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Oxygen, Oxidases, and the Essential Trace Metals

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Phil. Trans. R. Soc. Lond. B 1981 **294**, 119-128
doi: 10.1098/rstb.1981.0093

Phil. Trans. R. Soc. Lond. B **294**, 119–128 (1981) [119]
Printed in Great Britain

Oxygen, oxidases, and the essential trace metals

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The dominant function of dioxygen as the terminal electron acceptor in aerobic systems is well established; the roles of iron and copper in the terminal oxidases are less well understood. The minor, but crucial, part that dioxygen plays in other biological processes has recently attracted much attention. The chemistry of the reduction products of dioxygen is described and the possible relation of these products to the toxic properties of dioxygen is discussed. It is suggested that the uncontrolled reaction of dioxygen with reduced species, to give the superoxide ion, hydrogen peroxide, the hydroxyl radical and perhaps other entities derived from these, is potentially hazardous to the organism. Defences exist against these species, not least in the dismutases dependent on copper–zinc, manganese and iron, in catalase and in the selenium-dependent peroxidase. The effectiveness of these defences is examined and their integrities in situations of metal deficiency are examined. The employment of the reduction products of dioxygen during phagocytosis is discussed.

Introduction

The use of dioxygen as the terminal electron acceptor by aerobes is accomplished with the aid of a remarkable enzyme, cytochrome oxidase (Malmstrom 1980), which has been the subject of intensive investigation; some details of the molecular architecture are beginning to emerge. The redox proteins that precede it in the respiratory chain are one-electron transfer proteins. Cytochrome oxidase contains *four* redox centres, two containing copper and two containing haem. The spatial relation between these centres has not been completely established but there appears little doubt that haem a_3 and one of the copper sites are close, perhaps very close, even to the extent of sharing a ligand. The haem *a* centre, which is presumed (Malmstrom 1980) to accept the electron from cytochrome *c*, and the other copper site are some distance removed. These ideas (Chance *et al.* 1979; Blum *et al.* 1980) are depicted in figure 1.

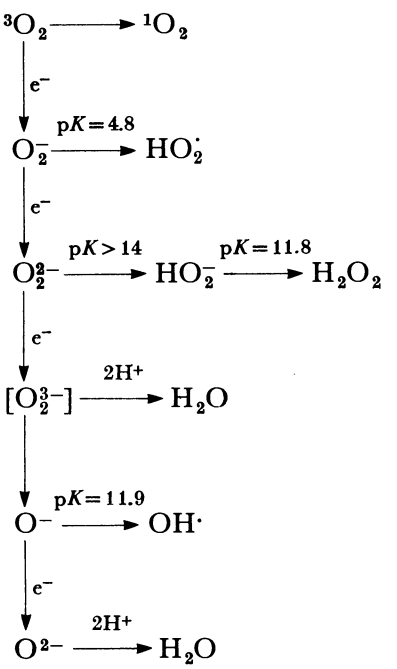
Cytochrome oxidase is embedded in a mitochondrial membrane with one face exposed to the outer surface, where it encounters the water-soluble cytochrome *c*. On the other side of the molecule rests the haem a_3–copper couple. When fully reduced, the latter captures dioxygen giving, perhaps, a Fe^{III}–O–O–Cu^{II} μ-peroxo-bridged species, within which subsequent reduction to water proceeds. In this way the cytochrome oxidase behaves as a molecular 'capacitor' specifically discharged by dioxygen and protons. Crucial to this role is the reduction of dioxygen without the concomitant release of the reduction products of dioxygen (other than water), at least in an unperturbed or undamaged system. Hence from this most crucial part of the respiratory chain, no potentially toxic or destructive entities are produced. This may not be true elsewhere in the cell. There is very good evidence (Chance *et al.* 1979) for the production of both hydrogen peroxide and superoxide, the principal source being the reaction of dioxygen with reduced ubiquinone. It is not immediately obvious whether such a process has, or could have, a function. In general, we should expect that the reaction of dioxygen with 'electrons', i.e. with reduced components of the respiratory chain, in an *uncontrolled* manner would present a hazard to any cell in which such a reaction took place. It would amount to a short-circuiting of electron

conduction or an uncontrolled discharge of the biological 'capacitor' referred to earlier. Reagents that promote or cause such short-circuiting would be expected to be particularly toxic to biological systems in which both oxygen and electron transport are present. The deleterious effect of the viologens on photosynthesizing organisms in which electrons are diverted from the electron transport system and passed via the reduced viologen to dioxygen is one such example (Dodge 1977).

