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UNIVERSITY OF ALBERTA

SUPEROXIDE DISMUTASE MODIFIED WITH EITHER ALBUMIN OR CATALASE INCREASES ITS ANTIOXIDANT EFFECTIVENESS

by

Guo Dong Mao

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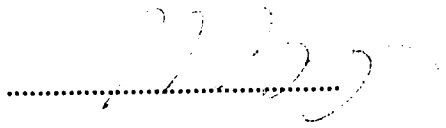

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The undersigned certify they have read, and recommened to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled SUPEROXIDE DISMUTASE MODIFIED WITH EITHER ALBUMIN OR CATALASE INCREASES ITS ANTIOXIDANT EFFECTIVENESS, submitted by Guo Dong Mao in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

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To my wife and parents

without whose love

this thesis would not have been written

Abstract

Superoxide dismutase (SOD) has been reported to offer important pharmacological advantages in decreasing oxygen toxicity as a result of its ability to scavenge oxygen free radicals. This has proven most exciting in reducing damage associated with reperfusion injury following ischemia. Unfortunately superoxide dismutase has a circulation life time of only a few minutes and exogenous SOD may be immunogenic. In order to overcome these limitations, we have crosslinked SOD with albumin to form water soluble polymers using glutaraldehyde as the coupling agent. The SOD-Albumin polymers have important advantages over free SOD in terms of stability, extended circulation time in vivo, enhanced resistance to inhibitor and reduced immunogenicity.

Xanthine oxidase and activated neutrophils have been hypothesized to be important sources of biological free radical generation. The xanthine oxidase generates superoxide radicals and has been widely used as a superoxide radical generating system, however the enzyme may also generate other forms of oxygen free radicals. The generation of superoxide radicals by neutrophils has been unequivocally shown to result from the "respiratory burst" with a number of stimuli. However, the generation of hydroxyl radicals by stimulated neutrophils remains in debate. In order to study the nature of oxygen free radicals generated from different sources, we have applied electron spin resonance (ESR) with the spin trap, 5,5'-dimethyl-1-pyrroline-N-oxide, to characterize the different free radical species generated by xanthine oxidase and phorbol myristate acetate stimulated neutrophils. Upon the reaction of xanthine with xanthine oxidase, both superoxide (DMPO-OOH) and hydroxyl

(DMPO-OH) signals are observed. In the presence of superoxide dismutase, all DMPO-OOH signals are inhibited but not the DMPO-OH signals suggesting that superoxide radicals are not required for the formation of hydroxyl radicals. In the presence of DMSO, a DMPO-CH_3 signal appears indicating the existence of hydroxyl radicals in the system. Catalase decreases the DMPO-OH signals while the combination of SOD and catalase completely scavenges all free radicals. These results indicate that xanthine oxidase can generate hydroxyl radicals as well as superoxide radicals. As a consequence of stimulation by phorbol myristate acetate, human neutrophils produce a continuous flux of superoxide radicals. However the DMPO-OH and DMPO-CH_3 signals derived from the stimulated neutrophils are not inhibited by catalase and do not accumulate following the stimulation. All the free radical signals generated by neutrophils can be scavenged by superoxide dismutase. These data suggest that neutrophils may not generate hydroxyl radicals; the DMPO-OH and DMPO-CH_3 signals obtained from the stimulated neutrophils may derive from the decomposition of DMPO-OOH rather than from direct generation by neutrophils.

A knowledge of the permeability of phospholipid membranes and biological membranes to superoxide radicals is necessary for a successful application of SOD as a therapeutic agent. For this purpose, we have used SOD-loaded lipid vesicles and SOD-loaded erythrocyte ghosts as model systems. The superoxide radicals are externally generated by the reaction of xanthine with xanthine oxidase. The ferricytochrome c assay and the electron spin resonance and spin trapping techniques are used to test the interaction of superoxide with the entrapped SOD. Superoxide dismutase trapped inside lipid vesicles does not eliminate extravesicular superoxide radicals unless deoxycholate is added. This suggests that superoxide radicals are unable to diffuse through the lipid

membrane freely. SOD-loaded erythrocyte ghosts, however, can scavenge externally generated superoxide radicals as effectively as free SOD. This indicates that superoxide can cross the biological membrane and react with the entrapped enzyme.

One of the hypotheses for the potential toxicity of high dose SOD is the over-production of hydrogen peroxide which may in turn produce toxic hydroxyl radicals via the Fenton reaction. In order to verify this mechanism, we have studied the effect of iron on the enhancement of hydroxyl radical production in vitro using electron spin resonance techniques. In the absence of SOD, the ESR spectra following the reaction of xanthine with xanthine oxidase indicate that the size of the DMPO-OH signal only slightly increases as iron concentration increases. However, a dramatic increase of DMPO-OH is observed in the presence of both iron and SOD. The intensity of the main DMPO-OH peak obtained from a sample containing 40 IU/ml SOD and 20 μm Fe^{2+} is about 30-fold of that obtained from the sample containing only SOD. The ferric and ferrous ions exhibit a similar effect on the enhancement of hydroxyl radical production in the presence of SOD. These results suggest that SOD may accelerate the production of hydroxyl radicals by providing more hydrogen peroxide, which may contribute to the toxicity of SOD. The dose-response study of SOD has shown that the intensity of the DMPO-OH signal increases as a function of SOD dose until a plateau is reached. This supports our proposal that high doses of SOD may become toxic if an active form of iron is available to induce highly toxic hydroxyl radicals.

In order to attenuate the potential toxicity of SOD, we have prepared SOD-CATALASE conjugates by a cross-linking reaction. These conjugates markedly

prolong the half-life of both SOD and catalase in vivo, increase the resistance of the enzymes to inhibitors and effectively diminish the production of hydroxyl radicals caused by the combined action of iron and SOD. SOD-CATALASE conjugates also appear to be more effective on inhibiting the production of hydroxyl radicals than the equivalent amount of free enzymes. This may be attributed to the "immobilized" catalase which can remove hydrogen peroxide before it diffuses away to react with iron and form hydroxyl radicals.

The property of SOD-CATALASE conjugates is further assessed in isolated working hearts. The rat heart is subjected to 30-minute global ischemia, followed by reperfusion with the conjugates or free enzymes. The results have shown that a low dose of free SOD does not improve the recovery of reperfused hearts in terms of heart rate and cardiac function. The high dose of free SOD exhibits an apparent toxicity on the reperfused hearts. In contrast, SOD-CATALASE conjugates, either low or high dose, significantly increase functional recovery of the reperfused hearts during the period of reperfusion. Therefore, SOD-CATALASE may have a great role in strengthening the antioxidant defense in vivo.

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Abbreviations

A	absorbance (optical density)
Ab	antibody
ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
Arg	arginine
Asp	aspartic acid
ATP	adenosine-5'-triphosphate
ATPase	Adenosine triphosphatase
BSA	bovine serum albumin
C°	centigrade degrees
cm	centimeter
cpm	counts per minute
DETAPAC	diethylenetriaminepentaacetic acid
DMPO	5,5-dimethyl-1-pyrroline-N-oxide
DMPO-CH_3	DMPO-methyl radical adduct
DMPO-OH	DMPO-hydroxyl radical adduct
DMPO-OOH	DMPO-superoxide radical adduct
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOC	deoxycholic acid (sodium salt)
E	molar extinction
EDTA	ethylenediaminetetraacetate
e.g.	exempli gratia, for example
eggPC	phosphatidylcholine (from egg yolk)
ELISA	enzyme-linked immunosorbentassay
ESR	electron spin resonance
et al.	et alii (and others)
g	grams or gravitational acceleration
G	gauss
GHz	gigahertz
GPX	glutathione peroxidase
GSH	glutathione (reduced)
GSSG	glutathione (oxidized)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

Hb	hemoglobin
HbO_2	oxyhemoglobin
His	histidine
12-HETE	12-hydroxy-5,8,11,14-eicosatetraenoic acid
12-HPETE	12-hydroperoxy-5,8,11,14-eicosatetraenoic acid
hr	hour
HR	heart rate
HRP	horse radish peroxidase
HSA	human serum albumin
i.e.	id est, that is
i.p.	intraperitoneal
IU	international unit
i.v.	intravenous
Kcal	kilocalorie
KHz	kilohertz
LDL	low density lipoprotein
M	molar concentration (moles of solute per liter of solution)
MDA	malondialdehyde
metHb	methemoglobin
metMb	metmyoglobin
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimole
MSA	mouse serum albumin
mw	molecular weight
mW	milliwatt
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
nm	nanometer
p	page
PBS	phosphate buffered saline
PEG	polyethylene glycol
PBN	N-tert-butyl-α-phenylnitrone
PGH_2	9,11-endo-peroxy-15-hydroxyprostaglandin

PGG_2	9,11-endo-peroxy-15-hydroperoxyprostaglandin
pH	negative logarithm of H^+ concentration
pKa	the negative logarithm of an equilibrium constant of a weak acid
PMA	phorbol-12-myristate-13-acetate
PMN	polymorphonuclear leukocyte
PSP	peak systolic pressure
psi	pound per square inch
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
sec	second
SEM	standard error of mean
SOD	superoxide dismutase
$T_{1/2}$	half life
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TPA	tissue plasminogen activator
TTC	triphenyl tetrazolium chloride
U	unit
µl	microliter
µM	micromole
vol	volume
w	weight

1. General Introduction

Oxygen is essential for higher life forms, yet it is also toxic to all aerobes. Few would question the first fact of this oxygen paradox, but participation of oxygen in toxic reactions and its etiological role have been viewed skeptically from the start. Perhaps the earliest suggestion to explain oxygen toxicity was that oxygen inhibited cellular enzymes, particularly those containing sulfhydryl groups such as 3-phosphoglyceraldehyde dehydrogenase, urease, and succinate dehydrogenase (1,2). Indeed, direct inhibition of cellular enzymatic activity by oxygen accounted for part, but not all, of the decreased growth rate of organisms at an elevated pressure (3,4). Concern expressed in respect to this explanation is that the rates of enzyme inactivation by oxygen are too low and too limited in extent to account for the rate at which toxic effects develop (5). In fact most enzymes are totally unaffected by oxygen (6). In 1954, Rebecca Gershman and Daniel L. Gilbert in the U.S.A. proposed that most of the toxic effects of oxygen could be contributed to the formation of oxygen free radicals (7,8), but initial interest to this idea was limited largely due to the very short lifetimes and the difficulty in studying these oxygen species. Research involving oxygen free radicals in biological systems had to await further development of technology.

The major advances in oxygen free radical research was brought about by the development of methodology in the last two decades. A great deal was learned from direct electron spin resonance (ESR) measurement of "frozen" radicals generated by radiation, enzyme action and products of free radical reactions in the aqueous phase under stationary-state conditions (9,10).

It was, however, the discovery of **superoxide dismutase (SOD)** in biological systems by McCord and Fridovich in 1968 that marked a turning point in oxygen free radical research in biology and medicine. It is clear now that human physiology is equipped to deal with free radicals and prevent their damaging effects. SOD and a range of other compounds defined in biological systems have now been described as essential antioxidants. If these protective mechanisms are breached, the living organism suffers from the resulting consequences (11). During the last decade, biochemists have linked those destructive species to countless diseases, and pharmaceutical companies are developing drugs to arrest oxygen free radical damage. This introduction deals with the chemistry and deleterious consequences of oxygen free radicals in biological systems. It also presents an essential picture of antioxidant defense and their potential therapeutical usage.

2. Introduction

2.1. Oxygen Free Radical Chemistry

In modern terminology, a " free radical " is any species that has one or more unpaired electrons. This definition embraces the atom of hydrogen, most transition metals and the oxygen molecule. Free radicals are generally produced by the homolytic fission of covalent bonds between two atoms. Gamma radiation, uv light, and environmental pollutants are among the many exogenous initiators of free radical reactions. However, because of the ubiquity of molecular oxygen in aerobic organisms, the most important sources of radical species in vivo are univalent, biochemical redox reactions involving oxygen. In the following sections, we will overview what oxygen free radicals are, where they come from and what they do.

2.1.1. Oxygen Molecule (O_2)

The oxygen molecule is itself a free radical with two unpaired electrons each located in a different π^* antibonding orbital. These two electrons have the same spin quantum number (parallel spin), and so if oxygen attempts to oxidize another atom or molecule by accepting a pair of electrons, both incoming electrons must be of parallel spin to fit into the vacant spaces in the π^* orbitals. This spin-restriction slows down the reaction of oxygen molecules with other nonradical molecules and keeps oxygen molecules in a stable state (12). Normally, the oxygen molecule is reduced to water by the addition of four electrons and four protons as energy is extracted from a substrate molecule for cellular use (Table 1, reaction 1).

Table 1

Formation of Oxygen Free Radicals and Their Metabolites

(1) Normal tetravalent reduction of oxygen to water

$$O_2 + 4e^- + 4H^+ \longrightarrow 2H_2O$$

(2) Univalent reduction of oxygen resulting in superoxide

$$O_2 + e^- \longrightarrow O_2^-$$

(3) Divalent reduction of oxygen resulting in hydrogen peroxide

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

(4) Trivalent reduction of oxygen resulting in hydroxyl radical

$$O_2 + 3e^- + 3H^+ \longrightarrow OH^- + \cdot OH + H^+ \longrightarrow H_2O + \cdot OH$$

(5) Dismutation of O_2^- (spontaneous or SOD-catalyzed)

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

(6) Haber-Weiss reaction

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

(7) Superoxide-driven Fenton reaction

(iron catalyzed Haber-Weiss reaction)

$$O_2^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2$$

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

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

(8) Hydrogen peroxide-halide-myeloperoxidase (MPO) system

$$H_2O_2 + Cl^- \longrightarrow OCl^- + H_2O$$

$$OCl^- + H_2O_2 \longrightarrow {}^1O_2 + Cl^- + H_2O$$

$$OCl^- + RNH_2 \longrightarrow RNHCl + OH^-$$

2.1.2. Singlet Oxygen ($^1O_2$)

Singlet oxygen is a strong electrophilic molecule and is formed when molecular oxygen absorbs a significant quantity of energy to cause a shift of one of its two unpaired electrons of similar spin to an orbital position with inversion of spin of one of the electrons (13). This movement of electron alleviates the spin restriction and increases the reactivity of molecular oxygen. When the excited electron forms an electron pair and occupies the same orbital, delta singlet oxygen (Δ^1O_2) is formed. Delta singlet oxygen is relatively stable (22.4 kcal energy above the ground state) and has a half-life in water of approximately 4 microseconds (14). When the excited electron remains unpaired (occupying a different orbital), a second form of singlet oxygen is formed, designated as sigma singlet oxygen (Σ^1O_2). Sigma singlet oxygen is less stable (37.5 kcal energy above the ground state) and usually decays to the delta singlet oxygen before it has time to react with anything (14). Delta singlet oxygen is not a free radical, since there are no unpaired electrons. However, in both forms of singlet oxygen the spin restriction is removed and so the oxidizing ability is greatly increased. The potent reactivity of singlet oxygen and its potential for indiscriminate interaction with other molecules are the result of its great instability. When singlet oxygen decays to the more stable form of molecular oxygen, energy is released as the excited electron returns to its thermodynamically stable configuration. This decay may result in chemical reactions with another molecule, release of thermal energy, or light emission at specific wave lengths (15,16). Under certain conditions chemiluminescence can be amplified through the interaction of singlet oxygen with a specific molecule, e.g. luminol, resulting in secondary excitation of that molecule (17,18).

