# SUPEROXIDE AND SUPEROXIDE DISMUTASE IN RED BLOOD CELLS

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## Superoxide and Superoxide Dismutase

Oxygen has a propensity for one-electron reduction to superoxide radica's which are potentially harmful in biological systems. The superoxide radical exists briefly as an anion ($O_2^-$) at physiological pH. It decays by dismutation to molecular oxygen and hydrogen peroxide:

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

In 1969 a previously obscure copper protein of red blood cells, erythrocuprein, was shown to catalyse the dismutation of superoxide radicals (McCord and Fridovich, 1969). Erythrocuprein thus became superoxide dismutase and the object of intensive study.

Superoxide dismutase is typically an enzyme of aerobic organisms which utilise oxygen as the major electron acceptor. The presence of superoxide dsmutase in microorganisms has been found to parallel their tolerance for oxygen (McCord **et al.**, 1971). Thus aerobes have the highest levels of superoxide dismutase, aerotolerant anaerobes contain intermediate levels and obligate anaerobes do not possess the enzyme with the exception of some sulphate reducing bacteria and clostridia (Hewitt and Morris, 1975; Hatchikian **et al.**, 1976).

Erythrocuprein was shown to be a copper-zinc protein by Carrico and Deutsch (1970). Eurkaryotes have a copper-zinc superoxide dismutase in the cytoplasm and a manganese enzyme in the mtochondria (Weisiger and Fridovich, 1973). Prokaryotes have a manganese superoxide dismutase in the cell matrix and an iron enzyme in the periplasmic space (Keele **et al.**, 1970; Yost and Fridovich, 1973). The luminous bacterium "Photobacterium leiognathi," which is a fish symbiont, is exceptional in that i+ contains iron and copper-zinc superoxide dismutases (Puget and Michelson, 1974).

The manganese and iron superoxide dismutases of **E. coli** B have been shown by sequence homology to be related to each other and to mitochondrial manganese superoxide dismuatase from chicken liver, but they are entirely different from eukaryote cytosol copper-zinc superoxide dismutases (Steinman and Hill, 1973). This supports the idea that present day mitochondria are the descendants of prokaryotes which entered into an endocellular symbiosis with protoeukaryotes (Fridovich, 1974).

A variety of oxidation-reduction reactions have been shown to produce superoxide radicals (Fridovich, 1975). The potential sources of these radicals in the cell are of three kinds. One source is the oxidation of the reduced forms of several compounds found in biological systems including hydroquinones, leucoflavins, catechol amines, thiols, tetrahydropteridines, ferredoxin and rubredoxin. A second analogous source is the oxidation of metabolites by certain enzymes including xanthine oxidase, aldehyde oxidase, dihydroorotic dehydrogenase and some flavoprotein dehydrogenases. A third source is the dissociation or displacement of superoxide from the oxygenated adducts of haem proteins including haemoglobin and oxyperoxidase. The production of superoxide radicals during autoxidation of oxyhaemoglobin and decay of oxyperoxidase to ferric peroxidase has been demonstrated (Misra and Fridovich, 1972; Rotilio **et al.** 1975). The autoxidation of oxyhaemoglobin is discussed below.

Toxic reactions follow the reduction of oxygen to superoxide radicals or hydrogen peroxide. The oxygen molecule has two

unpaired electrons with parallel spins, one in each of two anti-bonding ($\pi^*$) molecular orbitals. This state is the one of lowest energy and reactivity for oxygen, the ground or triplet state. Two excited or energetically higher singlet states are possible with spin pairing of the two electrons in one or over the two $\pi^*$ orbitals. Singlet oxygen is far more reactive than triplet oxygen although its life-time is extremely short (about one microsecond in water). Univalent reduction of oxygen to superoxide radicals is favourable because acceptance of an electron in one of the half-filled $\pi^*$ orbitals does not cause a need for spin inversion.

The ubiquity of superoxide in biological systems is clear but little is known about its reactivity with biological material. Deleterious effects may be due to superoxide itself, to other oxygen reduction products derived from superoxide, or to free radicals generated by reaction of superoxide or its products with cellular components. Superoxide can mediate reductions, giving up its extra electron, or oxidations, becoming reduced to hydrogen peroxide. In general free radicals are highly reactive. They can combine with one another to form covalent bonds or they can attack bonds in other molecules. This often leads to production of new radicals, setting up a chain reaction. Superoxide has limited bond breaking ability (Sutton **et al.**, 1976).

