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Superoxide Dismutases in Foods. A Review

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ABSTRACT

The importance of superoxide dismutase (SOD) in providing a defence against the superoxide radical in living systems has been increasingly established during the last twenty years. More recently, the occurrence and role of SOD in post-harvest and post-mortem foods and its significance as a natural antioxidant have been studied. This review highlights the importance of superoxide dismutase in foods. Current knowledge in aspects such as assay methods and the potential role of superoxide dismutase in the preservation of quality of harvested fruits and vegetables is reviewed.

INTRODUCTION

In 1969 a paper was published describing the isolation of a new enzyme from a mammalian source (McCord & Fridovich, 1969*a*). This enzyme, whose function is the disproportionation of superoxide radicals to oxygen and hydrogen peroxide (eqn (1)) was named superoxide dismutase (SOD) (EC 1.15.1.1):

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad E_0 + 1{\cdot}27V \qquad (1)$$

SOD was subsequently found to be a universal enzyme which exists in three different metalloforms where each form incorporates a transition metal ion at the active site. SOD is not known to react catalytically with any substrate other than the superoxide radical. Prior to the discovery of SOD many biochemists regarded the possible existence of oxygen radicals in the cellular

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environment with extreme scepticism. The proof of the existence of an enzyme which catalysed the removal of such a radical forced a change of view, and subsequently had profound and far reaching effects on the study of reactive oxygen species in living biological systems.

Oxygen is vital for life, yet it is also potentially toxic. To all aerobic organisms it is vital for the production of energy by its controlled reduction to water, yet to most forms of life it causes numerous manifestations of toxicity at greater than normal partial pressure. The biochemical mechanisms of this toxicity are not yet fully established. In fresh and processed foods oxygen is the main causative agent of well-known post-mortem and post-harvest changes—such as oxidative rancidity in fats and oils and the discoloration of meat, fruits and vegetables as well as many other less well-defined biochemical changes which are responsible for the loss of flavour and colour.

For the food scientist the presence of superoxide dismutase in foods has so far been generally ignored. This is partly due to the fact that superoxide dismutase is a relatively newly discovered enzyme and also because the enzyme seems only to catalyse the removal of a reactive oxygen species: the enzyme does not seem to directly bring about the oxidation or reduction of naturally important substances present in foods (though it may do so indirectly through the formation of hydrogen peroxide). Nevertheless, due to the ubiquitous nature of superoxide dismutase which is now known to be present in most biological materials with few exceptions, the possible role for the enzyme during redox reactions in food systems cannot be assumed to be negligible or unimportant.

Superoxide dismutase is an enzyme which might have potential use as a natural antioxidant in foods. Certainly it is known that the superoxide radical will rapidly oxidise important nutrients such as ascorbic acid and α-tocopherol. Although SOD has been patented as an antioxidant in foods (Michelson & Monod, 1975), its effectiveness as an antioxidant is untried and may be strictly limited by the conditions occurring in the food (Korycka-Dahl & Richardson, 1978). Schwimmer (1981) has outlined one possible drawback of this application; namely, the protection of anaerobic bacteria and phages by SOD.

FORMATION OF SUPEROXIDE AND ITS REACTIVITY IN BIOLOGICAL SYSTEMS

The stepwise addition of one electron at a time to O_2 results in the formation of free radical intermediates.

The first of these intermediate reaction products is either the superoxide

radical, O_2^-, or its corresponding acid, the perhydroxyl radical ·OOH (eqn (2)), depending upon the pH (pK_a 4·9, see Allen & Bielski, 1982).

$$O_2 \xrightarrow{e^-} O_2^- \xrightarrow{H^+} \cdot OOH \tag{2}$$

$$O_2^- \xrightarrow[2H^+]{e^-} H_2O_2 \tag{3}$$

$$H_2O_2 \xrightarrow[H^+]{e^-} \cdot OH + H_2O \tag{4}$$

$$\cdot OH \xrightarrow[H^+]{e^-} H_2O \tag{5}$$

The other products of the stepwise reduction are hydrogen peroxide (eqn (3)), a powerful oxidant, the hydroxyl radical (eqn (4)), which is one of the most chemically reactive species known, and ultimately, water (eqn (5)).

SUPEROXIDE IN BIOLOGICAL SYSTEMS

The superoxide radical is formed in all living cells exposed to oxygen, by various biochemical systems in the cells. These systems range from whole cells to small molecules, and some examples are presented in Table 1. Quantification of the amount of O_2^- produced in cells is difficult because of the instability of the radical in aqueous media and the ubiquitous presence

TABLE 1
Examples of Biochemical Systems Which May Produce O_2^- *In Vivo*

Source of O_2^-	*Examples*
Small molecules	Leucoflavins, hydroquinones, catecholamines, tetrahydropterines (by autoxidation).
Enzymes	Xanthine oxidase (degradation of purines to nitrogenous execretory products) Aldehyde oxidase Peroxidase (oxidative reaction) Dihydroorotic dehydrogenase Flavoprotein dehydrogenases
Cell organelles	Chloroplasts Mitochondria Micelles
Whole cells	Polymorphonuclear leukocytes (during the oxygen burst which leads to the destruction of invading bacteria and viruses)

(after Fee, 1980)

of superoxide dismutases which catalyse its removal. Estimations of steady-state cellular $O_2^{\cdot-}$ concentration vary from 6×10^{-9}M in chloroplasts (Asada *et al.*, 1977), to 8×10^{-11}M in rat liver mitochondria (Tyler, 1975).

In aerobically respiring cells most of the oxygen absorbed is catalytically reduced to water by cytochrome c oxidases or blue copper oxidases in the respiration cycle. However, as much as 17% of the O_2 has been estimated to be univalently reduced to $O_2^{\cdot-}$ by biological systems, such as those listed in Table 1 (Fridovich, 1984*a*).

The chemical reactivity of $O_2^{\cdot-}$ in aqueous solutions is dominated by its strong solvation by water molecules. The energy of solvation is approximately 400 kJ mol^{-1} (Fee, 1980). In this respect a chemical analogy can be made with F^- aq.; both F^- and $O_2^{\cdot-}$ are poor nucleophiles and the conjugate bases of weak acids: pK_a HF = 3·5, pK_a ·OOH = 4·8. In weakly solvating systems such as dimethyl sulphoxide, however, $O_2^{\cdot-}$ becomes a powerful nucleophile, as does F^-.