In vitro, excitation of O_2 to the singlet states can be achieved when several pigments such as chlorophylls, retinal, flavins or porphyrins are illuminated in the presence of O_2 (15). The pigment absorbs light, enters a higher excited state, and transfers energy into the O_2 molecule to make singlet oxygen (19,20). Singlet oxygen can also be formed as a result of the interaction of H_2O_2 with hypochlorite (Table 1, reaction 8), and this has been proposed as a mechanism for the chemiluminescence associated with phagocytic cells (21,22,23). Major biological sources of singlet oxygen can be grouped into (a) those systems involving interaction of organic oxygen radicals such as lipid peroxy radicals that arise from lipid peroxidation; (b) those system which involve reactions of reduced oxygen intermediates like O_2^-, H_2O_2, and $\cdot OH$; (c) those systems which probably involve an enzymatic activation of oxygen, i.e., cyclooxygenase activity during prostaglandin biosynthesis; (d) those systems which involve the photosensitization reactions (24).

2.1.3. Superoxide Radical (O_2^-)

Single electron reduction of oxygen forms a superoxide radical (Table 1, reaction 2). Superoxide is formed in almost all aerobic cells (25). In organic solvents, O_2^- is a strong base and nucleophile; for example, it can displace Cl^- from unreactive chlorinated hydrocarbons such as CCl_4 (26). In an aqueous solution, O_2^- has moderate reactivity, and it will act either as a reducing agent to reduce cytochrome c or nitroblue tetrazolium (27,28), or as a weak oxidizing agent to oxidize such molecules as adrenalin and ascorbic acid (29). Its rate of reaction with a given molecule can be greatly influenced by the microenvironment of the target. For instance, O_2^- does not react, at a significant

rate, with NADH in free solution, but reacts very effectively with NADH which is bound to lactate dehydrogenase (30). Superoxide radicals exist as a superoxide anion, O_2^-(basic form) or a protonated form, $HO_2\cdot$ (acidic form). Since the pKa of ionization is 4.8, at physiological condition the superoxide anion would predominate (31).

When two molecules of O_2^- react with each other, one is oxidized and the other is reduced ultimately forming hydrogen peroxide and oxygen in a dismutation reaction. Spontaneous dismutation of O_2^- occurs most rapidly at a pH equal to the pKa of ionization of O_2^- (pH 4.8), because at this pH and at equilibrium, O_2^- and $HO_2\cdot$ are of equal concentration so the rate of reaction is maximum (31). At physiological pH, however, the nonprotonated form of the superoxide anion predominates. The overall rate of spontaneous dismutation of superoxide radical is very slow (rate constant is about $5 \times 10^5 M^{-1} sec^{-1}$) and very little H_2O_2 is produced. Any reactions undergone by O_2^- in aqueous solution will thus be in competition with this spontaneous dismutation (32). Superoxide dismutase accelerates this dismutation reaction at neutral pH by four orders of magnitude (33) (Table 1, reaction 5), which results in generating more H_2O_2.

2.1.4. Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide is formed by the divalent reduction of oxygen or dismutation of superoxide (Table 1, reaction 3). It has no unpaired electrons and is not a free radical. Hydrogen peroxide mixes readily with water and acts as an oxidizing agent. Reports of the toxicity of H_2O_2 to cells and organisms are

variable; some bacteria and animal cells are injured by H_2O_2 at micromolar concentrations, whereas other bacteria and photosynthetic algae generate and release large amounts of it (34). The variability can be accounted for both by the activity of H_2O_2-removing enzymes and by the rate of conversion of H_2O_2 into a more highly reactive free radical.

Hydrogen peroxide can act as a precursor of the more reactive and toxic species—hydroxyl radical. Homolytic fission of the O—O bond in H_2O_2 produces two hydroxyl radicals, and this process can be induced by heat, ionizing radiation or metal ions (35,36). In the presence of myeloperoxidase, hydrogen peroxide can also react with Cl^- to form hypochlorous acid which can react with either amino acids or proteins (Table 1, reaction 8).

2.1.5. Hydroxyl Radical (·OH)

Besides homolytic fission of H_2O_2 to form hydroxyl radicals, direct reduction of H_2O_2 also results in hydroxyl radicals (Table 1, reaction 4). In 1934 a paper was published by Haber and Weiss in which O_2^- as an electron donor interacting with H_2O_2 to form hydroxyl radicals was first postulated (37). This reaction has become known as the Haber-Weiss reaction (Table 1, reaction 6). Unfortunately, this direct reduction of H_2O_2 by O_2^- with the formation of ·OH has since been shown to occur very slowly under most physiological conditions, and will not compete with the spontaneous dismutation of O_2^-, thus the classic Haber-Weiss reaction is an unlikely source of significant quantities of ·OH (38).

The main proposed mechanism of ·OH production is the metal-dependent breakdown of H_2O_2 or the Fenton reaction (39,40). This reaction can be driven by superoxide in which O_2^- first reacts with an oxidized form of a transition metal, then the reduced form of the metal reacts with H_2O_2, regenerating the initial oxidized metal and forming OH^- and hydroxyl radical (Table 1, reaction 7).

Reaction rate constants (Table 2) for ·OH determined by pulse radiolysis have shown that this radical reacts with almost every type of molecule found in living cells at high rates (41), and so any hydroxyl radicals produced in vivo would react at or close to its site of formation.

Reactions of ·OH can be classified into three main types (36):

(a) Hydrogen Abstraction. The hydroxyl radicals react with most C—H bonds with a high rate constant and "pull off" a hydrogen atom and combine with it to form water, leaving behind an organic radical which undergoes further reaction.

$R—H + \cdot OH \longrightarrow R\cdot + H_2O$

(b) Addition Reaction. In several reactions, mainly with organic compounds having double bonds or with an aromatic ring structure, ·OH prefers to add to the double bond or aromatic ring and yields an addition product which undergoes further reaction.

$RCH=CH_2 + \cdot OH \longrightarrow RCHOH\text{-}CH_2\cdot$

(c) Electron Transfer Reaction. The hydroxyl radical is a powerful oxidant, therefore the electron transfer reaction with inorganic and organic compounds is quite common, e.g. with the chloride ion, the following reaction will occur.

$Cl^- + \cdot OH \longrightarrow Cl\cdot + OH^-$

Table 2

Rate Constants for Reaction of the Hydroxyl Radical

Compound tested	pH	Rate constant ($M^{-1} s^{-1}$)
Carbonate ion, CO_3^{2-}	10.7	2.0×10^8
Bicarbonate ion, HCO_3	6.5	1.0×10^7
Fe^{2+}	2.1	2.5×10^8
H_2O_2	7.0	4.5×10^7
Adenine	7.4	3.0×10^9
Adenosine	7.7	2.5×10^9
AMP	5.4	1.8×10^9
Arginine	7.0	2.1×10^9
Ascorbic acid	1.0	7.2×10^9
Benzene	7.0	3.3×10^9
Catalase	-	2.6×10^{11}
Cysteine	1.0	7.9×10^9
Cystine	2.0	3.2×10^9
Cytosine	7.0	2.9×10^9
Deoxyribose	-	1.9×10^9
Glucose	7.0	1.0×10^9
Glutamic acid	2.0	7.9×10^9
Glutathione	1.0	8.8×10^9
Glycylglycine	2.0	7.8×10^7
Guanine	-	1.0×10^{10}
Hemoglobin	-	3.6×10^{10}
Histidine	6-7	3.0×10^9
Hydoxyproline	2.0	2.1×10^8
Lecithin	-	5.0×10^8
Methionine	7.0	5.1×10^9
Nicotinic acid	-	6.3×10^8
Phenylalanine	6.0	3.5×10^9
Ribonuclease	-	1.9×10^{10}
Ribose	7.0	1.2×10^9
Serum albumin	-	2.3×10^{10}
Thiourea	7.0	4.7×10^9
Thymine	7.0	3.1×10^9
Tryptophan	6.0	8.5×10^9
Uracil	7.0	3.1×10^9
Urea	9.0	7.0×10^5

Note: Values are taken from the reference by M. Anbar and P. Neta (1967) Int. J. Appl. Radiation and Isotopes 18, 493-523.

2.2. Sources of Oxygen Free Radicals

2.2.1. Extracellular Sources

There are many free radical sources in our living environment which range from air pollutants, hyperoxia, pesticides, and tobacco smoke to the general class of aromatic hydrocarbons and solvents. These agents either already exist as free radicals or can be converted to free radical species by intracellular metabolic and detoxication processes (42,43,44). Many anthracyclic, antineoplastic agents such as adriamycin, daunorubicin,dexorubicin and other antibotics that depend on quinoid groups or bound metals for activity are able to generate oxygen free radicals. The essential process involves the transfer of an electron to oxygen from a semiquinone moiety or from a metal ion (45,46). Irradiation of organisms with electromagnetic radiation (X—rays or γ—rays) and particulate radiation (electrons, protons, α and β particles) also generates primary radicals by transferring their energy to cellular components such as water (47). These extracellular free radical sources and initiators share many common mechanisms of inducing cell damage (48).

2.2.2. Intracellular Sources

Excellent articles have been published to show that a wide variety of cell components are quantitatively important contributors to intracellular free radical production (49,50,51,52). These sources mainly include:

(1) Autoxidation of Small Molecules

Small intracellular molecules such as thiols (53), hydroquinones (54), catecholamines (29), flavins (48), tetrahydropterins (56), and divalent metals (57,58) are capable of undergoing oxidation-reduction reactions in a neutral aqueous milieu. In all cases, O_2^- is the primary radical formed by the univalent reduction of oxygen by these molecules.

(2) Soluble Enzymes and Proteins

Numerous enzymes generate free radicals during their catalytic cycling. Xanthine oxidase, probably the most studied free radical-producing enzyme, catalyzes the oxidation of purine to hypoxanthine, of hypoxanthine to xanthine, and xanthine to uric acid (59). During its catalytic cycling, xanthine oxidase can cause both univalent and divalent reduction of oxygen to generate superoxide radicals and hydrogen peroxide. The ratio of O_2^- to H_2O_2 produced by xanthine oxidase varies with pH and pO_2 , normally elevated pH and/or pO_2 favor O_2^- production (60). Xanthine oxidase can also oxidize other compounds such as aldehyde to the corresponding carboxylic acid (61). Allopurinol, an analog of hypoxanthine, is a powerful competitive inhibitor of xanthine oxidase (62).

Aldehyde oxidase shares many of the same substrates as xanthine oxidase and also generates superoxide radicals (63). Dihydroorotate dehydrogenase, flavoprotein dehydrogenase, and tryptophan dioxygenase also generate and/or utilize O_2^- during their catalytic cycle. This is deduced from either the observation that superoxide dismutase will inhibit enzyme activity or from electron spin resonance measurement of free radical intermediates during enzyme catalysis (55,64,65). Hemoglobin (Hb) and myoglobin (Mb) can undergo autoxidation to form methemoglobin (metHb) and metmyoglobin

(metMb) wherein dioxygen may serve as a one-electron acceptor and the reduced heme iron (Fe^{2+}) provides the single electron required for conversion of O_2 to O_2^-. The quantitative identification of superoxide radicals as a product of autoxidation of Hb and Mb is confirmed by concomitant reduction of cytochrome c as metHb or metMb is produced (66). In human erythrocytes, the "spontaneous" formation of metHb from Hb is about 3% per day (67). However this autoxidation can be promoted by certain anions (L^-) and the effect of anion on promoting autoxidation depends on its nucleophilicity, e.g. $Cl^- < F^- < OCN^- < SCN^- < CN^-$. The mechanism for the anion-facilitating autoxidation of Hb and Mb may contribute to the ability of anions to stabilize the heme Fe^{3+} state in the metHb or metMb, and thereby altering the oxidation-reduction potential to favor oxidation of heme Fe^{2+} to Fe^{3+} (68). Autoxidation of Hb and Mb to generate superoxide radicals can also be promoted by an increase in hydrogen ion concentration. This effect can be related to the protonation near the anion binding site at low pH, which facilitates anion binding to Hb or Mb and in turn promotes protein autoxidation (69). Thus a simple scheme of oxygen free radical generation from autoxidation of Hb (or Mb) can be given as:

$$Hb + H^+ \longrightarrow Hb(H^+)$$

$$Hb(H^+) + L^- \longrightarrow HbL^-(H^+)$$

$$HbL^-(H^+) + O_2 \longrightarrow metHbL^- + O_2^- \text{ or } (HO_2^-)$$

(3) Mitochondrial Electron Transport System

A major intracellular source of O_2^- appears to be the electron transport chain located on the inner mitochondrial membrane (70). The study assisted with site-specific respiratory chain inhibitors, such as antimycin A, cyanide, and rotenone, has identified that the ubiquinone-cytochrome b region in the respiratory chain is the major site of O_2^- generation (71). The data suggested that although two

electrons are transferred from the ubiquinone to the cytochrome b complex, the reduction of ubiquinone caused by a one-electron transfer can also occur. The generated ubisemiquinone is, in large part, reoxidized to ubiquinone by the cytochrome b complex, but may also react with molecular oxygen to generate superoxide. The superoxide in turn serves as a precursor for hydrogen peroxide and hydroxyl radical (72,73). NADH dehydrogenase in the respiratory chain is another region responsible for a portion of mitochondrial O_2^- production. The superoxide radical may be generated by autoxidation of reduced NADH dehydrogenase (74), although this mechanism appears to be quantitatively less important than the ubiquinone reaction (75). The superoxide radical generated in the mitochondria is converted, in large part, to H_2O_2 by the action of mitochondrial superoxide dismutase (76) and the reaction of unscavenged O_2^- with H_2O_2 may account for the production of hydroxyl radicals in mitochondria (77,78).

(4) Endoplasmic Reticulum and Nuclear Membrane Electron Transport Systems

The endoplasmic reticulum and nuclear membrane share many of the same elements (79). Both of these intracellular membranes contain the cytochromes P_{450} and b_5 that can convert nonpolar compounds such as unsaturated fatty acids (80) and xenobiotics (81) to hydroxylated derivatives by electron transport reactions. NADH or NADPH are required cofactors for these reactions. During normal electron transport reactions, electrons are transferred from the reduced cofactor of cytochrome reductase to the cytochrome-substrate complex and then to molecular oxygen (82). However, in the absence of metabolic substrate, autoxidation of reduced flavin cofactor of cytochrome reductase or cytochrome

intermediate may occur to generate oxygen free radicals (83), analogous to the autoxidation of hemoglobin to methemoglobin.