The superoxide anion ($O_2^-$) is the conjugate base of a weak acid called the hydroperoxyl radical ($HO_2\cdot$) whose $pK_\alpha$ is 4.8. The rate constant for spontaneous dismutation of superoxide species at pH 7.0 is of the order of $10^5$ $M^{-1}sec^{-1}$, whereas the dismutaton catalysed by superoxide dismutase has a rate constant of the order of $10^9$ $M^{-1}sec^{-1}$ (Fridovich, 1975). The half-life of superoxide radicals can therefore be decreased by several orders of magnitude by the enzyme. Another favourable consideration is the concentration of superoxide dismutase in the cell which is estimated to be at least five orders of magnitude higher than the steady state concentration of superoxide (Fridovich, 1975). The probability of collision of a superoxide radical with enzyme is therefore much greater than that of collision with another superoxide radical.

The spontaneous reaction of superoxide with itself generates hydrogen peroxide and singlet oxygen. The dismutase reaction produces molecular oxygen of normal reactivity, i.e. triplet oxygen, which gives the enzyme an additional advantage. Hydrogen peroxide produced by the dismutation of superoxide radicals (or otherwise) can be decomposed in the cell by catalase and glutathione peroxidase. These enzymes probably act in concert with superoxide dismutase to circumvent the toxicity of oxygen by eliminating the primary products of oxygen reduction.

It has become common practice to suggest that superoxide might generate hydroxyl radicals ($OH\cdot$) in biological media **via** the Haber-Weiss reaction,

$$O_2^- + H_2O_2 \longrightarrow OH\cdot + OH^- + O_2,$$

originally postulated as a step in the breakdown of hydrogen peroxide by iron salts (Haber and Weiss, 1934). However, this ignores the fact pointed out by Fee **et al.** (1975) that even in a solution of 95% hydrogen peroxide, superoxide reacts only by dismutation (George, 1947). It is possible that where there has been strong evidence of hydroxyl radical production, as in the autoxidation of several cytotoxic agents with formation of superoxide and hydrogen peroxide (Cohen and Heikkila, 1974), hydroxyl radicals were formed by a non-Haber-Weiss reaction not involving direct decomposition of hydrogen peroxide by superoxide radicals. Cohen and Heikkila (1974) make this point in passing. It should be noted that the hydroxyl radical is a very powerful oxidant and could vastly augment deleterious effects of superoxide.

**Superoxide in Red Blood Cells**

The red blood cell is at increased risk of damage by superoxide and hydrogen peroxide because of the carriage of oxygen by haemoglobin. Some 3% of the haemoglobin in the erythrocytes is oxidized per day and reduced again by methaemoglobin reductase and other systems which keep methaemoglobin levels at about 1% of the total haemoglobin. Methaemoglobin

formation may either involve the loss of superoxide to leave an iron (III) complex, or the addition of an electron to bound oxygen to give an iron (III) peroxide. Misra and Fridovich (1972) showed that the autoxidation of shark oxyhaemoglobin caused the co-oxidation of adrenaline to adrenochrome. Part of this co-oxidation is due to a haemoglobin-catalysed peroxidation of adrenaline since it could be inhibited by catalase. The remainder of the co-oxidation was inhibited by superoxide dismutase, indicating that autoxidation of oxyhaemoglobin results in the generation of superoxide radicals. The production of superoxide in the process of autoxidation has been demonstrated in similar ways for bovine haemoglobin (Wever **et al.**, 1973), $\alpha$ and $\beta$ chains of human haemoglobin (Brunori **et al.**, 1975), human haemoglobin A and unstable haemoglobins (Winterbourn **et al.**, 1976). Isolated $\alpha$ and $\beta$ chains and unstable haemoglobins release superoxide at faster rates than Hb A.

Whether superoxide dissociates or needs to be displaced from oxyhaemoglobin depends on the electronic structure of the iron-oxygen complex. This has been considered to involve a fully developed iron (III) superoxide ion couple (Weiss, 1964) but recent studies have shown strong covalent bonding between iron and bent, end-on dioxygen, a type of bonding which precludes ready loss of superoxide from oxyhaemoglobin. For this reason Wallace **et al.** (1974) have proposed that superoxide is displaced from oxyhaemoglobin by anionic nuncleophiles in a proton-assisted reaction. The chloride ion is sufficiently active in this regard that with a normal human erythrocyte chloride concentration of about 0.1 M it could account for much, if not all, of the normal autoxidation of oxyhaemoglobin. Another feature of haemoglobin autoxidation is its catalysis by copper probably after specific binding to the haemoglobin (Rifkind, 1974). The copper-catalysed autoxidation of Hb A also involves superoxide production (Winterbourn **et al.**, 1976). Copper might facilitate the superoxide loss mechanism in the erythrocyte via the fraction in rapid exchange with serum copper.

