. coli* cells.[32] An unusual finding was that in *Neisseria gonorrhoeae* catalase protected against the O_2^- and H_2O_2 produced by the xanthine oxidase reaction but SOD did not.[109] Further, although they are obligate aerobes with active respiratory chains, strains of this pathogen with no detectable SOD have been reported.[110] Another method of generation of O_2^- is via electric discharges in air, with trapping of the resultant *negative air ions* in water. Superoxide generated in this fashion killed cells of *Staphylococcus albus*, and SOD gave nearly 100% protection while catalase was ineffective. Interestingly, in the absence of a deliberate production of extracellular superoxide, the addition of SOD to a suspension of *Campylobacter fetus*, a microaerophile, substantiallu increased its oxygen tolerance.[112] If extracellular O_2^- can seriously damage cells was important. While exposure to H_2O_2 and O_2^- from the xanthine oxidase reaction killed cells of *Sarcina lutea* and *Staphylococcus aureus* and both SOD and catalase protected, lethality varied somewhat with the O_2^- generating system employed.[113] Catalase produces substantially improved aerobic growth of many of the lactobacilli and streptococci which normallu release large amounts of H_2O_2.[114] From the foregoing, it seems reasonable to conclude that under some conditions the presence of O_2^- alone leads to cell injury and deth, while in others H_2O_2 is most important, and in still others both toxic species play an important role. However, whether the O_2^- and H_2O_2 tolerance of animal pathogens is an important virulence factor ramains an interesting but unanswered questions.

11. Ionizing radiation and oxygen

Early in this century it was noticed by radiotherapists that tissues are generally more radiosensitive when well oxygenated than when anoxic. By the 1950s it had been shown that sensitivity to × and γ-rays but not to ultraviolet light increased when dissolved O_2 was present in the medium or tissue water.[115] For example, when a strain of *E. coli* was exposed to 60 kilorads of γ-irradiation, the presence of air decreased survival 100-1000-fold.[116] This effect is called the oxygen enhancement ratio (OER) which is the ratio of the slopes of aerobic and anaerobic kill curves or

$$\frac{D_{37}\ (\text{anaerobic})}{D_{37}\ (O_2)},$$

D_{37} being that dose of ionizing radiation permitting survival of 37% of the cells. The oxygen effect is largely independent of the type of cell irradiated and the method of assessing radiation damage, but quite dependent on the linear energy transfer (LET) of the radiation in the medium used.[115, 116] For example 2 MEV beryllium deuterons gave OERs of between 1.3 and 1.8 for ascites tumor cells, *Shigella flexneri*, Saccharomyces cerevisiae and *E. coli* B in four different laboratories and using different damage criteria, while 200 KEV ×-rays gave OERs of 2.1-3.7 in the same cells.[117] Oxygen enhancement of radiation toxicity is also dependent upon the pO_2, increasing rapidly at low pO_2 values, but saturating in all cases at about 5% of atmospheric pressure. In addition to active cells in aqueous media, the oxygen effect is seen with fried spores od *Aspergillus* and *Bacillus*, desiccated purified enzymes, and nucleic acids.[117] DNA has been reported to have a particularly high OER, 3.7 compared to 1.5-2.0 for enzymes.[118] Other indications of the particular sensitivity of DNA to oxygen-mediated radiation damage are high OERs for intracellular bacteriophage inactivation[119] and loss of transformability by *Streptococcus pneumoniae* DNA.[118] In another study, however, the transfecting ability of phage DNA exposed to ×rays showed little oxygen effect.[116] In four strains of *E. coli*, as well as strains of *Bacillus subtilis* and *Pseudomonas aeruginosa*, efficient DNA repair was associated with increased OER leding to the speculation that non-reparable double-stranded breaks in the DNA are oxygen-dependent.[120] Interestingly, there is evidence that *Micrococcus radiodurans*, a highly radio-resistant microorganism is able to repair double--stranded breaks and in addition has high catalase and SOD levels.[116, 121]

The mechanism(s) responsible for radiation damage are still nor clear. There is a long-held hypothesis that the primary action of the radiation is to create evanescent organic radicals, which in the presence of O_2 form peroxides, i.e., damage to organic molecules is *fixed* via peroxidation which only occurs if O_2 is present. Alternatively, or in addition, oxygen radicals may be a primari cause of damage. It is known that when × or γ-rays strike water a variety of oxygen radical species are produced

$$H_2O \xrightarrow{h\nu} H\cdot + e^-_{aq} + H_3O^+, H_2O_2$$

however, H· and e^-_{aq} are very reactive and if any O_2 is dissolved in the H_2O

$$H\cdot + 0_2 \rightarrow HO_2 \rightleftharpoons H^+ + O_2^-$$

and

$$e^-_{aq} + O_2 \rightarrow O_2^-$$

Of course, as in other free radical systems, subsequent reactions may be expected to generate OH· and possible $^1O_2$. Are these reactions and radicals of real importance in radiation damage? Data are still scanty. In one study *E. coli* cell suspension were exposed to 170 KEV ×-rays and lethality was increased 2.4-fold by oxygen. Extracellular SOD, catalase, and the hydroxyl radical scavengers mannitol and histidine all substantially reduced the OER.[122] A second study with *E. coli* showed that the OER accompanying ×-ray exposure dropped from 2.35 to 1.4 when SOD was added.[123] A third report likewise found SOD to partially protect *E. coli*.[116] The mycoplasma *Acholeplasma laidlawii* is also reported to be protected by SOD against the oxygen effect.[124] In contrast, there is a report that aerobically grown *E. coli* showed little more radioresistance in O_2 than anaerobically grown cells with less endogenous SOD.[116] When a number of highly radioresistant micrococci were compared many, but not all had unusually high SOD and catalase levels.[116] However in another report, exogenous SOD did nor enhance the resistance of one of these organisms *Micrococcus radiodurans* to the oxygen effect.[120] There is also a study reporting that coliphage T-4 is not protected from ×-rays damage by the presence of exogenous SOD.[125] In Eucaryotes, SOD has been shown to protect isolated myoblasts[26] alveolar macrophages[126] hemopoietic and mature blood cells[127] and mice[128, 129] from ×-ray damage.

Thus, while at present we have only a poor understanding of the molecular mechanisms behing radiation sensitivity and the oxygen effect it seems clear that SOD, as well as catalase, peroxidase, intracellular reductants, hydroxyl radical and singlet oxygen scavengers and DNA repair enzymes must all be considered as potentially important factors.

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Address for Reprints: Frederick S. Archibald
Department of Biochemistry
Duke University Medical Center
Durham - North Carolina - U.S.A.