Though it might seem that the use of a respiratory chain in which direct reduction to water was the end result would be the most efficient method of embracing the free energy available, some organisms, principally plants (Bendall & Bonner 1971) and bacteria (Lloyd & Edwards 1978) find that the two-electron reduction to hydrogen peroxide suffices. Thus these intermediate reduction products are not toxic *per se*; it is presumably only when they are temporally or spatially misplaced, where control is not present or, failing that, when defences are absent or impaired, that problems arise. What are the properties of these intermediate reduction products?

The chemistry of dioxygen

The species directly derived from dioxygen are shown in scheme 1 (Hill 1978). A most



Scheme 1

important property of the intermediate reduction products is that they are all good oxidants with respect to reduction to water and, most important, they suffer no kinetic constraints in that they either react rapidly or their reactions are subject to ready catalysis. This seeming paradox may well be exploited by biological systems: to effect oxidations, prior *reduction* of enzyme or cofactor is required. The clearest examples of this exploitation of the oxidizing ability of the reduction products of dioxygen are probably the monooxygenases. Of course, with these enzymes the reactivity of the reduction products is much modified by coordination

to metal ions or incorporation in organic cofactors. The unambiguous identification of these reduction products in biological, and indeed chemical, systems has proved difficult. In addition to the usual problems of designing assay methods that specifically detect any given species, the 'incestuous' reactions between members of this family, of which dioxygen is one parent and the electron the other, pose a severe problem. Some of these complicating reactions are shown in table 1. The situation is even more complicated when one considers the possible perturbation

TABLE 1

	k/mol^{-1} s^{-1}
$HO_2 + HO_2 \longrightarrow O_2 + H_2O_2$	8.6×10^5
$H_2O_2 + O_2^- \longrightarrow O_2 + OH^- + OH^\cdot$	0.13
$OH^\cdot + O_2^- \longrightarrow O_2 + OH^-$	1×10^{10}
$OH^\cdot + H_2O_2 \longrightarrow HO_2 + H_2O$	$1\text{–}6 \times 10^7$
$OH^\cdot + OH^\cdot \longrightarrow H_2O_2$	5.2×10^9
$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2$	—

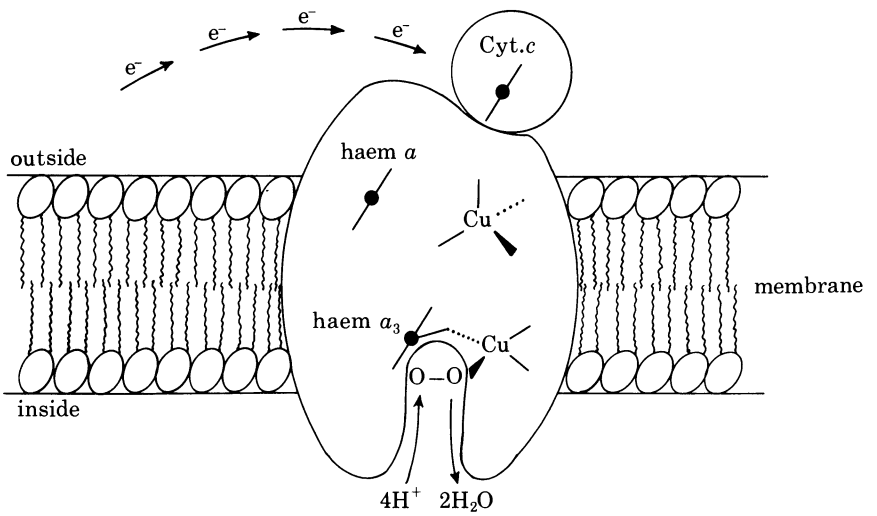


FIGURE 1. An illustration of some of the properties of cytochrome oxidase and its interaction with cytochrome *c*, based on a figure in Chance *et al.* (1979).