Release of superoxide can be regarded as the initial step in the autoxidation of haemoglobin. The superoxide can then oxidize more haem in competition with dismutation producing hydrogen peroxide, which itself gives further haem oxidation. Catalase decreases the autoxidation of Hb A by about 40%. Catalase and superoxide dismutase slow it down by about 65%. Superoxide dismutase alone has only a slight effect (Winterbourn **et al.**, 1976). This is not unexpected. Reactions which produce superoxide are not generally affected by superoxide dismutase. It is rather those reactions which are dependent upon superoxide which can be inhibited by the enzyzme (Misra and Fridovich, 1972). It is possible that in reactions where catalase has an inhibitory effect augmented by superoxide dismutase, the latter could be protecting catalase from reaction with superoxide to form the enzymatically inactive oxy form or Compound III (Fee **et al.**, 1975).

Superoxide can oxidize the haem groups in oxyhaemoglobin and reduce those in methaemoglobin. The oxidation of oxyhaemoglobin has a rate constant of the order of $10^3$ $M^{-1}sec^{-1}$ at pH 7. The rate constant for reduction of methaemoglobin is about 30% higher (Sutton **et al.**, 1976). In the erythrocyte the higher concentration of oxyhaemoglobin would favour the oxidation reaction but this is effectively suppressed by superoxide dismutase. The concentration of superoxide dismutase in normal erythrocytes is about 0.5 mg per g of haemiglobin or of the order of $10^{-6}$ M (Stansell and Deutsch, 1966). It is estimated that the erythrocyte has a molecule of superdxide dismutase for every 600 molecules of haemoglobin (Lavelle **et al.**, 1974). The concentration of the enzyme does not decrease in Wilson's disease even though serum caeruloplasmin is markedly reduced or absent (Alexander and Benson, 1975). The dismutase reaction in the erythrocyte is some 100 times faster than the oxidation of haemoglobin by superoxide taking into account the respective rate constants and the concentrations of superoxide dismutase and oxyhaemoglobin, the product of the rate con-

stant and concentration of reactant (**kC**) being about $10^4$ $sec^{-1}$ for the dismutase reaction and about $10^2$ $sec^{-1}$ for haemoglobin oxidation. The controlling or suppressing role of superoxide dismutase is therefore abundantly clear (Sutton **et al.**, 1976). Carrell **et al.** (1975) suggest that oxyhaemoglobin may act as a buffer by reaction with superoxide and hydrogen peroxide protecting sensitive components like the membrane lipids. If this is so, hypochromia would facilitate damage to the cell membrane and this might be particularly relevant in thalassaemia.

The oxidation of Hb A by superoxide does not give rise to irreversibly denatured haemoglobin derivatives in contrast to oxidation by hydrogen peroxide. There is no formation of haemichromes and precipitation. The $\beta$-93 cysteine residues are not oxidized. The unstable haemoglobins, Christchurch, Belfast and Köln react in a similar way to Hb A except for oxidation of the $\beta$-93 cysteines in Hb Köln. The methaemoglobins produced are quickly converted into haemichromes and precipitate, as expected for these haemoglobins. Haemichrome formation and precipitation is even more rapid after autoxidation of isolated $\alpha$ and $\beta$ chains (Winterbourn **et al.**, 1976).

With normal level of superoxide dismutase in the erythrocyte only about 1% of the superoxide generated may be expected to react with haemoglobin. If other reactions occur they must either have **kC** products around $10^2$ $sec^{-1}$ in order to compete with superoxide dismutase and haemoglobin for superoxide or else take place coterminously with superoxide generation (Sutton **et al.**, 1976).

The red blood cell has an efficient system for dealing with hydrogen peroxide generated by dismutation of superoxide radicals or otherwise. It is considered that glutathione peroxidase is the primary enzyme which eliminates hydrogen peroxide in red cells although present at much lower activities than catalase (Cohen and Hochstein, 1963). Oxidized glutathione is reduced by glutathione reductase with NADPH generated by the pentose phosphate pathway. This helps to rationalize the presence of reduced glutathione in the red blood cell apart from its availability for preferential oxidation.