(5) Plasma Membrane

One of the important biological sources of oxygen free radicals is the reaction catalyzed by NADPH oxidase in the plasma membrane of phagocytes. The mechanism involved in this process has been extensively reviewed (84,85,86). Briefly, upon recognition of a phagocytic or soluble stimulus, phagocytes undergo a so-called "respiratory burst", characterized by a rapid increase in oxygen consumption, and an increase in glucose metabolism via the activation of the hexose monophosphate shunt. In conjunction with an oxygen consumption, superoxide radicals are generated by the transference of a single electron to oxygen mediated by the phagocyte plasma membrane associated NADPH oxidase. Hydrogen peroxide is also formed by either dismutation of superoxide radicals or divalent reduction of oxygen via the reaction between glucose and glucose oxidase (87). These phagocyte-derived free radicals and derivatives including hydroxyl radical, singlet oxygen, and hypochlorous acid may be essential for the destruction of ingested microorganisms, but they also have the potential to damage both the source cell and cells in close apposition to stimulated phagocytes.

Lipoxygenase and cyclooxygenase, which are microsomal and plasma membrane-associated enzymes, catalyze the metabolism of arachidonic acid to form biologically potent products such as prostaglandins, thromboxanes, and leukotrienes. Recent studies have shown that free radicals participated in these enzymatic processes. For example, cyclooxygenase converts arachidonic acid to the unstable hydroperoxy endoperoxide PGG_2, followed by the peroxidase-

catalyzed reduction of PGG_2 to the endoperoxide PGH_2. The carbon-centered radical involved in the enzymatic conversion process has been shown to be a fatty acid free radical by electron spin resonance (88). This free radical is derived from cyclooxygenase-mediated abstraction of the methylene hydrogen of arachidonic acid. However, the nature of the oxygen-centered radical involved in the breakdown of PGG_2 is controversial. It has been suggested to be ·OH (89), while a recent report proposes that this oxygen-centered free radical is a cyclooxygenase hemoprotein radical, distinct from ·OH (90). Lipoxygenase metabolizes arachidonic acid to form 12-hydroperoxy-5,8,11,14-eicosatetraenoic acid (12-HPETE) which is a precursor of a range of biologically active chemicals known as leukotrienes. It has been noted that 12-HPETE and its 12-hydroxy derivative (12-HETE) are substrates for the peroxidase activity of cyclooxygenase and can lead to form oxygen-centered free radicals (91). It appears that these carbon- and oxygen-centered free radicals are capable of attacking the cyclooxygenase itself or subsequent enzymes in the prostaglandin pathway, causing inactivation of enzymes and inhibition of the whole cascade of prostaglandin biosynthesis. Hence, the addition of antioxidants can sometimes stimulate prostaglandin synthesis by preventing such inactivation (92).

2.3. Deleterious Consequences of Oxygen Free Radicals

Virtually all cell components are susceptible to free radical attack. The products of the reactions of proteins, polyunsaturated fatty acids, DNA and carbohydrates with free radicals are now becoming better characterized and more widely used as evidences of free radical damage in vivo (93).

2.3.1. Free Radical Effects on Protein

Because of the reactivity of unsaturated and sulfur-containing molecules with free radicals, proteins containing the amino acids tryptophan, tyrosine, phenylalanine, histidine, methionine, and cysteine can undergo free radical-mediated amino acid modification resulting in (a) protein conformational changes, fragmentation, and polymerization, (b) changes in susceptibility of protein to enzymatic hydrolysis, and (c) accumulation of denatured proteins (94).

It is likely that subtle changes in specific proteins may have significant biological consequences. Two examples serve to illustrate this point: (a) oxidation of an amino acid residue in the active site of α-protease inhibitor inactivates the protein and this would allow normally inactive α-protease to become active, resulting in tissue damage and possibly contributing to lung emphysema (95). (b) oxidation of specific amino acid residues on a subunit of glutamine synthetase inactivates the protein. The consequence of this may be a buildup of glutamate, a known excitatory neurotoxin, which may explain the damage that occurs in stroke (96).

2.3.2. Free Radical Effects on Membrane Lipids

The unsaturated bonds of membrane cholesterol and fatty acids can readily react with free radicals and undergo peroxidation. The lipid peroxidation process involves at least three distinct phases (97,98). The initiation step occurs when a free radical interacts with a polyunsaturated fatty acid and extracts a hydrogen atom, forming a fatty acid radical. This step is followed by the second

or propagation phase, in which the fatty acid radical reacts rapidly with molecular oxygen, generating a fatty acid peroxy radical. The fatty acid peroxy radical itself can abstract a hydrogen atom from another unsaturated fatty acid, leaving a fatty acid radical and a lipid hydroperoxide. The peroxidation reactions can be terminated in a number of ways. Glutathione peroxidase can reduce fatty acid peroxy radicals to nonreactive hydroxy fatty acids; free radical scavengers can quench the fatty acid peroxy radical; and two free radicals can destroy each other to terminate the chain reaction. Bond rearrangement of lipid hydroperoxide may cause formation of diene conjugates and degradation of it may produce malondialdehyde, ethane and pentane. These shortened-chain degradation products are often toxic and may play a key role in the development of certain pathological states. For instance, malondialdehyde has been shown to react with nucleic acids, possibly contributing to mutagenesis and carcinogenesis (99). Some lipid peroxidation products have been shown to react with low density lipoprotein (LDL) causing it to lose its high affinity for the LDL receptor, and thus prolonging LDL circulation and enhancing its uptake into macrophages. This could lead to foam cell formation in atherogenesis (100). The final consequences of extensive lipid peroxidation in biological membranes involve alteration in membrane fluidity, membrane potential, membrane permeability, activity of Na/K ATPase and eventual membrane rupture (101).

2.3.3. Free Radical Effect on Nucleic Acids and DNA

Oxygen free radicals have been implicated as the agent responsible for different lesions in DNA. Superoxide produced from the xanthine/xanthine oxidase system has been shown to cause DNA strand breaks (102). The

hydroxyl radical readily attacks the sugar portion, probably carbon 3' and 4', of the DNA, resulting in DNA strand scission (103). The other type of lesion is caused by base modification. At least three modified bases, 8-hydroxyguanine, 5-hydroxymethyluracil, and thymine glycol are formed by hydroxyl free radical attack on DNA (103). The formation of these modified bases may have serious consequences in terms of mutagenesis and carcinogenesis.

As a result of DNA strand breakage by free radicals, consequent repair process carried out by poly(ADP-ribose) synthetase will deplete nicotinamide nucleotides within the cells (104) and increase the probability of misincorporating the wrong base in the repaired DNA (105). These factors may further amplify free radical damage.

2.3.4. Free Radical Effect on Carbohydrates

Oxygen radical-induced oxidation of carbohydrates has been studied to a lesser extent. It has been postulated that monosaccaride autoxidation under physiological conditions may enhance protein cross-linking, causing protein aggregation and basement membrane thickening in the development of diabetic cataract and microangiopathy (106,107).

Oxygen radicals can also disrupt carbohydrate polymers such as hyaluronic acid (108,109). Hyaluronic acid, necessary for maintaining joint synovial fluid viscosity, is a glycosaminoglycan consisting of repeating units of glucuronic acid and N-acetyl glucosamine. It has been shown that in rheumatoid arthritis, the molecule is depolymerized and the effect can be attributed to oxygen free radicals generated by neutrophils at least in vitro (110).

Table 3
Clinical Conditions in Which Involvement of Oxygen Radicals Has Been Suggested*

- Inflammatory-immune injury
  - Glomerulonephritis
  - Vasculitis
  - Autoimmune diseases
  - Rheumatoid arthritis
- Ischemia: reflow states
  - Stroke/myocardial infarction
  - Organ transplantation
  - Inflamed rheumatoid joint?
  - Frostbite
  - Dupuytren's contracture?
  - Dysbaric osteonecrosis
- Drug and toxin-induced reactions
  - Iron overload
  - Idiopathic haemochromatosis
  - Dietary iron overload
  - Thalassemia and other chronic anemias
  - Nutritional deficiencies
- Alcoholism
  - Alcohol-induced iron overload
  - Alcoholic myopathy
- Radiation injury
  - Nuclear explosion
  - Accidental exposure
  - Radiotherapy
  - Hypoxic cell sensitizers
- Aging
  - Disorders of premature aging
- Red blood cells
  - Phenylhydrazine
  - Primaquine, related drugs
  - Lead poisoning
  - Protoporphyrin Photoxidation
  - Malaria
  - Sickle cell anemia
  - Favism
  - Fanconi' anemia
  - Hemolytic anemia of prematurity
- Lung
  - Cigarette smoke effects
  - Emphysema
  - Bronchopulmonary
  - Oxidant pollutants
  - Mineral dust pneumoconiosis
  - Asbestos carcinogenicity
  - Bleomycin toxicity
  - SO_2 toxicity
- Heart and cardiovascular system
  - Alcohol cardiomyopathy
  - Keshan disease
  - Atherosclerosis
  - Adriamycin cardiotoxicity
- Kidney
  - Autoimmune nephrotic syndrome
  - Aminoglycoside nephrotoxicity
  - Heavy metal nephrotoxicity
- Gastrointestinal tract
  - Endotoxic liver injury
  - Halogenated hydrocarbon liver injury
  - Diabetogenic action of alloxan
  - Pancreatitis
  - Oral iron poisoning
- Brain/nervous system/ neuromuscular disorders
  - Hyperbaric oxygen
  - Vitamin E deficiency
  - Neuronal ceroid lipofuscinoses
  - Allergic encephalomyelitis and other demyelinating diseases
  - Aluminum overload
  - Potentiation of traumatic injury
  - Muscular dystrophy
  - Multiple sclerosis
- Eye
  - Cataractogenesis
  - Ocular hemorrhage
  - Degenerative retinal damage
  - Retiopathy of prematurity
  - Photic retinopathy
- Skin
  - Solar radiation
  - Thermal injury
  - Porphyria
  - Hypericin, other photosensitizers
  - Contact dermatitis

*Reference: Barry Halliwell (1987) FASEB J. 1:358-364

2.4. Oxygen Free Radical and Human Diseases

Good evidence now exists for the role of oxygen free radicals in the pathology of several human diseases or conditions, including ageing (111), iron-overload disease (112), arthritis (113), infection with malaria or other parasites (114,115), neurological damage (116), diabetic cataract (117,118), dysbaric osteonecrosis (119), silicosis (120), asbestosis (121), Down's syndrome (122), atherosclerosis (123), and immune injury to kidney, liver, and lung (124). Table 3 lists some of the conditions in which an involvement of radicals has been suggested. In the following section, an overview of the role of oxygen free radicals in ischemia/reperfusion injury is included, because one of our primary research interest stems from this area.

2.5. Ischemia/Reperfusion Injury

Severe restriction of blood flow caused by blockage of an essential artery leads to complete deprivation of oxygen in the tissue. This is termed "ischemia". The extent of tissue necrosis depends on both the duration and severity of ischemia. Any tissue made ischemic for a sufficient period will be irreversibly injured. However, if the period of ischemia is insufficiently long to cause irreversible tissue damage, much tissue can be salvaged by reperfusing the tissue with blood and reintroducing O_2 and nutrients (125,126). In this situation, reperfusion appears to be a beneficial process overall. However, only recently has attention been focused on the idea that reintroduction of O_2 to an ischemic tissue can cause an additional insult even following a relatively brief period of ischemia (127,128). Ischemia/reperfusion injury may occur in the brain (129),

heart (130,131), skin (129), intestine (132), pancreas (132), liver (129), muscle (132), kidney (133,134), and lung (129). The interest in reperfusion injury has recently been heightened by the advent of thrombolytic therapy (135,136) and the performance of coronary angioplasty (137).

Ischemia/reperfusion injury has been attributed to many interrelated factors such as calcium overload of cells (138), cell swelling (139), and white cell plugging (140,141). One of the factors that has recently received considerable attention is oxygen free radical induced damage. This hypothesis was initially based on the observations of (a) free radical scavengers had a beneficial effect on reperfusion injury (142,143,144), and (b) xanthine oxidase is likely to generate a high concentration of oxygen free radicals at the moment of reperfusion in the tissues of certain species (145,146). Recently more extensive studies on the mechanism of reperfusion injury in ischemic myocardium have shown that during ischemia, some remarkable changes set the stage for major oxygen radical explosion following the reintroduction of oxygen. These changes in myocardium will be reviewed in the following section.

2.5.1. Xanthine Dehydrogenase/Xanthine Oxidase System

The xanthine dehydrogenase/xanthine oxidase system has been documented as an oxygen free radical source in certain ischemic tissues (147,148,149). This model was originally proposed by J. M. McCord in 1981 (145,150). Briefly, after the decline in tissue O_2 tension in ischemia, cells switch to an anaerobic metabolic pathway and depressed mitochondrial activity severely curtails the synthesis of ATP. The ATP stores thus become depleted as ATP is progressively converted to ADP and AMP with only a limited possibility of

rephosphorylation. The accumulated AMP is then metabolized to adenosine, inosine, and finally to hypoxanthine, a substrate of xanthine oxidase (150,151).

At the same time, since ATP is depleted very quickly in the ischemic tissue, the energy-dependent removal of Ca^{++} from the cytosol by the endoplasmic reticulum consequently drops. It is conceivable that several proteases are activated by the cytosolic accumulated Ca^{++}(152). One protease, probably calpain, converts the cytosolic enzyme xanthine dehydrogenase by limited proteolysis to a new enzyme, xanthine oxidase (153). Although xanthine oxidase is still capable of metabolizing accumulated hypoxanthine to uric acid, it uses oxygen instead of NAD to generate superoxide radicals via univalent reduction of oxygen. In the ischemia phase, because of the shortage of oxygen, this O_2^- generating reaction may hardly go forward to cause toxic effect. But at the moment of reperfusion, the reintroduction of oxygen drives the reaction of xanthine oxidase with hypoxanthine to completion with a burst of oxygen radical production. This model is supported by the observation that allopurinol, a competitive inhibitor of xanthine oxidase, is able to preserve the ATP and salvage tissue injury after reoxygenation (154,155).

Although the described xanthine oxidase pathway can account for reperfusion injury in certain tissues which contain an abundance of xanthine dehydrogenase and xanthine oxidase (156,157), recent studies have revealed a marked species difference with regard to tissue xanthine oxidase activity. For example, while rat and dog hearts have high xanthine oxidase activity (158), the rabbit heart has no detectable xanthine dehydrogenase or xanthine oxidase and thus, as would be expected, allopurinol has no effect on infarct size in the rabbit heart (159,160). It must be noted that other mechanisms for free radical

production exist in the rabbit heart since a free radical scavenger (e.g. SOD) does reduce the extent of injury following reintroduction of oxygen. The applicability of the xanthine oxidase hypothesis to myocardial ischemia in humans depends on whether the human heart contains xanthine oxidase. Unfortunately, the xanthine oxidase content in human heart remains controversial, ranging from levels in excess of that in rat and dog (161) to trace amounts (162,163) and to none (164,165). Although the data are sparse, it appears that different species have a fundamental difference in their response to ischemia/reperfusion.