of these reactions by metal ions (Groves 1980), especially redox-active metal ions. For example, the so-called catalysed Haber–Weiss reaction is often written as

$$M^{n+} + H_2O_2 \longrightarrow OH^\cdot + OH^- + M^{(n+1)+};$$
$$M^{(n+1)+} + O_2^- \longrightarrow M^{n+} + O_2.$$

In addition, one should consider the reactions of metal complexes of these reduction products of dioxygen, e.g.

$$M^{n+} + OH^\cdot \longrightarrow MO^{(n-1)+} + H^+$$
$$M^{n+} + O_2^- \longrightarrow MO_2^{(n-1)+}$$
$$M^{n+} + H_2O_2 \longrightarrow [MO_2H]^{(n-1)+} + H^+.$$

The species $MO^{(n-1)+}$ is exemplified by the postulated ferryl ions, FeO^{2+}, and possibly cupryl

ions, CuO^+. All of these species are more likely to be found in complexes containing other ligands, and the analogous haem derivatives have been proposed as intermediates in peroxidases (Dunford 1979) and monooxygenases (Blake & Coon 1979), i.e. [haem]$Fe^{IV}O$; [haem]$Fe^{III}O_2$ and [haem]$Fe^{III}O_2H$.

The redox-active metal ions are perhaps the major perturbations of the interfamilial reactions referred to above. Reactions of all the reduction products of dioxygen, or the metal complexes derived from them, with organic compounds could play an important part in expressing the toxicity of molecular oxygen. The more prominent products of such reactions are peroxides, hydroperoxides and epoxides. A less obvious reactant is chloride which, in the presence of hydrogen peroxide and myeloperoxidase (MPO) (Klebanoff & Clark 1978), a constituent of neutrophils, gives rise to hypochlorite. This then reacts further with O_2^- to give (Long & Bielski 1980) the hydroxyl radical:

$$H_2O_2 + Cl^- \xrightarrow{\text{MPO}} HClO + HO^-$$
$$HClO + O_2^- \longrightarrow OH^\cdot + O_2 + Cl^-$$
$$\text{overall: } H_2O_2 + O_2^- \longrightarrow OH^\cdot + O_2 + OH^-.$$

An important lesson emerges from these considerations, namely that it is difficult to predict the reactions of oxygen-derived species in biological systems from consideration of their reactions *in vitro*. The conjunction of different components of a cell is such that unless the distribution of these species with a cell is strictly controlled, many other reactions involving the principal reduction products of dioxygen may take place. The last reaction is a striking example of this as it represents a chloride ion promoted Haber–Weiss reaction, albeit catalysed by myeloperoxidase.

Thus the consequences of one-electron addition to dioxygen to form the superoxide ion appear to be that, unless the products are trapped within a metal complex, or some other environment within which they are constrained, reactions leading to the other reduction products of dioxygen may then ensue. Some examples of systems in which the superoxide ion has been detected are given in table 2. Estimates have been made (Chance *et al.* 1979) of the rate of production and steady-state levels of superoxide and hydrogen peroxide in intact cells or organelles. There is no doubt that, even in cells subject to the minimum perturbation, these reduction products are found, sometimes in considerable amounts. In conditions that might be described as pathological, i.e. where damage has been done to the cell or to its contents, it is likely that the amount of O_2^- and H_2O_2 produced will be greater. Both are sufficiently reactive to pose a threat to the cell. Of the reactivity of the hydroxyl radical there can be no doubt; it is difficult to think of a cellular component that would be safe from attack. In addition, all can act as precursors of less-reactive, longer-lived radicals subject to few constraints and able to diffuse to different parts of the cell. This has been suggested (Michelson & Durosay 1977) in relation to photohaemolysis and its enhancement by carbonate, with the indictment of the CO_3^- radical anion. We have found that haemolysis by phenylhydrazine, a so-called 'oxidant drug', is associated with the formation of the relatively long-lived phenyl radicals (Hill & Thornalley 1981).