Superoxide is a moderately strong reductant and a weak oxidant in aqueous solution (see Halliwell, 1984). It will often participate in rapid, one electron, reductions, but will only oxidise strong reducing agents such as ascorbic acid, or α-tocopherol. This may be because $O_2^{\cdot-}$ mediated oxidations are energetically unfavourable, the peroxy ion product O_2^{2-} being highly energetic and not readily formed. Therefore, only compounds which are able to donate H^+, to form the more reactive ·OOH will be oxidised by $O_2^{\cdot-}$.

In aqueous solutions (such as biological systems) superoxide radicals are poorly reactive towards organic molecules, at least as an oxidant (Larson, 1988). $O_2^{\cdot-}$ is probably not reactive enough to cause cell damage and there is no documented evidence of such a reaction occurring under controlled conditions. However, in post-harvest and post-mortem foods compartmentalisation of various substrates and enzymes is gradually lost and under these conditions chemically reactive species may be released or accumulate in aqueous and non-aqueous environments. Biochemical systems generate $O_2^{\cdot-}$ in all cells utilising oxygen (see Table 1) and fluxes of $O_2^{\cdot-}$ generated *in vitro* have been shown to damage cellular components in several instances. Induction of lipid peroxidation (Kellogg & Fridovich, 1975; Dhindsa *et al.*, 1981), depolymerisation of polysaccharides (Kon & Schwimmer, 1977), killing of bacteria and inactivation of viruses and enzymes (Michelson, 1977) are examples of damage caused by $O_2^{\cdot-}$ and the more reactive radicals derived from it.

THE ROLE OF SUPEROXIDE IN PRODUCTION OF A MORE REACTIVE SPECIES

Superoxide may contribute to oxygen toxicity within an aqueous environment in either living cells or post-harvest foods through its role as the

precursor of a more reactive species; namely, a reduced form of superoxide, the hydroxyl radical. (The formation of ·OH can usually be prevented by SOD or catalase.)

Equations (6) and (7) describe the possible mechanism for the production of the hydroxyl radicals in the presence of $O_2^{\cdot-}$, H_2O_2 and 'free' transition metal ions (i.e. not protein-bound). In both living cells and hence foods the most abundant transition metals are iron and copper. In foods either of these elements and possibly traces of other transition metals may react with $O_2^{\cdot-}$ and H_2O_2 to produce more reactive species.

$$O_2^{\cdot-} + M^{n+} \longrightarrow O_2 + M^{(n-1)+} \tag{6}$$

Superoxide acts as a metal ion reductant

$$M^{(n-1)+} + H_2O_2 \longrightarrow M^{n+} + \cdot OH + OH^- \tag{7}$$

Fenton Reaction

(N.B., the net reaction:

$$O_2^{\cdot-} + H_2O_2 \xrightarrow[\text{catalyst}]{\text{Fe-salt}} O_2 + \cdot OH + OH^-$$

is sometimes referred to as an iron-catalysed Haber–Weiss reaction).

In living cells, Fee *et al.* (see Halliwell, 1984) argue that $O_2^{\cdot-}$ can be replaced in the role as generator of ·OH in the presence of H_2O_2 by other compounds and it is unlikely that the $O_2^{\cdot-}$ makes a significant contribution to oxygen toxicity in such a manner. Good reducing agents such as glutathione and ascorbate are present at greater steady state concentrations than free $O_2^{\cdot-}$; so the role of $O_2^{\cdot-}$ in eqn (6) may be replaced by other metal ion reductants within the cell. However, foods, as we understand such materials, are non-living systems and therefore in foods there is only a limited ability to generate the natural reducing agents such as ascorbate and glutathione.

Thus the alternative metal ion reducing agents (e.g. ascorbate) would rapidly be consumed by such a system, whereas it is reasonable to assume that production of intracellular $O_2^{\cdot-}$ is continuous and would allow the production of OH to continue. Nevertheless, the reduced form of the transition metals may be generated by oxidation of ascorbate and glutathione and similar substances, or by the oxidation of superoxide radical; and in the presence of accumulating amounts of hydrogen peroxide they would be responsible for the production of hydroxyl radicals (eqn (2)).

Gutteridge & Quinlan (1986) have found evidence for an alternative mechanism for the production of ·OH in foods by the addition of carminic acid which is a major component of cochineal red food colouring, E.120. Carminic acid (CA) may undergo reduction to produce semiquinone

radicals (·CA); these radicals then act as metal ion reductants in the presence of trace amounts of iron salts and H_2O_2 (eqns (8) and (9)):

$$\cdot CA + Fe^{3+} \longrightarrow CA + Fe^{2+} \tag{8}$$

The semiquinone acts as a metal ion reductant.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \cdot OH \tag{9}$$

Fenton Reaction

O_2^- may compete with ·CA as the metal ion reductant and the role of SOD in this mechanism is unclear, but it is thought that O_2^- plays no part other than that of precursor of the H_2O_2 substrate required for eqn (9). O_2^- arises from reaction between carminic acid radicals and molecular oxygen ($\cdot CA + O_2 \rightarrow CA + O_2^-$).

The radical ·OH is considered to be a more reactive species than O_2^- in the cellular environment, but it comprises only a tiny proportion of the physiological O_2 concentration and is more likely to be present within living systems in organelles where the pH is low, e.g. in the phagocytic vacuoles (see Halliwell, 1984).

Although O_2^- is generally poorly reactive within an aqueous environment, some microenvironments within the cellular components in foods, such as the interior of phospholipid membranes, may resemble organic solvents in which O_2^- may become a reactive species. This activity may be due to the small size of O_2^- which enables it to diffuse easily through the phospholipid bi-layer.

The reactivity of O_2^- in cells and within the cellular compartments and components, and its contribution to oxygen toxicity, has not yet been unambiguously established. Oxygen toxicity is a complex phenomenon (Halliwell, 1984) with many manifestations which depend upon the exact nature of the stress applied. Although superoxide is not the sole cause of oxygen toxicity and SOD is not the sole mechanism by which O_2^- is removed, SOD has a significant role in defence against this toxicity; catalase, peroxidase, glutathione and ascorbate also scavenge the potentially harmful oxygen species. However, in stored and processed foods these scavenging enzymes, and possible substrates (e.g. glutathione and ascorbate) are not continuously regenerated and may indeed be lost during food processing and storage of even fresh fruits.