2.5.2. Arachidonic Acid Degradation Products

Cytosolic accumulation of Ca^{++} during ischemia also activates phospholipase A_2 that in turn frees arachidonic acid from the cell membrane (166,167). In normal states, arachidonic acid is either reacylated to phospholipids or further degraded via cyclooxygenase or lipoxygenase pathways (168,169). But in the presence of ischemia, recent studies suggest that the arachidonic acid reacylation pathway is defective leading to a buildup of arachidonic acid (170,171). Associated with this is the increased activity of the cyclooxygenase pathway with an accumulation of prostaglandins and free radicals (172).

The lipoxygenase capable of metabolizing arachidonic acid is found in leukocytes. During ischemia, some leukocytes may be trapped in the obstructed vasculature, thus through the interaction of lipoxygenase present in leukocytes and arachidonic acid, various leukotrienes and other metabolites that act as chemotactic factors can be formed (173). As a result more attraction and activation of leukocytes will occur in the ischemic region.

2.5.3. Leukocytes in Ischemia/Reperfusion

The complement system also appears to be activated by a protease during ischemia (174,175). The released component C_{5a} is a chemotactic factor (175) that amplifies the effects of leukocytes; the other released component C_{3b} attaches to the endothelial cell membrane where it acts as a receptor (C_{3bi}) (176,177). Leukocytes possess a surface glycoprotein complex (M_{o1}) that is stored in the cell before activation. After appearance of component C_{3bi} on the endothelial cell surface and activation of leukocytes by a chemotactic factor, M_{o1} undergoes a conformational change and the ligand site which can recognize and bind to the C_{3bi} receptor is exposed. The net result of these changes is an increased adherence of leukocytes at the endothelial surface (178,179,180,181).

The polymorphonuclear leukocytes (PMN) have an NADPH oxidase on the surface, which is activated at the same time as the leukocyte makes contact with the target cell (182,183). Activation of NADPH oxidase causes the local production of O_2^- via a single electron transfer to oxygen. Additional oxidant species can also be formed as a result of the activation of leukocytes. One of the pathways involves leukocyte myeloperoxidase (MPO) reacting with H_2O_2 to form an enzyme substrate complex. This complex has the capacity to oxidize various halides (184). Because of the wide distribution of chloride ions in biological systems, the very toxic hypochlorous acid (HOCl) thus can be formed (185).

Studies supporting the role of leukocytes in reperfusion injury are mostly indirect, e.g. a non-steroidal antiinflammatory agent, ibuprofen, has been found

to inhibit the leukocyte influx into the target tissue with a corresponding reduction of tissue insult (186). Similarly, leukocyte depletion by antileukocyte serum was associated with a significant reduction in reperfusion injury (187).

2.5.4. The Role of Oxygen Free Radicals in Ischemia/Reperfusion

2.5.4.1. Loss of Intracellular Antioxidant Defense during Ischemia/Reperfusion

Cells and plasma contain a variety of defenses against oxygen free radicals, helping the cell to maintain an oxidizing environment while preventing the deleterious effects of oxygen radicals on the cell membrane and organelles. Superoxide dismutase enzymatically transforms O_2^- to H_2O_2 and molecular oxygen (188). Hydrogen peroxide is metabolized to water and oxygen by catalase or glutathione peroxidase (189,190). The activities of these enzymes are known to be depressed during ischemia, which limits the ability of the cell to inactivate a variety of oxygen radical moieties (191,192,193). Therefore, the ischemic tissue is poorly prepared to cope with increased level of oxygen radicals and oxidant stress that occurs during reperfusion.

2.5.4.2. Interconversion of Oxygen Free Radicals

Once the O_2^- is formed in the tissue, it rapidly undergoes a series of interconversions leading to the formation of other harmful species. The following represents the sequence of possible biochemical reactions that can occur during the reperfusion of ischemic tissues.

(a) Dismutation of superoxide generating H_2O_2, SOD speeds up the reaction.

$$O_2^- + O_2^- \longrightarrow H_2O_2 + O_2 \quad (194)$$

(b) Fenton reaction generating ·OH, superoxide radical may participates in this reaction by reducing Fe^{3+} to Fe^{2+}.

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + \cdot OH + OH^- \quad (195)$$

(c) Lipid peroxidation generating lipid and peroxyl radicals*.

$$LH + \cdot OH \longrightarrow L\cdot + H_2O \quad (196,197)$$

$$L\cdot + O_2 \longrightarrow LOO\cdot$$

$$LOO\cdot + LH \longrightarrow LOOH$$

(d) Free radicals interact with each other to form nonradical products.

$$L\cdot + L\cdot \longrightarrow L\text{-}L \quad (198)$$

$$LOO\cdot + LOO\cdot \longrightarrow LOOL + O_2$$

$$LOO\cdot + L\cdot \longrightarrow LOOL$$

(e) Reaction of H_2O_2 with Cl^- generating toxic HOCl catalyzed by the leukocyte enzyme myeloperoxidase (MPO).

$$H_2O_2 + Cl^- \xrightarrow{MPO} OCl^- + H_2O \quad (199)$$

* LH lipid; L· lipid radical; LOO· lipid peroxy radical; LOOH lipid hydroperoxide

2.5.4.3. Deleterious Consequences of Free Radical Reactions

Lipid peroxidation is a common event following oxygen radical attack. This peroxidation process which begins when ·OH effectively removes a hydrogen atom from the lipid molecule could lead to the formation of defects or instabilities in the cell membrane (196,197). If enough of the cell membrane' s lipid bilayer is peroxidized, then the cell will not be able to maintain electrolyte and volume homeostasis, and cell destruction will ensue (200,201). One of the peroxidative end products, malondialdehyde, is often detected by the thiobarbituric acid (TBA) assay. Some laboratories have measured TBA-reactive materials during the reperfusion of ischemic tissues (202,203).

The leukocyte-produced HOCl can inactivate α_1-antiprotease, permitting the unchecked, destructive enzyme elastase to hydrolyze elastin and promote extension of tissue injury (204). Because of extreme reactivity of HOCl, similar reactions can also occur with proteins and with RNA and DNA, which may help perpetuate the original oxidative damage (205,206,207).

Recently, the involvement of oxygen free radicals in ischemia/reperfusion has been directly detected by some investigators in freeze-clamped myocardial tissue. These studies were performed with spin-trapping compounds and the electron spin resonance (ESR) technique (208,209). They found only a small change in free radical signals during the period of ischemia but a sustainable increase in total measured free radicals after reintroduction of oxygen. This post-ischemia change of free radical signals did not occur if reperfusion was performed with a nitrogen-containing solution rather than an oxygen solution.

Thus a major contributor to free radical production during reperfusion is the presence of oxygen in the reperfusion solution.

2.6. Antioxidants

2.6.1. Low Molecular Weight Antioxidants

A variety of lipid soluble molecules can reduce lipophilic free radical species to a less toxic form. Vitamin E (a series of isomers of tocopherol) is probably the best known antioxidant in biological systems. In vitro evidence suggests that vitamin E plays an important role in protecting and maintaining the integrity of cell membranes against lipid peroxidation caused by free radicals (210). This lipid peroxidation-preventing behavior of vitamin E may be aided by a specific interaction between the lipophilic side chain of vitamin E and the fatty acid chains of polyunsaturated phospholipids, providing a mechanism for anchoring vitamin E to membranes and facilitating the antioxidant function of vitamin E (211,212). Vitamin E terminates lipid peroxidation by competing for peroxy free radicals (LOO·) in contrast to the effects of water-soluble enzymatic antioxidants which inhibit the initiation of lipid peroxidation by ·OH. The result of vitamin E reaction with free radicals is to form tocopherol dimers or quinones (213,214). It is noteworthy that a definite protective role for vitamin E in the protection of free radical reactions, particularly in humans, has not been well established (215).

Ascorbic acid (vitamin C) serves as a water soluble reducing agent to maintain tocopherols in the reduced active form and detoxify various organic radicals to an inactive form by a similar reduction process (216,217). Ascorbate

reacts rapidly with oxygen radicals to give a semidehydroascorbate radical that is not particularly reactive and mainly undergoes a degradation reaction, helping to protect against oxygen radicals in vivo.

The tripeptide glutathione (GSH), in concert with GSH peroxidase, can reduce hydrogen peroxide, hydroxyl radical, lipid peroxides, disulfides and dehydroascorbate (218,219,220). The product of the reactions is glutathione disulfide (GSSG) or a GSH adduct of lipid or protein. Glutathione reductase will reduce GSSG back to GSH using NADPH as a cofactor. If a tissue is exposed to a large flux of hydrogen peroxide and/or hydroxyl radicals, the GSH/GSSG ratio might not be maintained at its normally high value. An accumulation of intracellular GSSG appears to inactivate a number of enzymes probably by disulfide interchange. Thus the depletion of intracellular NADPH that affects countless integrated metabolic processes is a secondary toxic manifestation of cellular free radical stress (221,222).

2.6.2. Enzymatic Antioxidants

2.6.2.1. Superoxide Dismutase

2.6.2.1.1. Discovery of Superoxide Dismutase

As early as 1938, scientists in England described a blue-green protein containing copper which was isolated from bovine red blood corpuscles, they named it hemocuprein (223). Later on, it was realized that a whole family of cupreins was present in various organs. H. Porter and J. Folch isolated a copper

protein from bovine brain and called it cerebrocuprein (224). A similar protein was also isolated from liver and named hepatocuprein (225). Besides copper, the cupreins were also found to contain zinc (226). However, the function of these proteins remained unknown until the work of J. M. McCord and I. Fridovich was published in 1969 (188). As a result of various observations, they found that cytochrome c was reduced by the superoxide radical generated from the xanthine/xanthine oxidase reaction, while the cuprein protein tended to competitively inhibit the reduction of cytochrome c. They proposed that the function of the protein was to facilitate the dismutation of the superoxide radical. The concept of superoxide dismutase (SOD) was thus born. Thereafter the copper proteins that had previously been isolated but were without a recognized function were now identified as SODs, which specifically catalyze the dismutation of superoxide radicals to hydrogen peroxide and oxygen:

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

Extensive investigation following the discovery of the copper/zinc SOD led to the realization that the enzyme exists as a family of metallo-proteins: copper/zinc containing SOD is essentially a eukaryotic enzyme, iron-containing SOD is essentially a prokaryotic enzyme, and manganese-containing enzyme is found in both prokaryocytes and eukaryocytes (227,228,229). Each isozyme has been found to have a particular characteristic sensitivity towards a number of reagents. Cyanide is found to inhibit the copper/zinc SOD, but not the manganese and iron SODs (230,231). Hydrogen peroxide inactivates both the copper/zinc and iron SODs but not the manganese SOD (232,233,234). Azide inhibits the enzymes in the following order: iron > manganese > copper/zinc SOD (235).

2.6.2.1.2. Copper/Zinc SOD

All the CuZnSODs so far isolated from eukaryotic cells have a molecular weight around 32,000 daltons and contain two protein subunits, each of which bears an active site containing one copper ion and one zinc ion. The two subunits are apparently identical and are associated solely by noncovalent interactions (236,237).

The copper ion in an active site appears to function in the dismutation reaction by undergoing alternate oxidation and reduction:

$$\text{Enzyme-Cu}^{2+} + O_2^- \longrightarrow \text{Enzyme-Cu}^{+} + O_2$$

$$\text{Enzyme-Cu}^{+} + O_2^- + 2H^+ \longrightarrow \text{Enzyme-Cu}^{2+} + H_2O_2$$

$$\text{Net reaction: } O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

In the first half-reaction, copper is reduced from the cupric to the cuprous state, and the first superoxide radical is oxidized to dioxygen. In the second half-reaction, copper is reoxidized to the cupric form, while the second superoxide is simultaneously reduced and protonated to form hydrogen peroxide (238,239).

The Zn does not function in the catalytic cycle (240,241), but Zn is ascribed a role in maintaining the stability of the enzyme. The evidence presented is (a) apoprotein, reconstituted with copper alone, is less thermostable than apoprotein reconstituted with both copper and zinc (242); (b) copper-free protein, containing zinc as the only metal, is more resistant to dissociation by sodium dodecyl sulfate than the apoprotein (243).

The complete amino acid sequence has been determined for 11 copper/zinc SODs (244,245,246,247). The alignments obtained indicated that the metal-binding sites appear to be conserved in all the enzymes. These are histidine residues 48, 50, 77, and 135 for the copper and histidine 77, 86, 95 and aspartic acid 85 for the zinc. Histidine 77 that bridges the copper and zinc may be involved in supplying the protons needed for the dismutation reaction. Although the two active sites on the enzyme are some distance from each other, the separated SOD subunits themselves catalyze O_2^- dismutation only slowly, if at all (248,249,250,251).

2.6.2.1.3. Manganese SOD

The superoxide dismutase first isolated from E. coli proved to be entirely unlike the CuZnSOD. It is pink rather than green and contains manganese at its active site (252). MnSOD has a molecular weight of 40,000 daltons and is not inhibited by cyanide or hydrogen peroxide, but MnSOD catalyzes exactly the same reaction as does the CuZnSOD.

MnSODs purified from a number of sources are not as uniform as CuZnSOD. Some are dimers, others are tetramers (253,254,255). Removal of the manganese from the active site causes loss of catalytic activity, and it can not in general be replaced by any other transition metal ion (256,257,258).

The amino acid sequences of MnSODs are extremely similar to each other and totally unrelated to those of the CuZnSOD. The ligands to the metal are histidine residues 26, 81, and 179 and aspartic acid residue 175. The manganese undergoes changes of its valency state during catalysis (259,260).

2.6.2.1.4. Iron SOD

FeSOD has been shown to exist in dimeric form with a subunit molecular weight of about 23,000 daltons. FeSOD usually contains 1 or 2 moles of iron per mole of enzyme. The iron in the resting state is Fe^{3+} and it probably oscillates between the Fe^{3+} and Fe^{2+} states during the catalytic cycle (261,262,263). There is as yet no complete amino acid sequence for a FeSOD, however, the amino acid residues of the manganese-binding site in MnSOD are also present in identical positions in the FeSOD (264). There are strong indications that the manganese and iron SODs are structural homologs (265).

2.6.2.2. Catalase

Catalase can be considered as a free radical scavenger, since it catalyzes the divalent reduction of H_2O_2, a precursor of more potent radical species, to water. Most catalases so far isolated from mammalian cells, bacteria and fungi consist of four protein subunits of equal size giving an approximate molecular weight of 240,000 daltons (266,267). Each of the subunits contains a haem (Fe^{3+}-protoporphyrin) group bound to its active site. Dissociation of the enzyme into its subunits causes loss of activity (268,269,270). The mechanism of the catalase reaction may be written as follows:

$$\text{catalase-Fe}^{3+} + H_2O_2 \longrightarrow \text{compound I}$$

$$\text{compound I} + H_2O_2 \longrightarrow \text{catalase-Fe}^{3+} + 2H_2O + O_2$$

The first stage in the catalytic process is the interaction of the first H_2O_2 molecule with heme, causing the enzyme to become compound I. The exact structure of compound I is uncertain, while it may be distinguished from the native enzyme by optical spectra. Reaction of compound I with a second molecule of H_2O_2 results in ferricatalase, O_2 and water. This reaction is less well studied and the H_2O_2 at this stage may be replaced by organic peroxides (271,272).