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Table 2. Biological sources of the superoxide ion

(*a*) *enzymatic product*
e.g. xanthine oxidase, aldehyde oxidase

(*b*) *enzymatic intermediate*
e.g. galactose oxidase, 2-nitropropane dioxygenase, cytochrome P-450

(*c*) *adventitious interaction of dioxygen with electron transport chains*
mitochondrion, cytochrome b_{566}

(*d*) *autooxidation*
e.g. haemoglobin, non-haem iron proteins, reduced flavins, reduced quinones

(*e*) *phagocytosis*

(*f*) *antibiotics*
e.g. bleomycin, streptonigrin

Defences against dioxygen and derived species

Hyperbaric dioxygen is toxic to most aerobes (Gerschman 1964; Fridovich 1975, 1978). In mammals it presents as convulsions followed by lung damage. In such conditions the body reacts by attempting to control the partial pressure of dioxygen in most organs (other than the lung) by means of the arterioles. A second line of defence seems to be simply increased respiration, that is detoxification by reduction to water via cytochrome oxidase, which might therefore be considered as part of the body's defensive mechanism. Are there further defences if these are not sufficient, if uncontrolled one-electron reduction takes place? The superoxide dismutases constitute a highly efficient defence against this potential precursor of reactive entities. There are three classes of superoxide dismutases (Fridovich 1979; Bannister & Hill (eds) 1980; Bannister & Bannister (eds) 1980) (table 3): the copper–zinc proteins found in eukaryotes, the manganese protein found in both eukaryotes and prokaryotes and the iron protein found mainly in prokaryotes. In eukaryotes, the copper–zinc protein is found in the cytosol and the manganese protein in the mitochondria though the distribution is species-related and can be altered by the nutritional state of the animal. [Recently there has been much discussion (Fee 1980*a*, *b*, 1981) of the functional role of the superoxide dismutases. There is no doubt that they *do* catalyse the disproportionation of the superoxide ion. So do other copper complexes, even aquated copper(II) itself, the latter just as well as the enzyme. This had caused problems for those who find it difficult to accept that simple metal complexes can ever have reactions that are as rapid as those catalysed by enzymes. This is manifestly not so for reactions with small substrates such as O_2 or O_2^-, in which the molecular architecture that must surround the metal ion to effect catalysis can be much less complicated than with large substrates, which require a corresponding complexity of the enzyme surface. Enzymes also have biological roles in addition to catalysis: they must be located, their synthesis must be controlled and competing reactions must be minimized. These will be hard to accomplish by using simple complexes. It has also been argued (Fee 1980*a*, *b*, 1981) that the superoxide ion is so unreactive that it could not be toxic. Even if this is true (but consider (Valentine 1979; Sawyer *et al.* 1980) the rapidity of its action as a one-electron reductant in water or its powerful nucleophilicity in aprotic solvents and the perturbation of its reactions by complexing to metal ions), the fact that it is the precursor of toxic species makes it toxic *de facto*.] Superoxide dismutase, on its own, cannot be considered an unalloyed blessing since it efficiently produces H_2O_2 from O_2^-. One therefore imagines that, if detoxification is its function, it must act in concert with enzymes that dispose of H_2O_2. The best known of these is catalase (Schonbaum & Chance 1976). This haem protein is found mainly

in the mitochondria and peroxisomes. At high concentrations of H_2O_2 it is a most effective catalyst. In other circumstances the detoxification of H_2O_2 may reside in the peroxidases. The mammalian glutathione peroxidase (Flohé *et al.* 1976), which contains selenium, resides mainly in the cytosol, with the remainder in the mitochondria. Glutathione peroxidase, though specific for glutathione, is catholic in its taste for peroxides. Whereas catalase will only accept H_2O_2 and small alkyl hydroperoxides, most hydroperoxides and H_2O_2 will act as substrates for glutathione peroxidase.