SOD exists as a metalloprotein which incorporates a copper, iron or manganese ion at the active site. (The enzyme is thought to catalyse the same reaction whatever the metal present.) The dismutase activity of Cu^{2+} and its complexes *in vitro* has led some authors, notably Fee (1982), to propose that the biological role of SOD is not dismutation of O_2^-, but some other activity not yet determined and that the dismutase activity of the

enzyme is merely a consequence of the fact that it contains copper (or another metal) (Fee, 1982). However, Cu^+ aq. functions poorly as a dismutation catalyst at physiological pH, compared to SODs. If the catalytic activity of Cu/Zn SODs is fortuitous then other Cu-proteins, e.g. cytochrome oxidase and ceruloplasmin, should perform the same function. None do so catalytically though it is possible that they do scavenge O_2^- in some non-catalytic fashion (see Goldstein *et al.*, 1979; Bannister *et al.*, 1980). Also SODs with iron or manganese as the prosthetic group are much more effective catalysts than free Mn or Fe ions. The true biological function of SODs is almost certainy dismutation, and SODs are the only efficient catalysts of this reaction *in vivo*. However, the superoxide theory of oxygen toxicity does not adequately explain the presence of SOD in anaerobic bacteria, or the ability of some aerobes to survive without SOD in an oxygen-rich environment.

Halliwell (1984) concludes that in the majority of organisms SODs perform a vital function as one of a range of defences against the toxic effects of oxygen. However, their significance in fresh and processed foods and their potential use as antioxidants in preserved foods has not been fully investigated or considered.

OCCURRENCE OF SOD IN FOODS

Superoxide dismutases (SOD) are ubiquitous in the plants and animals that comprise our food supply. In Table 2 examples are given of reports where SOD has been detected in, or purified from, food sources, particularly foods of plant origin. Superoxide dismutase is found in all fresh food sources and yet its influence upon the preservation and quality of foodstuffs is largely uninvestigated.

Some work has been carried out on mammalian sources of the SOD enzyme, and it has been shown that bovine milk SOD is resistant to pasteurisation at 71·7°C for 15 s (Hicks *et al.*, 1979), and may protect the milk from rancidity by removing superoxide radicals which initiate lipid peroxidation (see Korycka-Dahl & Richardson, 1978). In milk O_2^- is produced by enzymic and non-enzymic pathways, for example, by xanthine oxidase activity, or by reoxidation of photoreduced flavins. Superoxide dismutase may contribute to cheese flavour development, by controlling the concentration of superoxide (Schwimmer, 1981).

Loss of pigment in meat may occur through the following reaction:

$$\underset{\text{(red)}}{\text{Oxymyoglobin}} \rightleftharpoons \underset{\text{(brown)}}{\text{metmyoglobin}} \quad (+ O_2^-)$$

Autoxidation of myoglobin and haemoglobin releases O_2^- which, in turn,

TABLE 2
Superoxide Dismutase in Food Sources (D = detected; P = purified)

Food source	*Type of SOD*
Spinach leaves	Cu/Zn (P) (Asada *et al.*, 1973)
Tomato: fruit	Cu/Zn (P) (Baker, 1976)
Peas: seed	Cu/Zn (P) (Sawada *et al.*, 1972) (Giannopolitis & Ries, 1977*b*) (Duke & Salin, 1983)
Mustard leaves	Fe (P) (Salin & Bridges, 1980)
Mung bean	Cu/Zn (D) (Reddy & Vankaiah, 1984) Mn (D) (as above)
Wheatgerm	Cu/Zn (P) Mn (D) (Beauchamp & Fridovich, 1973)
Lentils	Cu/Zn (P) (Federico *et al.*, 1985)
Corn	Cu/Zn, Mn (D) (Giannopolitis & Ries, 1977*b*)
Oats	Cu/Zn (D) (Giannopolitis & Ries, 1977*a*)
Maize	Cu/Zn (P) (Baum *et al.*, 1983)
Cucumber, pepper	(D) (Rabinowitch & Sklan, 1981)
Apple, banana, avocado	(D) (Baker, 1976)
Cabbage	Cu/Zn (P) (Steffens *et al.*, 1986)
Jerusalem artichoke	Cu/Zn, Mn (D) (Arron *et al.*, 1976)
Bovine milk	Cu/Zn (D) (Hicks, 1980; Korycka-Dahl *et al.*, 1979)
Chicken liver	Cu/Zn, Mn (D) (Lonnerdal *et al.*, 1979)
Trout, salmon	Cu/Zn (D) (Sutton *et al.*, 1983)
Cow, pig, sheep	Cu/Zn, Mn (D) (see Steinman, 1982)
Yeast	Cu/Zn (P) (Weser *et al.*, 1972)

causes the oxidation of these pigments to the corresponding met states (Misra & Fridovich, 1972; Lynch *et al.*, 1976). However, superoxide dismutase may have a role in suppressing the autoxidation (see Rabinowitch & Fridovich, 1983). In spite of the high enzymic activity of SOD in muscle tissue the O_2^- may have time to initiate the chain reactions of fatty acid autoxidation that lead to rancidity of the fatty parts of the meat.

Free radicals, e.g. O_2^- which are potential precursors of off-flavour and off-colour compounds can be formed under some circumstances in the oxidatic reaction of peroxidase. Peroxidase activity is generally treated as deleterious to quality of plant foodstuffs and SOD may have a role in preventing loss of quality due to peroxidase activity. The role of SOD in the preservation of quality of harvested fruit and vegetables is unclear.

Lipid peroxidation, initiated either by a xanthine oxidase system production O_2^- or by lipoxygenase, is inhibited by the presence of SOD (Kellogg & Fridovich, 1975; Richter *et al.*, 1975; Dhindsa *et al.*, 1981). The autoxidation of anchovy oil was found to be delayed by the presence of SOD (Michelson & Monod, 1975).

As the biochemical role of the enzyme is still the subject of debate it is not surprising that there is little information on the importance of SOD in food. Although a brief comparison of SOD and peroxidase levels in fruit and vegetables has been carried out (Matkovics *et al.*, 1981), the interactive effects of SOD and peroxidase during the spoilage of fruits and vegetables is unknown. It remains to be determined whether SOD plays a protective or a reinforcing role in spoilage attributable to peroxidases but a product of SOD activity, H_2O_2, is required for peroxidatic activity.

Peroxidase can react with $O_2^{\cdot-}$ to form the relatively inactive form of the enzyme, compound III or oxyperoxidase. Compound III slowly decomposes to release $O_2^{\cdot-}$. It appears that superoxide radicals could play a dual role in the decomposition of a harvested plant, on the one hand aiding the production of undesirable compounds from the action of peroxidase, and on the other hand inactivating the peroxidase itself. Nevertheless, the role of SOD in preventing or aiding post-harvest plant degradation with respect to peroxidase activity has not yet been determined.