Catalase can be inhibited by azide, cyanide, and superoxide radical. A more useful inhibitor is aminotriazole. Its inhibitory action is exerted on compound I, so the extent of catalase inactivation by aminotriazole can indicate rates of cellular H_2O_2 production (273,274).

2.6.2.3. Glutathione Peroxidase (GPX)

Glutathione peroxidase has a molecular weight of approximately 85,000 daltons and is present in significant concentrations in the cytoplasm. The reaction catalyzed by glutathione peroxidase (GPX) can be written as follows:

$$LOOH + 2GSH \longrightarrow GSSG + LOH + H_2O$$

$$HOOH + 2GSH \longrightarrow GSSG + H_2O$$

At the expense of glutathione (GSH), GPX can reduce and detoxify H_2O_2 as well as fatty acid hydroperoxides to water and the corresponding hydroxy fatty acid derivatives (275,276,277).

GPX is made up of four protein subunits, selenium is probably present at the active site as selenocystein, the amino acid cysteine in which the normal sulphur atom has been replaced by a selenium atom (278). During the catalytic cycle,

the GSH apparently reduces the selenium and the reduced form of the enzyme then reacts with hydrogen peroxide or fatty acid hydroperoxide forming glutathione disulfide (GSSG) and detoxified hydroxy derivatives.

The action of GPX is dependent on the availability of GSH which is naturally regenerated from GSSG by glutathione reductase in the presence of the cofactor NADPH. Therefore, a decrease in glutathione reductase or a decrease in any of the four enzymes involved in the hexose monophosphate shunt, known mechanism for generation of NADPH, would impair the function of GPX and potentially would result in oxidative damage to tissues (279).

2.6.3. Applications of SOD

The list of human diseases for which antioxidant therapy is being recommended continues to grow based primarily on inferential evidence presuming a potential role for oxygen radicals in a wide range of pathophysiology. The therapy may include xanthine oxidase inhibitors (280,281), free radical scavengers (282,283), iron chelators (280,284), leukocyte depletors (285,286) and nonspecific antioxidants (287,288). Among these agents, superoxide dismutase has received the most attention. The section below will briefly review the literature emphasizing SOD as a potential modulator of inflammation, pulmonary disease and ischemia/reperfusion injury.

2.6.3.1. SOD in Inflammatory Disease

When a host tissue is challenged by a pathologic insult of either an immunologic or nonimmunologic nature, an inflammatory reaction may occur,

with subsequent clearance of the unwanted and potentially dangerous foreign particles, such as bacteria, by phagocytes. The acute inflammatory response, as a component of the defense mechanism, is beneficial to the organism and is normally a self-limiting event. However, anything causing abnormal activation of phagocytes will have the potential to provoke a devastating and potentially propagating response. For instance, in chronic phagocyte-mediated inflammation, superoxide radicals released into the extracellular fluid by neutrophils is deleterious to host tissue, connective tissue, and ground substance, particularly since higher organisms have evolved with little SOD in their extracellular fluids (289). Furthermore, these free radicals will promote the formation of aggregates of IgG which may provide a pathway to autoimmune disease (290,291,292).

The antiinflammatory properties of SOD were discovered by Huber and Schulte long before the enzymatic nature of the metallo-protein was recognized (293). The effect of SOD has been observed in several animal models of inflammation. Multiple i.v. injections of SOD have been found to inhibit the prostaglandin phase of carrageenan-induced foot edema in normal rats, in which neutrophils comprise about 70% of the inflammatory cells at the site of carrageenan injection, as well as in agranulocytic rats, in which the inflammatory cells are almost all mononuclear phagocytes (294). According to Huber et al. (295), native SOD was effective in the carrageenan and Arthus model of inflammatory response (the 'Arthus reaction' is the name given to a local inflammatory response that results when an antigen is injected into the skin of the animal that has a high level of circulating antibody against that antigen) by any parenteral route of administration. Hirschelman and Bekemeier (296) also observed inhibition of carrageenan induced edema with native SOD injected

intravenously. However, McCord et al. showed inhibition of carrageenan-induced edema with i.v. injections of Ficoll-coupled SOD but not with native SOD. They proposed that native SOD had little antiinflammatory effect because SOD is cleared from the circulation within minutes by the kidney (297).

Direct injection of SOD into inflamed joints has been reported to partially relieve the symptoms of rheumatoid arthritis. A number of double blind clinical trials with Orgotein (the generic name in the U.S. for drug version of CuZnSOD) have compared its effectiveness with that of aspirin (298), methylprednisolone (299), and gold (300). At the end of treatment, clinical improvement of the Orgotein groups was found to be statistically significant in all the parameters in terms of morning stiffness, use of analgesics, grip strength, pain and the articular index.

2.6.3.2. SOD in Pulmonary Disease

Bronchopulmonary dysplasia (BPD) is a complication of intensive respiratory therapy in low birth weight neonates receiving high concentrations of oxygen under pressure. Such infants are also subject to retinopathy, nervous system hemorrhage, and bowel disorders. Under the rationale that oxygen radicals could form in such patients and that a scavenging defense would likely be primitive, Rosenfeld et al. conducted a randomized, blind trial of subcutaneous SOD in 45 neonates (301). Bovine SOD was administered every 12 hours by injection, and subjects were monitored for both their requirement for continued oxygen therapy as well as the development of clinical and/or radiologic findings of BPD. The trial seemed to be quite encouraging. By a variety of parameters in

both groups, the SOD trial appeared to have been most effective. Detectable serum SOD levels were noted 2.5 hours after injection, persisting for several hours, and no toxicity was observed. However, there are also some obvious conceptual problems in the Rosenfeld approach. One is the disparity between their observations of detectable circulating SOD for several hours versus the very short half-life of SOD expected from the rat studies. Secondly, it is hard to visualize SOD in serum effectively trapping radicals at the level of pulmonary epithelial (or endothelial) cells. Finally it might be noted that there is a possible role for iron as a contributor to neonatal oxygen pathology, based on the decreased ceruloplasmin and transferrin levels in such patients (302).

Other pulmonary conditions also suggested a potential therapeutic role for SOD. A recent study of radiation-induced pulmonary damage in rats, following a single dose of 30 Gy to the thorax, concluded that intraperitoneally administered SOD increased the proportion of rats surviving, and reduced the extent of pulmonary fibrosis (303). Acute pulmonary injury in rabbits induced by activated leukocytes was also suppressed by SOD (304). In dogs, the pulmonary edema induced by α-naphthylthiourea was inhibited with SOD by preventing an increase in capillary permeability to plasma proteins (305).

2.6.3.3. SOD in Ischemia/Reperfusion

Since many laboratories have reported the successful reduction of infarct size by SOD after ischemia and reperfusion in animal models (306,307), SOD may become the first of the antioxidants to be used in clinical trials in patients undergoing reperfusion by thrombolysis or percutaneous transluminal

angioplasty of acute myocardial infarction. However, the initial enthusiasm for SOD as a means of attenuating the damage to the heart from sustained ischemia has unfortunately been tempered by recent conflicting results in laboratory animal models of myocardial reperfusion injury. The reports have been mixed with approximately half showing little or no effect and the other half exhibiting excellent protection (308,309,310,311,312,313,314). Several possible reasons may explain the disparate results as discussed below.

(a) Marker of Cell Viability

Triphenyl tetrazolium chloride (TTC) was used to demarcate viable myocardial tissue in some reperfusion experiments, while in other experiments, the infarcts were inspected by histological techniques. TTC is thought to stain viable tissue because it is reduced by dehydrogenases and NADH in the tissue (315). Following ischemic injury, reperfusion apparently washed the enzymes and cofactors out of the tissue quickly because the permeability of both the sarcolemma and the capillary has been increased (316). Thus, necrotic tissue loses its ability to stain with TTC. However, recently some investigators have proposed that using TTC staining as the only marker of cell viability was unreliable, especially when SOD was used as an antioxidant and the ischemic tissue was reperfused for a short period (317). A likely explanation was that SOD appeared to retain the TTC staining ability of necrotic tissue by presumably preventing an increase in capillary permeability (318). SOD has been shown to very effectively preserve capillary permeability following an ischemic insult in the kidney (319), intestine (320), and lung (321). Therefore if the capillary integrity was preserved by SOD, then enzymes and cofactors released from the dead cell might persist for a longer period of time in the interstitial space and react with TTC resulting in the overestimation of the healthy tissue (318). Instead,

histological staining delivered a sharp resolution of the borders between necrotic and viable tissues, and the extent of necrosis and histological evaluation was correlated. Histology thus appears to be an adequate marker for gross inspection of extent of necrosis (322).

(b) Baseline Predictors

The dependance of myocardial necrosis on collateral flow is believed to be a linear, inverse relation and is considered to be a critical factor in the ultimate determination of the extent of infarct size (323). However, several SOD trials of dog models from which positive results were reported did not control for the effect of collateral blood flow (306,307,324). Thus in some of these studies, the treatment and control groups may not have had equivalent degrees of ischemia. If animals with higher collateral blood flow were inadvertently assigned to the treatment group and animals with lower flow were assigned to the control group, in the absence of measurement of collateral flow, difference in infarct size between two groups would be falsely attributed to an effect of treatment (325).

(c) Reperfusion Technique

It is possible that the technique by which reperfusion was induced could modify the amount of free radicals produced. In this regard, reperfusion through a critical stenosis (one that limits reactive hyperemia but does not compromise normal blood flow) has been used in studies with positive results in an attempt to model the situation in patients reperfused with thrombolytic therapy (306,307,311). In contrast, some studies with negative results reperfused abruptly, allowing full reactive hyperemia to develop. Richard et al. suggested that critical stenosis might exacerbate reperfusion injury either by limiting the washout of toxic metabolites such as hypoxanthine and xanthine, or by

increasing neutrophil aggregation in the previously ischemic tissue (322). Therefore, the reperfusion technique shows another potential source of difference between the studies with positive and negative results.

(c) Duration of Reperfusion

The duration of reperfusion is likely to be a significant factor in the disparate results. In most cases, infarct size limitation was observed when reperfusion lasted up to 6 hours (307,310,324), whereas with a reperfusion period of 1-7 days, no limitation of infarct size was observed (312,313,326). The possible explanations are: (a) in the experimental studies with 1-7 days reperfusion, the SOD was usually infused at first 1 hour of reperfusion. Since the half-life of SOD in circulation is less than 30 minutes (312,327), the adequate levels of SOD in vivo in those studies might only protect against the "burst" of superoxide from xanthine oxidase, mitochondrial leak, and early neutrophil-produced superoxide. SOD levels might have not been sufficient for more sustained sources of superoxide such as arachidonate metabolism, continuous infiltration and persistant production of free radicals by neutrophils (328,329); (b) there are multiple mechanisms of reperfusion injury. Even if SOD could scavenge "all" of the superoxide from all the sources, other mechanisms with later action such as neutrophil degranulation, progressive capillary plugging, edema, and protease effect may cause an equivalent degree of injury in longer periods of reperfusion (330,331); (c) treatment with SOD only delays free radical-induced cell death. The initially salvaged tissues undergo necrosis later in the reperfusion phase after the SOD level decreases below their therapeutic levels (delayed reperfusion injury). This possibility warrants further investigation.

(d) Potential Toxicity of SOD

Recent studies have shown that the effect of SOD on reducing the incidence of ischemia-reperfusion damage exhibits an asymmetric U-shaped curve, i.e. SOD is protective over a range of doses, but low and high doses beyond this range are ineffective (417). Furthermore, some studies have reported that high dose SOD tends to exacerbate the free radical damage, i.e. high dose SOD may be toxic (418). A likely interpretation of SOD toxicity has not been available. However, some hypotheses and our work related to this topic will be discussed in section 6.

2.6.3.4. Implications

From the above discussion, it is clear that the design of future laboratory trials of SOD should consider: (a) advantages of employing prolonged infusion of SOD or special preparation of SOD with a long half-life to eliminate oxygen free radicals; (b) antioxidant effects on different sources of free radicals with initial or sustained duration; (c) experimental protocol and all appropriate covariates including the time of administration of SOD relative to the period of ischemia, the dosage of SOD, the route of administration and the method used to assess tissue damage.

2.7. Modification of Enzyme

2.7.1. Problems Associated with the Use of Enzymes in Medicine

In recent years it has been suggested that superoxide dismutase as well as other enzyme preparations could play a greater role in medicine (332).

However, the everyday clinical use of enzymes has been hindered by a number of factors. One such limitation has been the short duration of enzyme activity once introduced into the organism under physiological conditions. For instance, SOD has a half-life in the circulation that has been reported to be as low as 5 minutes. This limitation has increased greatly the quantity of preparation to be used in the course of treatment. Another limitation has been the antigenic properties of enzymes that appear as foreign proteins to the organism and may increase the problems of immunogenicity, and thus limit the amount or frequency of administration of the enzyme. Other limitations include possible nonspecific toxicity, enzyme accessibility to the site of substrate accumulation, and the high price and shortage of particular enzymes suitable for therapeutic use.

The problems to be solved in paving the way for wider use of enzymes in therapy are basically to decrease the total dose of the enzyme in use, to counter the problem of any potential immunogenicity of enzyme, and to increase the duration of enzyme activity in vivo. It is now evident that modification of enzymes by covalently binding to biological macromolecules may solve these problems, particularly in regard to retarding bioinactivation, and avoiding immunological consequences due to administration of a foreign protein (372).

2.7.2. Approach to Modify Enzyme for Medical Application

There are two general ways of modifying enzymes and specifically binding them to macromolecules (carrier molecules); (a) water-soluble enzyme derivatives or chemical modification and (b) water-insoluble and biodissolving

particles. If the enzyme is intended for long term circulation in vivo to affect macromolecular substrate having no strict localization, it is reasonable to utilize water-soluble enzyme derivatives. These derivatives differ from the native enzyme by having higher stability against denaturation and lower clearance rates in vivo. In other cases where the enzyme is necessary only in certain locations, for example, in local thrombosis like lesions, enzyme derivatives may be made of biodissolving particles that can easily localize in the lesion zone and discharge the active material at a given rate. We chose to produce a water-soluble SOD derivative based on the consideration that SOD should be able to attenuate oxygen radicals generated from various sources and different locations within the organism. In the following section, the characteristics of water-soluble enzyme derivatives will be reviewed.