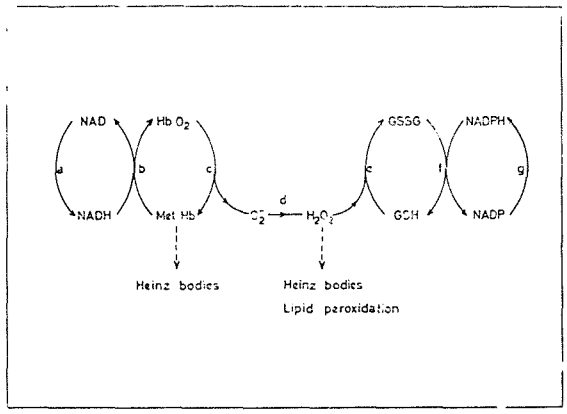


**Figure 1.**
**Superoxide production and elimination in the red blood cell. a, Glycolysis, b, Methaemoglobin reductase; c, Oxidation of oxyhaemoglobin; d, Superoxide dismutase; e, Glutathione peroxidase; f, Glutathione reductase; g, Pentose shunt. Interrupted arrows show reactions leading to haemolysis.**

A scheme of superoxide production and elimination in the red blood cell is shown in Fig. 1 after Carrell **et al.** (1975). The oxidation of oxyhaemoglobin and reduction of methaemoglobin constitutes a cycle producing a continuous supply of superoxide. Any factor that augments the production of superoxide or diminishes the elimination of hydrogen peroxide after superoxide dismutation is a potential threat to the red blood cell leading to haemolysis. Increased production of superoxide via oxidation of haemoglobin occurs with oxidative drugs, unstable haemoglobins and free $\alpha$ and $\beta$ chains (as in thalassaemia). Build up of hydrogen peroxide can become acute in failure of reduction of oxidized glutathione through lack of NADPH generation in glucose-6-phosphate dehydrogenase deficiency. Direct toxic effects of superoxide are not shown in Fig. 1. The activity of superoxide dismutase in red blood cells in very high and superoxide dismutase deficiencies have yet to be de-

monstrated. Superoxide-dependent peroxidation of membrane lipids has been inferred from protection exerted by superoxide dismutase. A free radical or radical-like agent is involved in lipid peroxidation. Superoxide and hydrogen peroxide give rise to this agent (Zimmermann et al., 1973; Tyler, 1975).

Exposure of red blood cells to superoxide radicals does not cause haemolysis (Fee **et al.**, 1975). Dialuric acid (2,4,5 6-tetrahydroxypyrimidine), which autoxidizes rapidly to alloxan with production of superoxide and hydrogen peroxide, produces lipid peroxidation and haemolysis of erythrocytes from vitamin E-deficient rats. Catalase gives considerable protection against haemolysis if present during the brief period of oxidation of the dialuric acid. A mixture of catalase and superoxide dismutase is more effective than catalase alone. Superoxide dismutase by itself is not effective. Hydrogen peroxide in the concentration produced by oxidation of the dialuric acid has no haemolytic effect. Dialuric acid and oxygen probably react to form some highly reactive substance which either attacks the red cell membrane directly or serves as a precursor to the reactive substance. Catalase might react with the reactive species rather than hydrogen peroxide. Superoxide might block the effect of catalase by formation of Compound III and superoxide dismutase might prevent this (Fee **et al.**, 1975).

## Erythrocyte Superoxide Dismutase — Erythrocuprein

The first preparation of erythrocuprein was from bovine erythrocytes (Mann and Keilin, 1939). Human erythrocuprein was first isolated by Markowitz **et al.** (1959). Thus erythrocuprein was known for several years before its superoxide dismutase activity was found by McCord and Fridovich (1969). Interestingly superoxide dismutase activity of tissue extracts or haemolysates was reported unknowingly in 1967 as tetrazolium oxidase activity. Two isozymes called A and B were found. A deviating phenotype of isozyme A was not reflected in isozyme B. This suggested that the two isozymes were under separate genetic control (Brewer, 1967). In fact isozyme A corresponded to cytosol or copper-zinc superoxide dismutase and isozyme B corresponded to mitochondrial or manganese superoxide dismutase (Beckman **et al.**, 1973).

Erythrocuprein is cytosol superoxide dismutase. This form of superoxide dismutase has a molecular weight of about 32,000 and consists of two subunits. Each subunit contains one atom of copper and one atom of zinc. The catalytic activity is due to the copper. The zinc probaly helps to stabilize the tertiary structure of the enzyme.