HEAT STABILITY OF SOD IN FOODS

Little information is available on the heat stability of SODs or their fate during thermal processing. Cu/Zn-SOD in bovine milk can withstand pasteurisation at 63°C (Korycka-Dahl *et al.*, 1979), but at temperatures higher than 70°C the degree of enzyme inactivation increases rapidly with temperature. Raw milk undergoing HTST pasteurization at 80°C loses approximately half of its SOD activity (Hicks *et al.*, 1979). In crude extracts of corn and oats the SOD activity has been found to be more resistant to boiling than peroxidase activity (Giannapolitis & Reis, 1977*a*). The SOD activities of crude extracts of corn and peas (Giannapolitis & Reis, 1977*a*) and cabbage (Walker, 1987) are known to be more heat-stable than that of partially purified enzymes, indicating that cellular components may protect the protein from thermal denaturation.

The SOD activities found in cabbage extracts and sprout extracts are stable to 45°C, and at higher temperatures the cabbage SOD is the more heat-sensitive; heating at 70°C for 2 min destroys virtually all the SOD activity in crude extract of cabbage (Walker *et al.*, 1987). In contrast with cabbage, one of the two main isoenzymes in Brussels sprout crude extract was found to be highly heat-stable (demonstrated by the activity remaining after 30 minutes at 80°C) and thus the differential stabilities of the isoenzymes may account for the non-linear pattern of inactivation with heating time.

It is of interest that Mn-SOD purified from pea leaves gives sigmoidal heat-inactivation curves (Sevilla *et al.*, 1980). It is not yet known whether

these differences are characteristic of the metalloform of SOD and could therefore be exploited.

There is no evidence to support the possibility of SOD activity regeneration following thermal denaturation, as is the case with peroxidases (Moulding *et al.*, 1988), and it is therefore unlikely that SOD will be active in the majority of foods that have been heat-treated for food processing.

FOOD IRRADIATION AND SOD

The damage caused to DNA, proteins and lipids in living cells by high energy irradiation results from the formation of free radicals such as ·OH and $O_2^{\cdot-}$. The effects of radiation on living tissue are enhanced by oxygen. Superoxide dismutase has been found to exert a protective effect against radiation in viruses, suspensions of bacteria, mammalian cell cultures and even whole mice (Fridovich, 1978). A radiation dose of only 5 kGy was found to cause extensive inhibition of the enzyme alcohol dehydrogenase. This inhibition was enhanced by oxygen, and lessened by the presence of SOD (Gee *et al.*, 1985). Irradiation of living plants results in increased *in vivo* concentrations of SOD, catalase and peroxidase (Chakraborti & Chatterjee, 1983).

The preservation of food by radiation doses of up to 10 kGy was concluded by the World Health Organisation in 1981 to present no toxicological hazard. 'Low' levels of irradiation, up to 1 kGy, used to control pests and prevent sprouting and ripening of food plants, are certain to produce some reactive oxygen radicals in the foodstuff that may account for the claimed destruction of small amounts of some vitamins and other micronutrients. Both naturally occurring and, in some instances, deliberately added, superoxide dismutases, could play a vital role in preserving the nutritional status of irradiated fruit and vegetables, although, as stated by Schwimmer (1981), it may also afford protection to pathogenic organisms.

Foods with a high fat content, for example, dairy foods, are unsuitable for radiation treatment because of the adverse flavours and tastes produced by subsequent lipid oxidation. It seems conceivable that SOD may be able to reduce the production of these undesirable compounds and allow successful irradiation of such foods.

THE BIOLOGICAL DISTRIBUTION OF SOD (Enzyme types)

Surveys of SODs from many different organisms have led to some generalisation and classification of the enzymes relating to their biological distribution (see Steinman, 1982). Thus, the prokaryotes, i.e., blue–green algae and bacteria, contain Fe-SOD and Mn-SOD, often both enzymes

occurring together in the same organism. Recent research has provided evidence that the bacterium *Streptococcus mutans* uses the same apoenzyme to form either Fe-SOD or Mn-SOD depending on the metal availability. Martin *et al.* (1986) have commented that such 'cambialistic' enzymes (i.e. those capable of making a cofactor substitution) may represent a previously unrecognised family of SODs. Stallings *et al.* (1984) have also cited recent reports of cases where the selectivity of dismutase apoproteins for metals is not absolute, at least for enzymes that usually contain iron or manganese, Fe-SOD, found in prokaryotes, is the most frequently occurring form of SOD in obligate anaerobes, although most of these organisms contain no SOD at all. Mn-SOD has been found to be present in the mitochondria and peroxisomes of eukaryotes. Cu/Zn-SOD has been found in the cytosol of all eukaryotic cells, but is generally absent from prokaryotic organisms.

Although this is still the general pattern of distribution of SODs, exceptions have since been found which break most of these rules. Eukaryotic organisms have been found which lack Cu/Zn-SOD, from primitive eukaryotes such as green algae to higher organisms such as water lilies (Bridges & Salin, 1981), and the fungus *Pleurotus olearius* (Steinman, 1982). Three bacteria have been found to contain Cu/Zn-SOD. In the case of *Photobacterium leiognathi* the presence of Cu/Zn-SOD has been proposed to be the result of a symbiotic host–gene transfer (Martin & Fridovich, 1981), but this explanation does not suffice for the other two cases, which are both free living bacteria; and in any case the most recent evidence, from amino acid sequence data of *P. leiognathi* SOD, does not support this hypothesis (Steffens *et al.*, 1986).

Fe-SOD has been found in primitive eukaryotes which lack Cu/Zn-SOD, such as *Euglena gracilis* (Kanematsu & Asada, 1979), and more recently it has also been detected in several families of higher plants. Water lilies, mustard, ginkgo trees (a primitive fern) (Bridges & Salin, 1981), tomato plants (Kwiatowski *et al.*, 1985) and lemon trees (Sevilla *et al.*, 1984) are plants which contain an Fe-SOD.

Eukaryotic Mn-SOD is not confined to mitochondria, since it has been claimed to be present in the cytosol of baboon liver and bakers yeast (Steinman, 1982). Several reports of Mn-SOD in chloroplast fractions have also been published, but this activity may be due to contamination by other membranous fractions, such as mitochondria (Jackson *et al.*, 1978). Mn-SOD has also been found in peroxisomes of pea plants (Del Rio *et al.*, 1983; Sandalio *et al.*, 1987; Palma *et al.*, 1987).

PROPERTIES OF THE METALLOFORMS OF SOD

The properties which characterise and differentiate the three forms of SOD are summarised in Table 3.