Table 3. The superoxide dismutases

	source	10^{-3} M_r	number of subunits	metal content per subunit
copper–zinc	eukaryotic organisms	30–35	2	1 Cu, 1 Zn
manganese	bacteria, blue-green algae, red algae	40–45	2	1–2
	bacteria, eukaryotic mitochondrion, cytoplasm	70–100	4	2–4
iron	bacteria, blue-green algae	37–43	2	1–2
	bacteria	85	4	4

Where, in a given cell, lies the threat from the reduction products of dioxygen? The location of dioxygen and its reduction products has been gauged by investigating the distribution of the enzymes presumed to act as a defence against them. Much elegant use has been made of the formation of compound I from catalase and H_2O_2 (Chance *et al.* 1979). Ideally one would like to estimate the local concentrations *independently* of the location of enzymes for which they act as substrates. A reasonable assumption is that the reduction products of dioxygen are likely to occur where dioxygen is used. In the eukaryotic organism this is presumably mainly in the mitochondrion, the peroxisome and the endoplasmic reticulum. Do the cytosolic enzymes protect the latter against the deleterious effects? There have been reports (Conover & Siebert 1965; Conover 1967) of respiratory activity in the nucleus; which enzyme defends the nucleus is not well understood (Patton *et al.* 1980). Even if dioxygen is not utilized in a particular organelle, there remain two additional threats: the reduction products may diffuse from, for example, the many oxidases in the endoplasmic reticulum. If dioxygen itself diffuses to other parts of the cell, the presence of high-energy radiation, which can generate all the reduction products, may initiate cellular damage. This could be of the utmost importance in connection with the mutagenic effects of radiation; if the nucleus lacks adequate defences, the genetic material is at risk.

Consequences of alterations in the defensive capability

As might be expected, the activity of copper–zinc superoxide dismutase is decreased in copper deficiency (Bohnenkamp & Weser 1976), that of manganese dismutase in manganese deficiency (DaRosa *et al.* 1978) and that of glutathione peroxidase in selenium deficiency (Boyne & Arthur 1979). However, it is difficult to point to causal relations between the reduced amounts of these enzymes and any physiological consequence. It may be that there is sufficient redundancy that, unless the enzymes are completely absent, no damage is caused. That is not to say that there are no effects of copper, zinc, manganese or selenium deficiencies; evidently there are, but it has not yet been established whether these are directly related to the increased production of oxygen-derived radicals.

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Various conditions related to genetic defects may be relevant. For example, in acatalasaemia, catalase is absent (Takahara 1952). The only apparent consequence is expressed in an increased tendency to gingivitis. Another genetic defect yields activities of glutathione peroxidase one-quarter of normal; again, there are no apparent pathological effects (Necheles 1974). No congenital defect resulting in an absence of the superoxide dismutases has been reported; it is possible that such a defect would be lethal. However, in trisomy 21 (Down's syndrome), in which chromosome 21, which contains the gene for the copper–zinc superoxides dismutase (Tan *et al.* 1973), is triplicated, there is a 50% increase in the concentration of the copper–zinc protein (Sinet 1977). Interestingly, the manganese dismutase concentration (the gene for which is on chromosome 6 (Smith *et al.* 1978)) is decreased by 30% and amazingly, the concentration of glutathione peroxidase (coded for on chromosome 3 (Donald *et al.* 1979)) is increased by 50% (Sinet *et al.* 1975). This is a striking reflection of the interrelation between the defensive forces of the body. Again, when, in copper deficiency, the copper–zinc dismutase activity decreases, that of manganese dismutase increases. In the fungus, *Dactylium dendroides*, grown under conditions of copper deficiency, the manganese dismutase, normally found in the mitochondrion, appears in the cytosol (Shalzman & Kosman 1979).

The generation of oxygen-derived radicals during phagocytosis

The potential toxicity of oxygen-derived radicals may be turned to good use in phagocytosis. The respiratory burst associated with phagocytosis by leucocytes is now believed to yield oxygen-derived species, O_2^-, H_2O_2, $OH^{\cdot}$, ClO^- and perhaps singlet dioxygen (Klebanoff & Clark 1978). These are thought to be employed to rupture the cell walls of target organisms or host debris. We have shown (Green *et al.* 1979; Okolow-Zubkowska & Hill 1980) that the superoxide ion is the precursor of the hydroxyl radical in this sequence of events and that consequently superoxide dismutase and copper complexes act as good inhibitors of the production of the hydroxyl radical. The steady-state concentration of the species produced depends on the method by which the respiratory burst is stimulated and the temperature at which the experiment is conducted. A question arises as to how the neutrophil protects itself against these radicals, which, under the conditions that most experiments have been reported, are released into the extracellular medium. Though the consequences of decreased superoxide dismutase levels in the neutrophils have not been examined, it has been shown (Boyne & Arthur 1979) that selenium-deficient neutrophils, which presumably have lowered amounts of glutathione peroxidase, are less able to kill *Candida albicans* and yield only about 50% of the amount of hydroxyl radicals as the control.