2.7.2.1. Water-Soluble Enzyme Polymer

When using a native soluble enzyme in the organism, the instability of the enzyme over long periods can be disadvantageous. Using a water-soluble enzyme polymer can overcome this disadvantage because carrier molecules with high molecular weight will greatly increase the stability of enzyme in vivo by slowing down the loss of enzyme in metabolism and providing an improved electrostatic microenvironment around the native enzyme (333,334).

The first reports on water-soluble enzyme polymers were by Katchalski and Sela in 1958 (335), who had prepared them in order to elucidate the interrelationship between the electrostatic potential of the polymer chain and the displacement of the optimal pH of the enzyme. In other reports (336,337), the

water-soluble enzyme polymers have been prepared in order to increase the effective molecular size of the enzyme to prevent its release from membrane dependent devices, and to improve the mechanical properties and operational stability of the enzyme.

Generally speaking, the macromolecules that can bind enzymes and thereby improve enzyme properties range from synthetic polymers (poly-L-lysine), to biological macromolecules like albumin and immunoglobulins, to lipoprotein particles, to intact viable cells (leukocytes), the list seems to be almost endless. Dextrans, due to their high biocompatibility and easy utilization by organisms, are effective natural enzyme carriers. For example, α-amylase or catalase. bound to dextrans activated by the traditional cyanogen bromide method, have significantly increased their circulation life in experimental animals as compared to the native enzyme (338). Abuchowski and Davis have succeeded in covalently attaching polyethylene glycol (PEG) to different enzymes and greatly diminishing both the immunogenicity and the antigenicity of the native enzyme (339,340,341). They have reported increased circulation lives for the PEG-enzyme for arginase, uricase, asparaginase and a number of other enzymes. In a number of cases although the modified enzyme yields appear poor, the advantages in terms of circulation lives and resistance to bioinactivation easily compensate for the loss in activity during the cross-linking process. Sela (342) demonstrated that multichain poly-DL-alanine preparations provided a large degree of protection against immune reaction to protein conjugated with the polyamino acid. Uren and Ragin used this property to prepare nonimmunogenic preparations of L-asparaginase that also had increased plasma life ranging from 4 hours for the native enzyme to 28 hours for the polymer conjugated enzymes, and the conjugated preparations were much more effective in lowering plasma

L-asparagine levels than equivalent amounts of native enzyme (343). Poly(N-vinylpyrrolidone) has been conjugated to β-D-N-acetyl-hexosaminidase A (344), resulting in a conjugated enzyme with a greatly enhanced circulation half-life. A number of other synthetic polymers have been used as enzyme carriers including polyvinyl alcohol (345), carboxymethylcellulose (346), polyacrylic acid and polymaleic acid (347). While these preparations exhibit certain advantages over native enzyme with respect to stability and resistance to biodegradation, no testing of either immunogenicity or antigenicity has been reported.

In contrast to the use of biologically inert carriers, it may be feasible to use carriers that by themselves show biological activity or which may be able to accentuate the action of the conjugated enzyme. Examples of such carriers include fibrin (348) and collagen (349), which are essentially void of antigenic properties. The natural anticoagulant heparin is a rigid polysaccaride macromolecule containing a sugar residue which can be used for enzyme binding via periodate oxidation or cyanogen bromide activation. Heparin is suitable for the modification of enzymes intended for thrombolytic therapy such as urokinase, since the patients undergoing such therapy often are given heparin simultaneously. Studies have shown that after conjugating urokinase with heparin, the urokinase polymer exhibited increased thermostability, resistance to inhibitor and prolonged circulation half-life in rats (350).

2.7.2.2. Principles of Cross-Linking Reaction

The modification of an enzyme by covalent attachment to a macromolecule must involve functional groups of the enzyme that are not essential for catalytic action, and therefore no reagent must be used which could affect the binding

and catalytic sites of the enzyme. In order to achieve higher activities in the modified enzyme and prevent inactivation reactions with the essential amino acid residues of the enzyme active site, a number of methods have been devised (351): (a) covalent attachment of the enzyme to the carriers in the presence of a competitive inhibitor or substrate; (b) making a reversible covalently linked enzyme-inhibitor complex before conjugating to the carrier; (c) using a chemically modified soluble enzyme whose covalent linkage to the carrier is achieved by newly incorporated residues; (d) covalent attachment of a zymogen (enzyme precursor) to the carrier molecules, then converting the zymogen to an active enzyme form.

Most of the couping processes involve the reaction of a carbonyl group of the coupling reagent with nucleophilic groups of the protein (amino, thiol, or hydroxyl). In terms of nucleophilic reactivity and stability of the resulting bond, the most convenient residues of protein for involvement in coupling reactions are, in descending order, L-lysine, L-cysteine, L-tyrosine, L-histidine, L-aspartic acid, L-glutamic acid, L-arginine, L-tryptophan, L-serine, L-threonine, and L-methionine (352). The majority of carriers have hydroxyl, amino, amide, or carboxyl groups which require activation before they are used for a coupling reaction. The bonding of enzymes to soluble carriers may be achieved by one of the following procedures: reactions of the enzyme with an activated soluble carrier, reaction of the enzyme with an activated insoluble carrier followed by solubilization of the enzyme polymer, or copolymerization of monomers with enzyme. The covalent coupling reactions between enzyme and carriers which have been developed to date can be mainly classified into the following: (a) diazotization; (b) amide bond formation; (c) alkylation and arylation; (d) Schiff base formation; (e) amidination reactions; (f) thiol-disulphide interchange.

2.7.2.3. Albumin: a Natural Enzyme Carrier

The idea that using albumin, a natural plasma protein, as an attractive enzyme carrier was proposed by Thomas and colleagues in 1974 (353). They demonstrated that covalent binding of an excess of albumin with either hog liver uricase or Escherichia coli L-asparaginase to form a soluble polymer with significantly increased resistance of the polymer to heat denaturation. These studies have been greatly extended by M. Poznansky in the 1980's. The initial studies in Dr Poznansky's laboratory served primarily to determine if the use of homologous albumin might serve to: (a) reduce the immunogenicity of foreign proteins (enzymes) and thus reduce the risk of hypersensitivity reactions and (b) increase the ability of the enzyme to remain in the circulation following intravenous administration. Both of these objectives were met as polymers of hog liver uricase and albumin were shown to be non-immunogenic and non-antigenic when compared to the native enzyme (354). At that time, they were careful to point out that the reduced antigenicity following cross-linking with homologous albumin was limited to the enzyme uricase. However, later Poznansky and co-workers conjugated a number of different enzymes with albumin, and in each case similar characteristics have been demonstrated, which included increased resistance of the polymer to heat denaturation and to proteolytic inactivation, prolonged circulation half-lives, and reduced immunogenicity of the enzymes while retaining enzyme activity (355,356,357,358 359).

In this thesis, our research project has continued this idea that water-soluble enzyme polymers can serve a specific and useful purpose. We have also chosen albumin as a carrier for SOD in an attempt to improve the therapeutic efficiency of SOD. The choice stems from a number of specific advantages that albumin affords: (a) albumin is a natural and abundant plasma protein and so its conjugation to other proteins may allow the foreign enzymes to be accepted into the plasma seemingly as simply another plasma protein; (b) albumin has a relatively long circulation time with a turnover rate in the order of 30 hours; (c) albumin is inexpensive, readily available and it has multiple reactive sites on which to attach enzymes; and (d) the cross-linking reaction between albumin and enzyme is relatively gentle (using either glutaraldehyde or water-soluble carbodiimide) at physiological pH.

2.8. Research Objectives

Since oxygen free radicals have a deleterious effect on macromolecules, subcellular components, cells and tissues, it seems logical that oxygen free radical scavengers, such as SOD and catalase, could be used to prevent the tissue injury under certain pathological conditions. However, therapeutic application of SOD and other scavengers at the present stage is not generally successful . The reason for this is that some crucial questions related to SOD therapy are waiting to be answered. These include (a) how can the circulation half-life of superoxide dismutase be extended? (b) do oxygen free radicals act only where they are produced or can they diffuse across cellular membranes to attack other cellular components and/or neighboring cells? (c) can SOD be toxic under certain conditions and what is the optimal dose of SOD? (d) what

factors influence the conversion of superoxide radical to the very toxic hydroxyl radical and how can this conversion be suppressed ?

The objectives of the our research have mainly addressed above questions. The main experimental approaches of our work include: (a) extending SOD half-life in the circulation by cross-linking SOD with albumin and characterizing the SOD-Albumin polymer; (b) encapsulating SOD into liposomes and erythrocyte ghosts to examine the permeability of oxygen free radicals through lipid bilayers and biological membranes; (c) using electron spin resonance and spin trapping techniques to directly examine behavior of oxygen radicals from different sources and determine the factors that facilitate the conversion of the superoxide radical to hydroxyl radical; (d) elucidating the mechanism underlying the apparent SOD toxicity; (e) investigating the properties of a new SOD-CATALASE conjugate in both in vitro and in tissue models.

3. Improving the Pharmacological Properties of Superoxide Dismutase by Conjugation with HSA

3.1. Introduction

Superoxide dismutase was first reported in 1968 by McCord and Fridovich (188). The biological function of the enzyme is the dismutation of the superoxide free radical to oxygen and hydrogen peroxide which in turn can be reduced to oxygen and water by the enzyme catalase. Superoxide dismutase is a ubiquitous enzyme found in all tissues in two major forms: as an intracellular enzyme found free in the cytoplasm and a second form which exists as part of the extracellular matrix (360).

The therapeutic potential of exogenously administered SOD for the prevention of oxygen radical damage has been given a great deal of attention (361). Although work with SOD as a free radical scavenger has been pursued in the treatment of models of radiation damage, atherosclerosis, skeletal muscle ischemia (363) and others, its role as an agent to protect against postreperfusion injury following myocardial infarction has perhaps received the most attention (307,362). However, the reports have been mixed with disparate results (313). It would appear that the major discrepancies can be attributed to the time of administration of the SOD relative to the period of ischemia and to the rapid clearance of the SOD from the circulation following its administration. A more recent suggestion has been that the dose of SOD is also critical, SOD at a high dose tends to exacerbate the oxygen free radical damage (418).

In this part of our work, we have attempted to alter the properties of SOD to make it more amenable to use as a therapeutic agent. Our approach has been to modify the SOD by cross-linking it with an excess of human serum albumin, thereby altering its size and subsequently its mode and rate of clearance from the circulation and altering its degree of immunogenicity by possibly masking its antigenic site with the non-immunogenic albumin. This approach has proven to be successful and the SOD-Albumin polymer we have prepared has greatly increased circulation half-life and greatly reduced immunogenicity compared to the native enzyme.

3.2. Materials and Methods

3.2.1. Materials

Yeast superoxide dismutase was the product of Carlsberg Biotechnology Ltd (Copenhagen, N. Denmark). On SDS-PAGE, SOD exhibited a single band with an apparent molecular weight of 33,000 daltons which was confirmed with molecular sieve chromatography using Sephadex G-100. $Na^{125}I$ was provided by the Edmonton Radiopharmaceutical Center (Edmonton, AB Canada). Iodo-beads were purchased from Pierce Chemical Company (U.S.A.). Sprague-Dawley rats and Balb C mice were provided by the Animal Services, Health Sciences Laboratory (University of Alberta, Canada). Sephadex G-150 and Gel Filtration Calibration Kit were from Pharmacia Fine Chemicals (Sweden). Human serum albumin, 25% glutaraldehyde, ferricytochrome c, xanthine, xanthine oxidase and other reagents were purchased from Sigma Chemical Company (St. Louis, MO U.S.A.).

3.2.2. SOD Activity Assay

The reduction rate of ferricytochrome c by superoxide radicals was monitored by measuring the absorbance at 550 nm utilizing the xanthine/xanthine oxidase system as the source of O_2^- (188). The assay reaction was carried out at 25° C in 3 ml of 0.05 M potassium phosphate buffer, pH 7.8 containing 50 μM xanthine, 0.01 mM ferricytochrome c, 0.1 mM DETAPAC and sufficient xanthine oxidase to give a ferricytochrome c reduction rate of 0.025 absorbance unit per minute at 550 nm without SOD (≈6 nM). Addition of SOD would compete for superoxide radicals and decrease the reduction rate of ferricytochrome c. One unit of SOD was defined as that amount of enzyme which inhibited the rate of ferricytochrome c reduction by 50% (i.e. to a rate of 0.0125 absorbance unit per minute). $\Delta A\ min^{-1}$ was calculated from the linear part of the reaction.

3.2.3. Radioiodination of SOD

Radioiodination of SOD was carried out according to the method of Markwell (364) as follows. Iodo-beads which consist of the oxidant N-chloro-benzenesulfon-amide immobilized on 2.8 mm diameter nonporous polystyrene spheres were washed in PBS, and then blotted dry on filter paper. To a mixture of SOD and $Na^{125}I$, the iodination was initiated by the addition of the iodo-beads and allowed to proceed for 20 minutes at room temperature in an iodination hood. The reaction was stopped by adding unlabelled NaI and the reaction solution was loaded on a Sephadex G-25 column for separation of free ^{125}I and

the ^{125}I-SOD. Invariably, a significant amount of loosely bound ^{125}I remained associated with the SOD which was subsequently removed by extensive dialysis against a solution containing 0.01 M PBS, 0.01M unlabelled NaI and 0.9% NaCl at pH 7.4. The specific activity of the labelled SOD was determined by mixing 10 µl of the SOD solution with 1 ml of 0.1% (w/v) bovine serum albumin, precipitating all of the proteins by the addition of cold trichloroacetic acid to a final concentration of 10% (w/v), and determining the amount of radiolabel associated with the precipitated protein and that associated with the supernatant.

3.2.4. Preparation of SOD-Albumin Polymer

SOD-Albumin polymers were prepared as previously described (365,371). 5 mg of SOD was dissolved along with 50 mg of human, bovine, or mouse serum albumin in 5 ml of 0.1 M PBS at pH 7.0. The mixture was brought to 4°C and 15 µl of fresh reagent grade 25% glutaraldehyde was added and the reaction allowed to proceed with stirring for an additional 5 hours. The reaction was stopped by the addition of 50 mg of glycine.

The preparation was dialyzed overnight (with 2 changes) against 0.02 M PBS containing 0.15 M NaCl and 0.1% glycine prior to separation of native SOD and SOD-Albumin polymer by Sephadex G-150 column chromatography which was standardized with ferritin, catalase, aldolase, and bovine serum albumin. Fractions were collected and assayed for protein (366), SOD activity and ^{125}I if ^{125}I-SOD was used. The appropriate fractions (m.w. 200,000-330,000) were further concentrated using an Amicon ultrafiltration cell with an XM100A

(100,000 m.w. cutoff) filter membrane. In an attempt to stabilize the Schiff base following the glutaraldehyde reaction, 100 µl of 0.5 mM sodium borohydride was added to the reaction mixture just prior to the addition of glycine. However, we did find any significant difference in the properties, stability or activity of the SOD-Albumin polymer using borohydride and so far all of the experiments reported in this thesis only glycine was used to quench the cross-linking reaction.