TABLE 3
Properties of the Metalloforms of SOD

Property	*Cu/Zn-SOD*	*Mn-SOD*	*Fe-SOD*
Molecular form	Dimer identical subunits	Prokaryotic dimer Eukaryotic tetramer Identical subunits	Dimer, occasionally tetramer Identical subunits
Metal ion prosthetic group	One Cu(II) One Zn(II) per subunit	One Mn(II) per subunit	One Fe(II) per subunit
Molecular weight	32 000	42–46 000 (dimer)	40–46 000 (dimer)
Inhibition by CN^-	At 1 mM CN^-	No effect	No effect
Inhibition by H_2O_2	At 0·5 mM H_2O_2	No effect	At 0·5 mM H_2O_2
Inhibition by chloroform/ ethanol	No effect	Inhibited	Inhibited
Effect of SDS	Stable to 4% solution SDS	Unstable to 1% solution SDS	Unstable to 1% solution SDS
Effect of pH on enzymic activity	No effect pH 5–10	Activity decreases at pH > 8	Activity decreases at pH > 8

In their general chemical properties, Mn-SOD and Fe-SOD differ only in their response to H_2O_2. It is of interest that Fe-SOD (Asada *et al.*, 1975) and Cu/Zn-SOD (Hodgson & Fridovich, 1975) are slowly but irreversibly inactivated by one of the products of the enzyme catalysed reaction; namely, hydrogen peroxide.

MECHANISM OF ENZYME CATALYSED DISMUTATION

In all SODs the metal ion prosthetic group is vital for catalytic activity. In Cu/Zn-SOD the Cu^{2+} ion is responsible for catalysis and the role of zinc is probably structural. Removal of Cu^{2+} from Cu/Zn-SOD causes loss of enzymic activity which is restored only by its replacement. Zn^{2+} can be replaced in Cu/Zn-SOD by Ag^{2+}, Co^{2+} or Cd^{2+} with no loss of enzyme activity (Forman & Fridovich, 1973).

At physiological pH superoxide exists predominantly as O_2^- (rather than the perhydroxyl radical ·OOH), and so catalysis of dismutation must overcome electrostatic repulsion between the negatively charged radicals and consequent slow rate of reaction:

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \tag{10}$$

At the active site the enzyme bound copper acts as an electron carrier between two superoxide radicals in the two successive half-reactions, the two radicals never actually meet. In the first half-reaction (eqn (11)) Cu^{2+} is reduced to Cu^+, and O_2^- oxidised to molecular oxygen. In the second half-reaction (eqn (12)) Cu^+ is oxidised to Cu^{2+} and a second O_2^- is simultaneously reduced and protonated to form hydrogen peroxide.

$$E - Cu^{2+} + O_2^- \longrightarrow E - Cu^+ + O_2 \tag{11}$$

$$E - Cu^+ + O_2^- + 2H^+ \longrightarrow E - Cu^{2+} + H_2O_2 \tag{12}$$

(E = one SOD subunit).

The two half-reactions have rate constants identical to each other and to the overall rate constant of $2 \times 10^9\ M^{-1}\ s^{-1}$. The large rate constant for catalysis, and the dependence of the reaction rate on viscosity (Rotilio *et al.*, 1972) suggest that the reaction rate is diffusion limited. Thus it is one of the fastest enzymic reactions known.

The two active sites on the Cu/Zn-SOD enzyme function independently although separated subunits have not been found to be active. It is thought that the reaction of one copper-site with the substrate prevents the site on the other subunit from catalytic function, i.e. antico-operative interaction between the two sites (Rotilio *et al.*, 1977). Cyanide completely inhibits Cu/Zn-SOD by binding to the inner co-ordination sphere of the copper (Fee & Gaber, 1972).

SOD is powerful enough to increase the rate of dismutation of O_2^- by several orders of magnitude at physiological pHs. The catalytic activity of Cu/Zn-SOD has been shown by direct assay to be constant over the pH range 5–9·5 (Rotilio *et al.*, 1972; Klug *et al.*, 1972) and to decrease rapidly above pH 10. Above pH 10 positive charges are lost from the lysine residues on the enzyme surface. The positive charge on the surface, in combination with the electrostatic repulsion by negatively charged areas on the surface, serves activity to guide O_2^- radicals to the active site 'channel'. This effect is also demonstrated by the decrease in enzyme activity with increasing ionic strength (Cudd & Fridovich, 1982).

THE PROTECTIVE ROLE OF SOD IN PLANTS

Growth, development and senescence

The SOD content of plants varies greatly with the age and the part of the plant examined (it is present in roots, stems, leaves and seeds). Cu/Zn-SOD in leaves is almost exclusively associated with the chloroplasts. Oxygen, a product of photosynthesis, is produced in high concentration in the chloroplasts, where the presence of many strong reductants makes the formation of O_2^- unavoidable. Chlorplasts therefore have a higher concentrations of O_2^-, approximately 6×10^{-9}M (Asada *et al.*, 1977) than the other parts of chlorophyll-containing cells. The SOD content of seeds increases dramatically with germination and the early stages of growth (Giannopolitis & Ries, 1977*b*; Matkovics, 1977).

In the dry seed of mung bean, one of the SOD isoenzymes (SOD 1) is undetectable and as the seedling grows the level increases; it then becomes the major SOD of the plant (Reddy & Venkaiah, 1984). Bhattacharjee & Choudhuri (1986) have shown that pretreatment of jute seeds with chemicals such as cinnamic acid stimulates the activities of free-radical scavenging enzymes including SOD. This treatment may enhance the storage potential of seeds and render the seedlings tolerant against water deficit stress.

The distribution of free radicals in maize plants was found to correlate with metabolic activity, thus the young and actively dividing parts of the plants had the highest concentration of radicals (Michalov & Placek, 1982). This was parallelled by findings of higher SOD activity in young leaves than in mature and senescing leaves (Dhindsa *et al.*, 1982; Rabinowitch & Fridovich, 1983) and of high SOD content in order metabolically active parts of plants such as root nodule mitochondria in soybeans (Puppo *et al.*, 1987). Gillham & Dodge (1987) have used pea plants to show that although the levels of ascorbate and other antioxidants within chloroplasts display seasonal variation (grown in glasshouse conditions), SOD activities vary little throughout the year. It seems likely that SOD activity in chloroplasts is independent of light intensity and possibly other environmental factors.

An early survey of the SOD content during apple, banana, avocado and tomato fruit ripening found little difference between pre- and post-climacteric fruit (Baker 1976). Later studies on tomatoes (Rabinowitch & Sklan, 1980) and peppers and cucumbers (Rabinowitch & Sklan, 1981) found the SOD content to vary with the different stages of ripening in the same way in all three fruits. The susceptibility of a fruit to sunscald, a form of photooxidative injury resulting in damage to the skin of the ripe fruit, was found to be related to SOD concentration in the fruit. When the SOD content was low, sunscald tolerance was low, and vice versa, suggesting a

protective role for SOD against insolation damage in green tissues (Rabinowitch *et al.*, 1982).