There has been much discussion (Bannister & Bannister (eds) 1980) of the relation between the production of oxygen-derived species during the respiratory burst and various inflammatory conditions, of which rheumatoid arthritis (Salin & McCord 1974) has attracted the most attention. It has been suggested that the oxygen-derived species, as well as taking part in killing, are also involved in the synthesis both of prostaglandins and of chemotactic factors (McCord *et al.* 1980). If so, exogenous sources of the normally extracellular enzymes that have these oxygen-derived species as substrates might well be used to ameliorate their destructive effects. A number of experiments that strongly suggest that this might have some promise have been reported (Bannister & Bannister (eds) 1980). An elegant demonstration of the destructive effects of the reduction products of dioxygen on the microcirculatory system and the protective effects of

superoxide dismutase, catalase and hydroxyl radical scavengers has been described (delMaestro *et al.* 1980).

Conclusions

It is a truism that dioxygen is essential for aerobic organisms. Is it a two-edged sword, an obligatory toxin? It appears that it must be considered a hazard against which an elaborate defence has been mounted. As with the best defence it is essential to attack early and, as summarized in figure 2, this can take place by controlling the local partial pressure of oxygen,

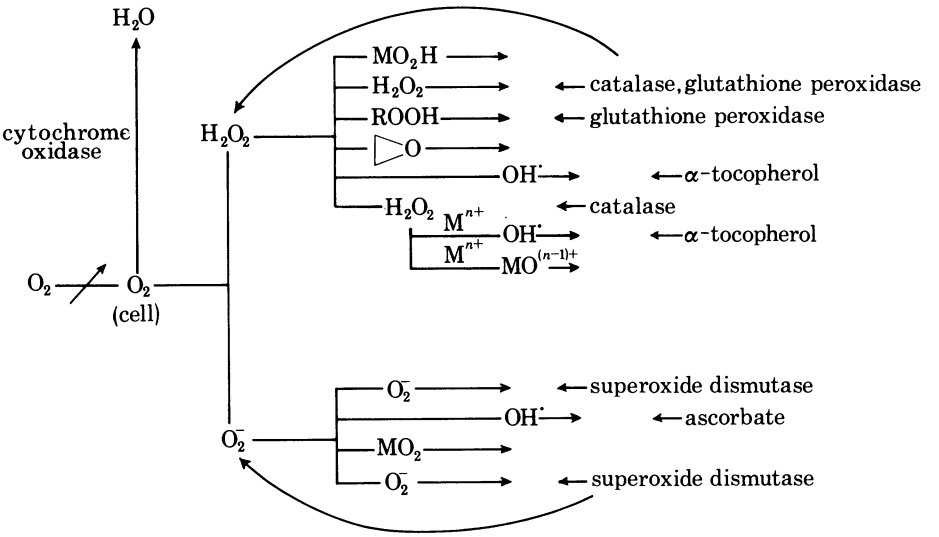

Figure 2. A schematic description of the interaction between dioxygen and species derived from them and components of the cellular defence.

by diverting it to cytochrome oxidase (a highly efficient defence) and, failing that, by destroying the resulting reduction products by a combination of the superoxide dismutases, catalase and the peroxidases. If these too fail, and, for example, the hydroxyl radical results, one is left with non-specific defence mechanisms involving α-tocopherol or perhaps ascorbate, or relies on the destruction of cellular components with subsequent repair. Inorganic elements may likewise have a dual influence on tissue integrity, first as essential components of defence when their activities are controlled and circumscribed, but, if not, they may join forces with the attacking oxygen radicals to amplify their destructive power.

I wish to thank the British Heart Foundation for support, and my colleagues, Professor W. H. Bannister, Dr J. V. Bannister, Dr M. P. Esnouf and Dr A. E. G. Cass, Mr P. J. Thornalley and Miss M. J. Okolow-Zubkowska, for their help.

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