Knowledge of human erythrocuprein has lagged behind that of the bovine enzyme. The complete amino acid sequence of bovine erythrocuprein is known (Steinman **et al.**, 1974) and theh three-dimensional structure as revealed by X-ray diffraction analysis is known to a resolution of 3 Angströms (Richardson **et el.**, 1975). The subunits are identical. The most prominent structural feature of the enzyme is a cylinder like a barrel whose walls are composed of eight strands of the peptide chain in an antiparallel $\beta$ structure. The helical content is very low. These findings have vindicated earlier predictions of the secondary structure from circular dichroism and infra-red spectra (Wood **et al.**, 1971; W.H. Bannister **et al.**, 1973). The metals share a histidine residue at position 61 of the amino acid sequence. The copper, which is more exposed than the zinc, is liganded to three other histidines. The histidine ligands of this metal had been predicted by nuclear magnetic resonance spectra (Stokes **et al.**, 1973) The zinc is liganded to two more histidines and an aspartic acid residue.

It has been suggested that the subunits might not be identical in human erythrocuprein (Hartz and Deutsch, 1972). This is at variance with the observed polymorphism of the enzyme. Human cytosol superoxide dismutase (SOD–1) is polymorphic in northern Sweden and northern Finland with genetically controlled electrophoretic variation. The common phenotype, SOD-1 1, shows one major zone

and one or two minor anodically faster moving zones in gel electrophoresis. The rare phenotype, SOD-1 2, shows a similar isozyme pattern but with an overall slower electrophoretic mobility. The phenotype, SOD-1 2-1, shows the SOD-1 1 and SOD-1 2 zones and in addition a hybrid enzyme with intermediate electrophoretic mobility. The observation of this hybrid enzyme implies that the superoxide dismutase is at least a dimer formed through free recombination between two equal polypeptide subunits (Beckman **et al.**, 1973).

Most preparations of human erythrocyte superoxide dismutase have contained a minor component with somewhat faster anordic mobility in gel electrophoresis (Bannister **et al.**, 1972) We recently described the isolation and properties of two forms of superoxide dismutase from human erythrocytes, SOD I and SOD II (Bannister **et al.**, 1976). These can be obtained from a haemolysate of red blood cells after precipitation of the haemoglobin with a mixture of ethanol and chloroform. They are separated by ion-exchange chromatography on QAE-Sephadex with buffers of low ionic strength and decreasing pH. SOD I and SOD II are homogeneous in polyacrylamide gel electrophohresis. SOD II has a slightly higher anodic mobility than SOD I. Both SOD I and SOD II contain 2 g atoms of copper and 2 g atoms of zinc per mole of protein. The rate constant for the dismutase reaction estimated with a xanthine-xanthine oxidase-cytochrome c assay system (Sawada and Yamazaki, 1973) is $1.2 \times 10^9$ $M^{-1}$ $sec^{-1}$ for SOD I and $1.3 \times 10^9$ $M^{-1}$ $sec^{-1}$ for SOD II at pH 7.8 in good agreement with the value of $1.4 \times 10^9$ $M^{-1}$ $sec^{-1}$ at pH 7.5 determined in a previous pulse radiolysis study on human erythrocuprein (J.V. Bannister **et al.**, 1973). The amino acid compositions of SOD I and SOD II are essentially similar. The percentages of secondary structure as computed from far-ultraviolet crcular dischroism spectra are closely simlar for SOD I and SOD II. Little helical structure (4-9%) and about 50% (47-58%) $\beta$-sheet secondary structure is indicated.

Human erythrocuprein has a well-resolved band at 322 nm in the absorption spectrum (Bannister **et al.**, 1972) which is not shown by bovine and other known cytosol superoxide dismutases. The band is optically active giving a strong positive contribution in the circular dichroism spectrum. The chromophore is present in SOD I and lacking in SOD II. We have previously shown that a persulphide group, R-S-SH, is the most likely structure for the chromophore on the basis of its chemical reactivity. Four g atoms of labile sulphur per mole of protein were found (Calabrese **et al.**, 1975). Labile sulphur may be related to some as yet unknown physiological function of human erythrocyte superoxide dismutase.

The amino acid composition of human erythrocyte sueroxide dismutase shows some difference with respect to the bovine enzyme. Tryptophan is present and methionine is notably absent. The presence of tryptophan originally inferred from ultraviolet fluorescence spectra (Bannister **et al.**, 1968), has been confirmed by magnetic circular dichroism spectra. Two residues are indicated. The catalytic or copper site is closely similar to that in bovine enzyme (Roberts **et al.**, 1974). Nuclear magnetic resonance spectra indicate a histidine ligand field for the copper as in the superoxide dismutase of bovine erythrocytes.

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