Fruit ripening involves many complex metabolic changes including lipid peroxidation, hydrogen peroxide formation and the breakdown of cell membranes and cell walls. Superoxide is involved in many of these processes, and SOD might therefore have an important role, the nature of which has yet to be determined, in the control of fruit ripening.

Plant polysaccharides have been found to be depolymerised as the result of a system producing superoxide and hydrogen peroxide and their subsequent interaction to produce damaging free radical species. In experiments by Kon & Schwimmer (1977), SOD and catalase gave complete protection from this production system. SOD may also function in the control of lignification of plant tissues, in which O_2^- has been shown to participate (Rabinowitch & Fridovich, 1983). Protection of plants against toxic concentrations of heavy metals (in particular copper) has been suggested as the role of Mn-SOD which, together with catalase, is more active in peroxisomes of Cu-tolerant than Cu-sensitive pea plants (Palma *et al.*, 1987). SOD may also have a role in response to wounding of tissue. Work on pea plants by Olsen & Cook (1987) has indicated the swift protein turnover of this enzyme. These researchers found evidence for a significant loss and dramatic recovery of activity of SOD (within 1 h) following wounding of etiolated stem tissue; catalase and peroxidase do not seem to follow such a rapid pattern of loss and recovery.

STRUCTURE OF SUPEROXIDE DISMUTASE

Cu/Zn-SOD

X-ray diffraction has shown the subunit structure of bovine erythrocyte Cu/Zn-SOD to be largely composed of anti-parallel β-sheet. Eight stands of β-sheet fold into a cylinder (the β-barrel) which has two large non-helical loops protruding from opposite edges. These loops enclose the active site. There is one intra-chain disulphide bond. Intersubunit bonding is on the opposite side of the barrel to the active site, and is non-covalent and largely hydrophobic. This accounts for the stability of the enzyme to denaturing organic solvents such as chloroform and ethanol (Table 3).

Figure 1 describes the active site groups of Cu/Zn–SOD. The Cu^{2+} ion is ligated to four histidine residues in a roughly planar arrangement. The Zn^{2+} ion has one aspartic acid and three histidine ligands, one of which it shares with the Cu^{2+}, in a tetrahedral formation. The common histidine ligand is referred to as the imidazole bridge.

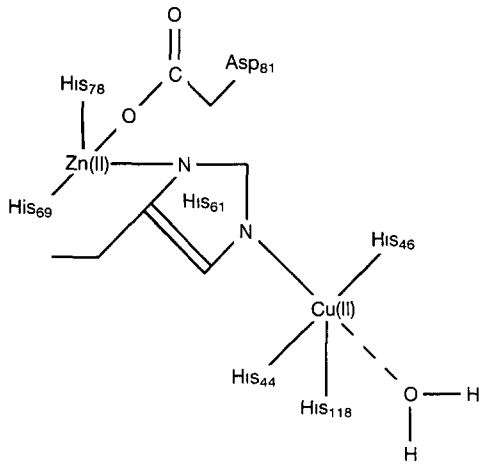

Fig. 1. Schematic diagram of the active site groups of Cu/Zn–superoxide dismutase (adapted from Fielden & Rotilio, 1984).

At the active site, Cu^{2+} is at the bottom of a 'channel', and is only partially exposed to the solvent. An arginine residue (Arg 141) at the outer end of the 'channel' assists in guiding the substrate, O_2^-, down to the Cu^{2+} at the active site. Solvents or reagents which affect the charge on arginine, such as phosphate buffer (Mota de Freitas & Valentine, 1984) or butane-dione (Malinowski & Fridovich, 1979) cause some diminution of enzyme activity.

The Zn^{2+} is wholly buried within the protein structure and is thought to play a structural role only, aiding protein stability.

Mn-SOD and Fe-SOD

The secondary and tertiary structures for Mn-SOD and Fe-SOD have not been as extensively investigated as those of Cu/Zn-SOD. However, results so far achieved (see Steinman, 1982) show that Mn-SODs and Fe-SODs from a variety of prokaryotic sources are structurally similar to each other. Both have extensive areas of α-helix in the tertiary structure, but have little β-sheet. The folded proteins are roughly rectangular in shape, with the metal binding site close to, or even part of, the subunit interface (Stallings *et al.*, 1984). For both Mn-SOD and Fe-SOD there is one metal binding site per subunit.

Sequence homology of SOD

Sequence homology is notable between Mn-SODs and Fe-SODs from different sources, including Mn-SOD from eukaryotic mitochondria. However, there is no homology with the Cu/Zn-SODs beyond that expected

by chance, leading to the assumption that the Cu/Zn and Mn/Fe SODs have evolved independently and do not share a common ancestral protein.

Extensive sequence homology, between such disparate sources of Cu/Zn-SOD as bovine erythrocytes, cabbage and yeast, indicates a high degree of evolutionary conservation of the secondary structure of the enzyme (see Steffans *et al.*, 1986). Cu/Zn-SODs from many sources have been found to have a high content of glycine, which allows for the extensive β-sheet formation and sharp bending of the protein chain, and consistently low tryptophan and tyrosine content. Amino acids vital for enzymic activity, such as the metal ion ligands, 'channel' residues and arginine-141, are conserved in all the Cu/Zn-SODs examined.

MEASUREMENT OF SOD

Most assay methods for enzyme activity depend on measurement of either the rate of disappearance of a substrate, or appearance of a product. However, SOD is unique in that its substrate $O_2^{\cdot-}$ is unstable in aqueous solution at physiological pH; this means that traditional methods of assay are inappropriate. Consequently, there has been much research aimed at a convenient assay system (see Greenwald, 1985) and this has resulted in the publication of many papers each year, describing novel methods of circumvention of the instability of the substrate radical (see Fridovich, 1982). Table 4 lists some of the published methods, a few of them are direct assays (Nos 1–3 in the table), but since their application is hampered by the requirement of special apparatus, the majority are indirect assays, which rely on the ability of SOD to inhibit a superoxide driven reaction.

Direct assays

These assays allow the determination of kinetic parameters and involve $O_2^{\cdot-}$ radical detection and quantification. They are problematic because they require a high concentration of $O_2^{\cdot-}$ and this is difficult to achieve because of the instability of $O_2^{\cdot-}$ in aqueous media at physiological pH.