3.2.5. Animal Experiments

All experiments involving the pharmacokinetics of SOD and SOD-Albumin polymer were performed in Sprague-Dawley rats under anaesthesia with Inactin. One jugular vein was cannulated for the injection and one femoral vein was cannulated for sampling. After injection of ^{125}I-SOD or ^{125}I-SOD-Albumin, blood samples were taken as desired and assayed for enzyme activity and ^{125}I radioactivity. At the designated time, rats were sacrificed and the tissue ^{125}I was determined.

Immunological experiments to test the immunogenicity of the native SOD and its polymer were performed in Balb C mice. The mice were given subcutaneous injections of the SOD (500 µg of the SOD in each injection dissolved in 100 µl of buffer). Injections were made in three sets of three on days: 1, 2, and 3; 8, 9, and 10; and 16, 17, and 18. The first series was given in combination with Freund's Complete Adjuvant and the second two series in plain buffer. The mice were bled on day 25, and the antisera were examined for antibodies to SOD by ELISA technology.

3.2.6. SOD ELISA Assay

Enzyme-linked immunosorbentassay (ELISA) exploits the interactions of antigens with antibodies for measuring proteins. The assay is carried out by immobilizing one of the components of the assay on a substrate and using an enzyme-linked detection system for quantitative results. In most cases ELISA is very sensitive, since enzymatically catalyzed reactions can proceed to near completion and in principal the signal generated by even one molecule of enzyme can be amplified by providing sufficient substrate.

In our assay, native SOD was bound to a solid phase, generally a well in a polystyrene microtiter plate, by passive absorption (367). The unbound SOD was washed away with PBS-Tween and the binding capacity of the wells were further saturated by goat serum. The bound SOD was then exposed to the SOD or SOD-Albumin immunized sera with different dilutions. Normal mouse serum was used as negative control. After incubation of SOD with antisera at room temperature for 1 hour, the wells were washed with PBS-Tween. The bound antiSOD antibody was detected by adding an enzyme-linked second antibody developed in goat (horse radish peroxidase-antimouse Ab conjugate). After incubation for 1 hour, the excess conjugate was washed away with PBS-Tween. The substrate solution containing 2,2'-azinobis[3-ethylbenzthiazolinesulfonic acid] (ABTS), citric acid and H_2O_2 was then added to the wells for development and quantification. The antiSOD concentration was obtained by reading the absorbance at 405 nm in a spectrophotometer.

3.3. Results

3.3.1. Chromatography Profile of SOD-Albumin Polymer

Glutaraldehyde is an effective reagent for covalent linking proteins. The reaction works by Schiff base formation between the aldehyde group on the glutaraldehyde and the primary amino group on the enzyme at the right acidity. For most enzymes, primary amino groups are often associated with catalytic function, therefore substrate is to be added during the coupling reaction to protect the enzyme active site. However, the active site of SOD is mainly formed by the imidazolate rings of L-histidine residues that bridge copper and zinc (368,369). Following our cross-linking reaction recipe (5 mg SOD : 50 mg albumin, 15 µl glutaraldehyde, 5 hours reaction time), all the SOD activity has been retained.

Figure 1 demonstrates the data from molecular sieve chromatography (Sephadex G-150) of the SOD-Albumin polymer containing a 1:5 ratio of SOD to human serum albumin. Note the coincidence of SOD activity as measured in either enzyme units or radiolabel (^{125}I). There was also no suggestion that the polymer contained any free SOD. This particular preparation has not undergone any previous purification by either molecular sieve chromatography or pressure ultrafiltration. The peak activity of the polymer (fraction #30) coincided with a molecular weight of 295,000 daltons. The elution peak for the free SOD eluted at molecular weight of 32,000 daltons, very close to the established molecular weight. All of the subsequent experiments were performed with SOD-Albumin polymer that had been already purified by molecular sieve chromatography.

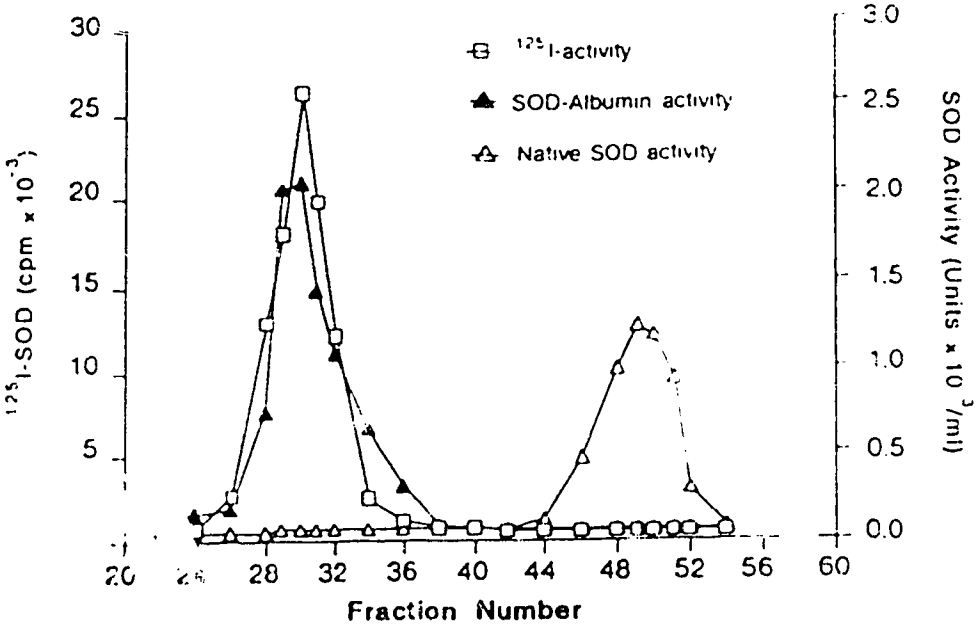


Figure 1: Molecular Sieve Chromatography of SOD-Albumin polymer. The Sephadex G-150 column was standardized using ferritin, catalase, aldolase and bovine serum albumin. Each fraction contained 3 ml of eluant. Units were expressed as ^{125}-I (cpm/ml) or SOD activity (units/ml).

The molecular weight of polymer is determined by the factors such as the amount of cross-linking reagent, ratio of reaction components, and reaction time. Larger molecular weight polymers can be obtained by increasing the ratio of albumin to enzyme and the concentration of glutaraldehyde at a constant reaction time.

3.3.2. Comparison of Pharmacokinetics of Native SOD and SOD-Albumin Polymer

Figure 2 and table 4 demonstrate some of the pharmacokinetics of native SOD and SOD-Albumin polymer. The altered clearance rates of SOD from the circulation following intravenous administration was most striking. Whereas native SOD had a $t_{1/2}$ for clearance as little as 10 minutes, the SOD-Albumin polymers extended the clearance rates ($t_{1/2}$) to about 4 hours. We have essentially identical data for the clearance in terms of removal of ^{125}I and actual SOD activity from the circulation following the administration of rather substantial amounts of the enzyme in either free or polymer forms.

Table 4 indicates the sites of uptake of the SOD and SOD-Albumin polymers. The native SOD was largely removed from the circulation 30 minutes following its intravenous injection and a substantial amount was found associated with the kidney and urine. This probably reflected the fairly low molecular weight of the native SOD and its ability to pass the glomerular endothelium. The SOD-Albumin on the other hand appeared to be cleared largely by the liver. The very small fraction of the radioactivity taken up by the thyroid indicated that little free ^{125}I was present in the injected material.

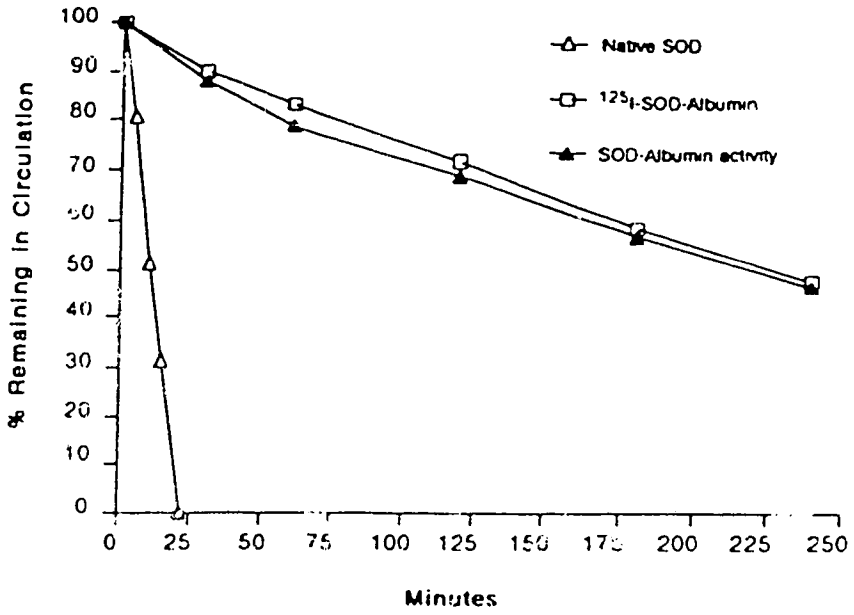


Figure 2: Pharmacokinetics of SOD and SOD-Albumin in Sprague-Dawley rats weighing between 250 and 350 gm. One femoral vein and one jugular vein were cannulated for sampling and injection. We routinely use 2 million cpm ^{125}I activity and a protein load of between 1 and 2 mg depending on whether the SOD was being administered alone or in the polymer form. Samples were taken at regularly intervals and the animal was then sacrificed. All counts were made directly in a Gamma Counter. We also precipitated the blood samples with 10% TCA in order to determine whether the ^{125}I label was in a free or precipitable (protein) form.

Table 4

Tissue Distribution of SOD and SOD-Albumin Polymers

	SOD	SOD-Albumin Polymer	
	30 min	30 min	300 min
Tissue			
Blood	8	81	48
Liver	6	8	23
Kidney(s)	30	1	2
Urine	14	-	2
Spleen	2	1	3
Heart	1	-	-
Lung	3	2	2
Muscle	2	-	1
Thyroid	1	-	3

^{125}I-SOD in the free (M.W. 32,000) or polymer form with albumin (M.W. 250,000) was injected into 250 gm male Sprague-Dawley rats kept under anesthesia using Inactin. Blood samples were drawn at regular intervals (Figure 2). At the designated time, rats were sacrificed and tissue ^{125}I was determined.

3.3.3. Resistance to Hydrogen Peroxide Inhibition

Since H_2O_2 is a powerful inhibitor of SOD (370) and many of the preparations examining the effect of SOD on ischemia had been tested in closed systems where H_2O_2 might be expected to accumulate (in the absence of sufficient amount of catalase), we have examined the effects of varying the concentration of H_2O_2 on the enzyme activity (Figure 3) . As expected, H_2O_2 was found to be an effective inhibitor of SOD, but the concentration required for 50% inhibition increased from 0.12 mM to 0.68 mM when SOD was used in the polymer form with albumin. A possible explanation is that large albumin molecules form a "shell" around the SOD active site and thereby effectively preventing the histidine residue and copper ion in the active site from being oxidized by H_2O_2. When SOD was polymerized with catalase, it was not surprising to find that the hydrogen peroxide was without an inhibitory effect probably because the H_2O_2 was immediately being converted to water and oxygen without being able to exert its effect on SOD.

3.3.4. Storage Properties of Enzyme Preparations

Table 5 demonstrates some of the storage properties of free SOD and SOD-Albumin polymers under a variety of storage conditions. SOD itself is a very stable enzyme when compared to most others and free SOD retained up to 75% of its initial activity even after being stored for up six months at 4° C. The enzyme was even more stable when stored frozen at -20° C or under lyophilized conditions stored at room temperature or even better at -20° C. Each of the preparations of SOD-Albumin polymers had much superior storage stability, retaining virtually 100% activity when stored either frozen or lyophilized and

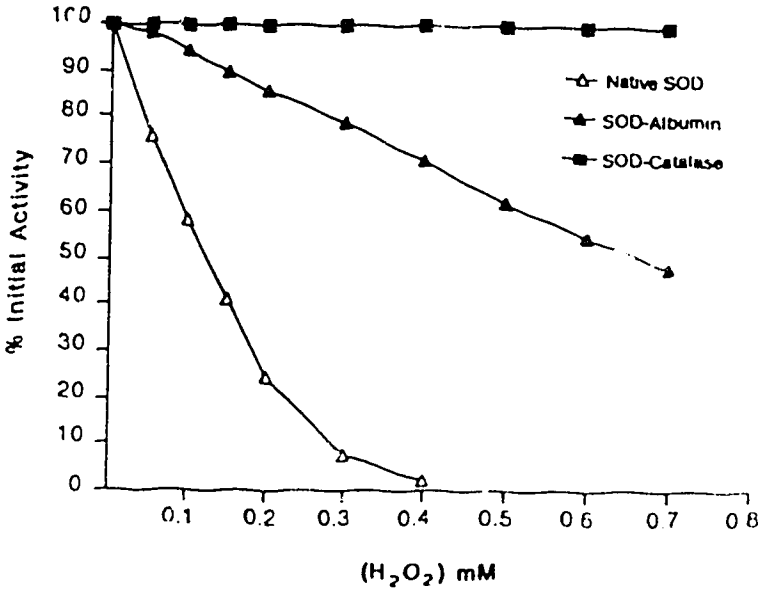


Figure 3: The effect of increasing concentration of hydrogen peroxide on SOD activity was measured using free SOD or SOD polymerized with either albumin or catalase. Solutions were made up with the normal buffer, substrates and various amount of hydrogen peroxide were added prior to the addition of the SOD and the initaiation of the normal timed reaction. Very similar results were also obtained if the enzyme preparations were incubated with the hydrogen peroxide prior to the addition of the substrate.

Table 5

Storage Properties of Enzyme Preparations

Enzyme Preparation	Activity (units/mg Protein)		
	Time 0	1month	6month
Free SOD Solution (4°C)	6200	5600	4650
Free SOD Solution (-20°C)	6100	5850	5600
Free SOD (lyophilized, RT)	6000	5875	5775
Free SOD (lyophilized, -20°C)	6000	5875	5775
SOD-Albumin (4°C)	775	770	760
SOD-Albumin (-20°C)	780	780	765
SOD-Albumin (lyophilized, RT)	750	745	735
SOD-Albumin (lyophilized,-20°C)	740	745	735
	Retention of Activity		
α-Glucosidase Solution (4°C)	100%	5%	–
α-Glucosidase Solution (-20°C)	100%	45%	–
α-Glucosidase-Albumin Sol'n (4°C)	100%	50%	–
α-Glucosidase-Albumin Sol'n (-20°C)	100%	85%	–

The α-Glucosidase-Albumin polymers were prepared as described in reference 371.