Stable solutions of $O_2^{\cdot-}$ are difficult to prepare and handle under normal laboratory conditions, but this can be overcome, e.g., by the use of elevated pH to slow the spontaneous dismutation sufficiently to allow its direct observation (Marklund, 1976) or by performance of the assay over a millisecond timescale, using pulse radiolysis (Klug *et al.*, 1972). Together with the problems of monitoring the rate of decay of $O_2^{\cdot-}$ this requirement necessitates the use of considerable expertise, reagents of a high purity and expensive apparatus, not commonly available in a typical laboratory. (For

TABLE 4
Methods used to Assay for SOD Activity

Source of O_2^-	*Detection systems*
1. Pulse radiolysis	Spectrophotometric
2. Polarography	Measurement of limiting current
3 Solution of O_2^-	Spectrophotometric
4 ENZYMIC	
Xanthine oxidase + xanthine	Cytochrome c reduction
	Adrenalin oxidation
	Hydroxylamine oxidation
	Sulphite oxidation and polarographic measurement
5 PHOTOCHEMICAL	
Riboflavin + TEMED or	Nitroblue tetrazolium reduction
Methionine	Peroxidase + dianisidine
Phenazine methosulphate + NADH	Cytochrome c reduction
	Nitroblue tetrazolium reduction
Flavin mononucleotide + TEMED	Cytochrome c reduction
Riboflavin + dianisidine	Dianisidine oxidation
6 AUTOOXIDATION	
Adrenalin	Adenochrome
Pyrogallol	Chromophoric products
6-hydroxydopamine	Quinoids
Hydroxylamine nitrate	NBT reduction
Sulphite	Sulphate
Haematoxylin	Haematin
	Nuclear magnetic relaxation of ^{19}F (binds to active site of enzyme)

example, a direct assay may involve the use of an electron spin resonance spectrometer—ESR). Therefore direct assays are unsuitable for routine use, but as Fridovich (1982) has stated, they are important for the validation of the more convenient indirect assay systems and studying the mechanisms of SODs.

Indirect assays

The common feature of the many published indirect assay methods is competition between SOD and some indicating scavenger compound of O_2^-. These assays work on the principle of uniting a generating system for O_2^- with a measurable indicating system to detect their presence.

Although more convenient than direct assays, they preclude kinetic

studies and only provide an estimation of the relative SOD concentration in samples of comparable composition. For example, Oyanagui (1984) has developed a kit for measurement of SOD in blood for clinical studies, based on the hydroxylamine detector system.

In most cases, SOD measurement depends on solving the $O_2^{\cdot-}$ instability problem, and usually this is overcome by the continuous generation of a flux of $O_2^{\cdot-}$ by electrical, chemical, photochemical or enzymic means. The presence of the superoxide radicals is then detected by reaction with an indicating scavenger compound to form a quantifiable product. A common drawback to all SOD assays is that substrate saturation of the enzyme can never be achieved, and in most steady state systems the concentration of $O_2^{\cdot-}$ is very small.

In the absence of SOD, the rate of $O_2^{\cdot-}$ production is equal to the rate of the reaction of $O_2^{\cdot-}$ with the scavenger, and a steady state exists. When SOD is present, the steady state concentration of $O_2^{\cdot-}$ is reduced by the enzyme-catalysed dismutation; this results in a lower rate of reaction between $O_2^{\cdot-}$ and the scavenger; thus SOD inhibits the measured rate of product formation.

The sensitivity of indirect assays and the balance of competition for $O_2^{\cdot-}$ depends on many factors, such as the rate of $O_2^{\cdot-}$ production, the concentration of indicating scavenger compound and the pH of the reaction mixture, due to their effects on spontaneous dismutation and reduction of the detector compound. Beyer & Fridovich (1987) have investigated the effects of experimental variables on the two more commonly used indirect assays, which are based on the use of ferricytochrome c and nitroblue tetrazolium. The principle of these two assays is described below.

Xanthine oxidase/cytochrome c method

The reactions occurring in the assay are presented in schematic form in Fig. 2.

$O_2^{\cdot-}$ is generated by the xanthine oxidase (XOD) reaction and the rate of reduction of ferricytochrome c (cyt c^{3+}) to ferrocytochrome c (cyt c^{2+}) by $O_2^{\cdot-}$ is followed spectrophotometrically, by measuring the increase in absorbance at 550 nm. At the pH of the assay, pH 7·8, the rate of spontaneous dismutation is slow and can be disregarded.

In this 'standard' assay SOD is quantified by the degree to which it inhibits the measured rate of cyt c^{3+} reduction (McCord & Fridovich, 1969*a*). One unit of SOD activity is defined as the concentration of SOD required to produce 50% inhibition under specified conditions (Fridovich, 1984*b*).

This is possibly the most widely used assay method, but it is subject to interference from several groups of compounds. Fridovich (1985) has

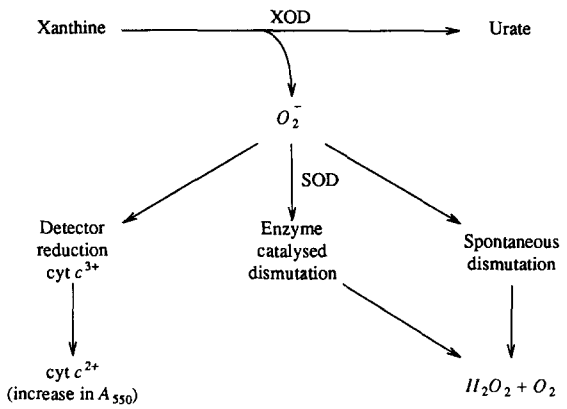

Fig. 2. Scheme of reactions occurring in the xanthine oxidase/cytochrome c assay.

outlined some of the potential artifacts which may be recognised, avoided or corrected for when assaying tissue extracts. Interference can occur from many sources in crude plant extract samples, resulting in inhibition of XOD, direct reduction of cyt c^{3+} by reductants other than O_2^- and reoxidation of reduced cyt c^{2+}. Xanthine oxidase is inhibited by heavy metals. This may be overcome by including a chelating agent, e.g. EDTA, in the assay. Lactoperoxidases, which may be present in the XOD preparation, are capable of reoxidising the reduced cyt c^{2+}, thus mimicking SOD activity. Cytochrome oxidases and peroxidases which may be present in the extract or homogenate assayed might interfere in the same way. Deflavine xanthine oxidase, a possible contaminant of XOD preparations, is capable of directly reducing cyt c^{3+}. This reduction of cyt c^{3+} is not inhibited by the presence of SOD and is referred to as 'SOD-resistant reduction'. Compounds such as flavin mononucleotide in the extract assayed may act as an alternative to O_2^- in carrying electrons between XOD and cyt c^{3+} and rise to SOD-resistant reduction. It is therefore essential that rather than total cytochrome c reduction, the SOD-inhibitable component of that reduction is measured; this requires the careful use of controls.

The XOD system for generating O_2^- may be coupled to other detector systems for the assay of SOD activity (see Table 4). Superoxide-initiated sulphite oxidation, detected polarographically, was used to determine SOD activity in turbid suspensions which contained cytochrome oxidase and had O_2^- -independent reducing activity (Tyler, 1975).

Riboflavin/nitroblue tetrazolium assay

In the presence of light, and a suitable hydrogen donor, such as methionine or TEMED, riboflavin is photoreduced and, by subsequent spontaneous

reoxidation, generates $O_2^{\cdot-}$. Nitroblue tetrazolium (NBT) is used as an indicating scavenger and is reduced by $O_2^{\cdot-}$, the stepwise addition of electronics forming first the monoformazan then the deformazan with radical intermediates. NBT reduction causes an increase in absorbance at 560 nm, which is inhibited by the presence of SOD. As for the xanthine oxidase/cytochrome c assay, one unit of SOD activity is defined as the amount of SOD required to produce 50% inhibition under specified conditions. The assay reactions are represented schematically in Fig. 3.

Formazan is only slightly soluble in aqueous solution; gelatin (Fried, 1975) and Triton X-100 (Nishikimi, 1975) have been proposed as solubilising reagents. Giannopolitis & Ries (1977*a*), however, did not find that a solubilising agent was necessary for the assay of SOD in crude plant extracts, but its use does provide one advantage: peroxidases present in the extract assayed may mimic SOD activity by reoxidising formazan or an intermediate to NBT, hence reducing the apparent rate of NBT reduction. We have found that the addition of Triton X-100 (0·013% w/v) to the assay medium is useful in the prevention of reoxidation to NBT by peroxidase which may be present in crude extracts (unpublished data).

This assay may be subject to similar interferences to the xanthine oxidase/cytochrome c assay. Other substances present in the extract may be capable of photoproduction of $O_2^{\cdot-}$, such as flavin mononucleotide, thus altering the steady-state concentration of $O_2^{\cdot-}$ in the presence of the extract. Figure 4 highlights some of the possible interferences that may occur when assaying crude extracts and demonstrates the need for control assays. Many

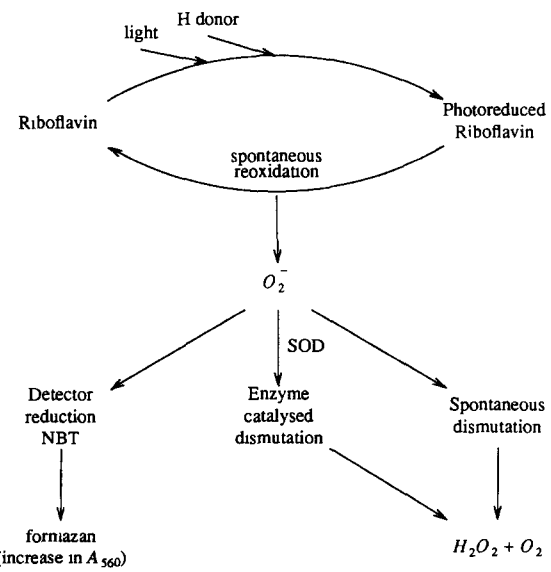

Fig. 3. Scheme of reactions occurring in the riboflavin/NBT assay.

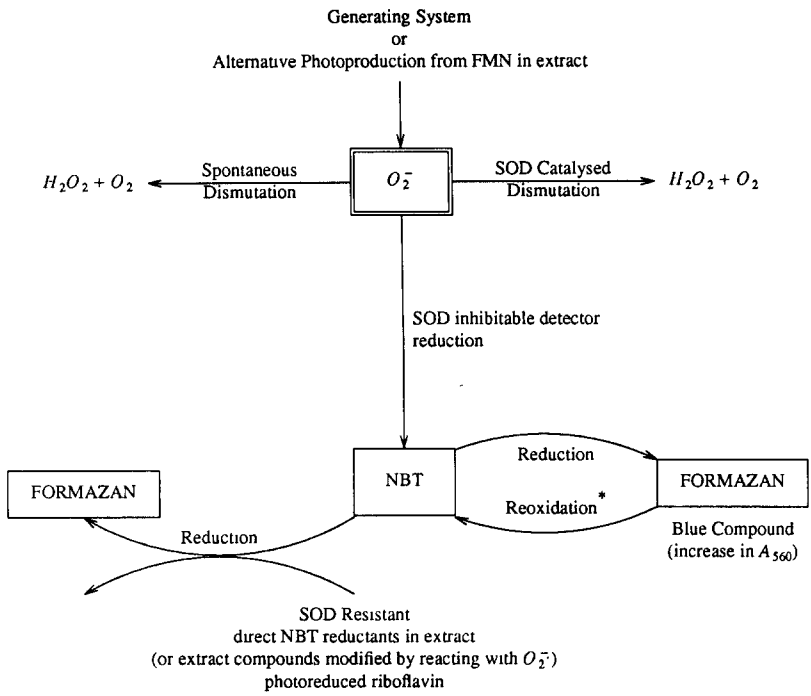

Fig. 4. Detector system using NBT as an O_2^- scavenger compound. * One of the reactions in this pathway is catalysed by peroxidase. (FMN = Flavin mononucleotide).

small molecules can mimic the SOD reaction; e.g. metallocomplexes of amino acids or oligopeptides, metal-chelate complexes (e.g. iron-EDTA) and ascorbate. To differentiate these activities from true SOD activities in crude extracts, it is necessary to compare the activities of the untreated extract (total 'SOD'), a heat-treated extract (i.e. non-enzymatic 'SOD') and an extensively dialysed extract (enzymatic SOD).

Beauchamp & Fridovich (1971) found that in control assays, when no SOD was present, 6% of the rate of NBT reduction was not mediated by O_2^-. and was therefore resistant to inhibition by SOD. They defined a unit of SOD activity as the amount required to produce 50% of the maximum *achievable* inhibition. We have found the riboflavin/NBT assay method to be more reliable and sensitive than the XOD/cytochrome c method. Smaller amounts of SOD from a purified bovine source and crude extract of cabbage are required to achieve 50% of the maximum inhibition of NBT reduction than are required to achieve 50% of the maximum inhibition of cyt c reduction (Walker, 1987). Another advantage of the NBT assay is that there are no proteins or enzymes in the assay mixture other than those present in the sample assayed and it is therefore subject to less interference from these sources; it is also less subject to interference by peroxidases (Walker, 1987).

Taking these factors into consideration, the NBT assay is preferable for the quantification of SOD activity in crude extracts.

The riboflavin/NBT system is also widely used to stain polyacrylamide gels for SOD activity. Blue formazan is formed in regions of the gel devoid of SOD activity, and areas of enzyme activity thus appear colourless against a dark blue background.

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