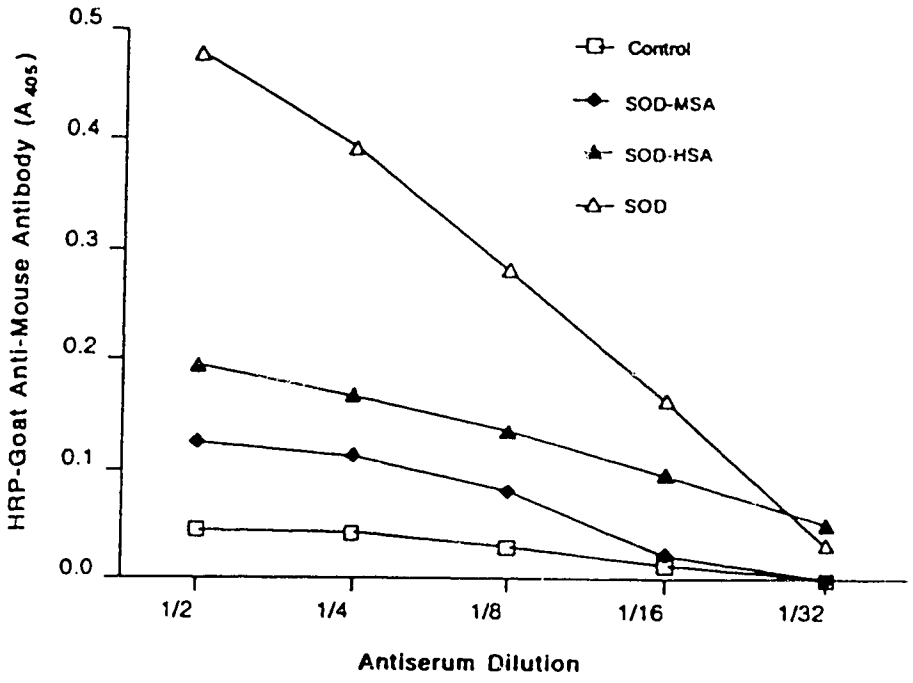


Figure 4: Standard enzyme-immunoassay of sera from mice which has been immunized under standard protocol with SOD,SOD-HSA, SOD-MSA and controls over a period of 4 weeks. Microtiter wells were coated with SOD for 24 hours at 4°C, washed with buffer and detergent and then exposed to the various antisera for an additional 2 hours at 37°C after which they were washed extensively before the addition of the enzymelabelled goat anti-mouse antiserum. The plate were then rewashed and the amount of attached antibody was measured by reading at 405 nm.

90-95% activity when stored in liquid form at 4° C. These values were compared to the storage properties of the enzyme α-glucosidase. The loss of enzyme activity of α-glucosidase was 95% when stored at 4° C for 1 month (55% loss when stored frozen) while only a 45% loss of activity when in the polymer form with albumin at 4° C and only a 15% loss when stored frozen.

3.3.5. Immunogenicity

Figure 4 presents some of the immunological data from mice that had been given repeated injections of native SOD, SOD-Mouse serum albumin (MSA) polymers and SOD-Human serum albumin polymers. Sera were collected from groups of five mice (along with control mice) and tested for antibodies against SOD using ELISA techniques. The SOD was clearly immunogenic whereas the SOD-HSA was much less so (identical amounts of SOD were given to the mice in each case) and the SOD-MSA injected mice demonstrated an antibody titre to SOD which was much closer to the control mice which did not receive SOD in any form. The explanation for the difference in immunogenicity between SOD-HSA and SOD-MSA in mice is addressed in the discussion.

3.4. Discussion

It is suggested that SOD in its native form may have limited therapeutic potential in light of its very rapid clearance from the circulation following intravenous administration largely as a result of its removal by the kidney and loss in the urine. The limited time frame for its activity in the circulation may serve to explain the many failures of SOD to exert protection where free radicals

were thought to influence pathology. In the case where SOD has been suggested to protect against reperfusion injury following a myocardial infarction, the window of opportunity has been suggested to be very narrow. It is suggested that the SOD has to be administered in a time frame which closely matches the period of maximum free radical formation. Clearly a mechanism to increase the effective life time of the enzyme would be of considerable interest.

It is of some interest that we have been able to extend the circulation life time of the SOD to the range of 4-5 hours since this is thought to coincide with the major period during which time post-reperfusion injury is thought to occur following myocardial ischemia (347,348). It might therefore be possible to administer the SOD as a single dose (in the polymer form with albumin) at the same time the perfusion is being reestablished either through surgical (angioplasty) or chemical means (TPA or streptokinase).

The other demonstrated properties of the SOD-Albumin polymer are of considerable interest from the point of view of the pharmaceutics. While the native form of SOD is in itself a very stable enzyme , studies have shown for this and other enzymes (371,372) that chemical cross-linking with an excess of homologous albumin renders the enzyme much more resistant to proteolytic degradation and invariably extends the shelf life of the enzyme significantly. In the case of SOD, the possibility of storing the enzyme-albumin polymer in a powder form at room temperature is a very significant practical advantage.

The ability to avoid inhibition of SOD by the product of its reaction (H_2O_2) by chemically cross-linking catalase to either SOD directly or through an albumin molecule may be of some practical interest. Although catalase is a fairly

ubiquitous enzyme present in both intracellular and extracellular compartments, it is clear that if levels of catalase are insufficient to rapidly remove accumulating H_2O_2, this product can potentially inhibit SOD as well as other cell functions. A greater understanding of the physiological significance of SOD as a therapeutic agent would be required before answering this question.

The ability of homologous albumin to render a foreign protein non-immunogenic has been demonstrated in this work. It is of some interest to note that in mice while the SOD-MSA polymers appear to be virtually non-immunogenic, the SOD-HSA polymers do elicit an immune response although to a much lesser extent than the free SOD. One possibility is that in addition to diminishing the antigenicity of the SOD by partly masking some of the antigenic determinants (following the chemical cross-linking) the homologous albumin (mouse but not human in this case) is acting as a toleragen for the attached hapten (SOD) thus rendering it non-immunogenic (332).

4. Detection of Oxygen Free Radicals by Electron Spin Resonance and Spin Trapping Techniques

4.1. Introduction

In order to make SOD therapy more effective, one should identify the sources and nature of free radicals generated in biological systems. Electron spin resonance (ESR) is a technique that permits the investigator to detect and, in favorable cases, to characterize molecules with unpaired electrons (free radicals) without altering or destroying the molecules. This chapter describeds our studies on the application of electron spin resonance and spin trapping techniques to detect and identify oxygen free radicals from different sources. We used the reaction of xanthine with xanthine oxidase as an enzymatic source and human neutrophils as a cellular source in vitro. The main objective of this study was to elucidate the nature of oxygen free radical species produced from these two sources. The following is a brief overview of electron spin resonance techniques and the production of oxygen free radicals by human neutrophils.

4.1.1. Electron Spin Resonance

Electron spin resonance (ESR) spectroscopy, like other forms of spectroscopy, monitors the net absorption of energy from a radiation field when molecules change their energy state. For the purpose of constructing a simple picture the electron can be thought of as a spinning negative charge. Since a moving charge generates a magnetic field, the axis of each spinning electron has been associated with a magnetic moment; that is, the electron acts like a tiny

bar magnet with a north and a south pole. When electrons are paired in chemical bonds or elsewhere, their spins are opposed and the associated opposite magnetic moments effectively cancel each other out. Most chemical and biochemical substances which contain only paired electrons have no net electron magnetic moments. But the "odd" or unpaired electron of a free radical will carry an uncanceled electron-spin magnetic moment. In the absence of an internal magnetic field, the free radicals and their spin magnetic moments are randomly oriented and are in the same average energy state. When an external magnetic field is applied, the "electron magnets" will become aligned either parallel or antiparallel to the external magnetic field and thus have two energy levels. If electromagnetic radiation of the correct energy is applied, some parallel electrons will absorb a quantum of radiation energy to "jump" from the lower to the higher energy states (antiparallel level) and some of the antiparallel electrons will flip over to the parallel state, thus releasing the same amount of energy to the electromagnetic field. Particles thermally distributed among quantized energy levels, however, always preferentially populate the more stable state. Therefore at resonance there are more parallel than antiparallel electrons; hence the resonance will give rise to a net absorption of energy from the field. It is this net absorption of electromagnetic energy at resonance that is detected and amplified to provide the sample signal in ESR spectroscopy.

4.1.2. Spin Trapping Technique

ESR is considered to be the least ambiguous method for the detection of free radicals. However, in many instances even when the concentration of free radicals exceeds those normally required for detection (10^{-8} M) (373), no ESR

signal is observed in an aqueous solution under physiological conditions. This is due to the very short relaxation time of the free radical resonance (due to strong 'spin orbital coupling') and the high reactivity of free radicals. Perhaps the best example of this is the hydroxyl radical, which reacts with itself or with most organic molecules at diffusion controlled rates (374). Thus it is only possible to detect stable free radicals which accumulate to measurable concentration or unstable radicals which reach a sufficiently high steady state concentration. A number of techniques are available to detect the presence of unstable radicals. One can use flow systems whereby the radicals are continuously generated in the spectrometer so as to maintain a steady-state concentration. With this means Yamazaki and Piette were able to detect the ascorbate semiquinone free radical in ESR studies of ascorbate oxidase (375). Another similar approach is to slow the rate of the radicals colliding with other species by rapidly freezing the sample. Such a "rapid-freezing" technique was used in 1969 by P.F. Knowles in England to identify the superoxide radical produced by the xanthine/xanthine oxidase system by observing its ESR spectrum (376). But this usually negates any physiologically significant kinetics and as well as structural aspects of the ESR spectrum. This technique is further limited by the concentration of the radical present before freezing. Another important approach to detect unstable free radicals is spin trapping. A highly reactive radical, difficult to observe by normal ESR method, is allowed to covalently react with a compound (spin trap) to form a long-lived radical which can be observed at room temperature using conventional ESR equipment. The hyperfine splitting of the spin adduct provides the identification and quantification of the original radical. Since the stable free radical accumulates, spin trapping is an integrative method and is more sensitive than procedures which measure only instantaneous or steady-state levels of free radicals (377).

The "ideal" spin trap agent should react rapidly and specifically with the radical, and produce a stable product that has a highly characteristic ESR spectrum. Nitrones and nitroso compounds are the spin traps most commonly used. With both of these traps, the adduct is a nitroxide free radical, a long-lived radical. Table 6 shows some of the trapping molecules that have been used. Nitrones have been the principal compounds used for spin trapping oxygen-centered free radicals, because the spin adduct derived from the reaction of nitroso compounds with oxygen free radical (e.g. hydroxyl radical) decomposes rapidly (378). Among the several nitrones used as spin traps, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) has received the most attention and has become the preferred spin trap for detecting hydroxyl and superoxide radicals in biological systems (379), since the ESR spectrum of DMPO spin adduct of superoxide and hydroxyl radicals are readily distinguished by their hyperfine splitting constants. The typical computer stimulation of ESR spectra of DMPO spin trapped adducts are shown in Table 6.

4.1.3. Production of Oxygen Free Radicals by Human Neutrophils

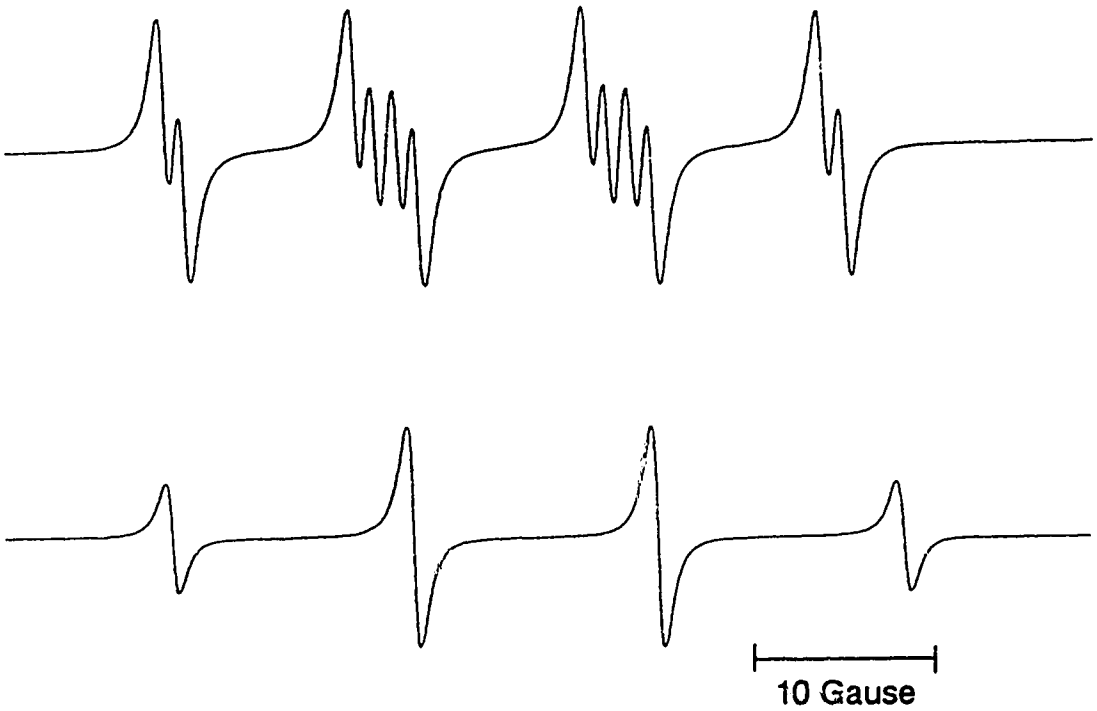
Neutrophils (polymorphonuclear leukocyte, PMN) in response to activation by particulate and/or specific soluble mediators, undergo a respiratory burst. This is associated with a 2 to 20-fold increase in oxygen consumption and increased glucose metabolism via the hexose monophosphate shunt. In conjunction with an increase in oxygen consumption, neutrophils have been shown to secrete superoxide radicals (380,381,382). The activating substances have been shown to include bacteria (383), opsonized zymosan (384), immune complexes (385), chemotactic peptides (386), synthetic oligopeptides such as N-formal-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe) (387), and phorbol myristate

Table 6

ESR Parameters of Various Spin Adducts

Spin Trap	Radical Trapped	A_N/G	A_b^H/G	A_d^H/G
DMPO	$O_2^-/HO_2\cdot$	14.3	11.7	1.25
DMPO	·OH	14.9	14.9	
DMPO	$\cdot CH_3$	16.4	23.3	
PBN	$O_2^-/HO_2\cdot$	14.8	2.75	
PBN	·OH	15.3	2.75	
PBN	$\cdot CH_2OH$	16.2	3.6	

Note: A more complete table is to be found in reference 377.



Computer simulation of ESR spectra of DMPO spin adducts. The splitting constants for DMPO-OOH (top spectrum) are: A_N=14.3 G, A_b^H=11.7 G, A_d^H=1.25 G; DMPO-OH (botterm spectrum) are: A_N= A_b^H=11.7 G.

acetate (PMA) a nonspecific membrane activator (388). The most potent of these activating agents are opsonized phagocytic particles and PMA.The enzyme system responsible for the increased oxygen consumption and superoxide radical generation of neutrophils has been identified as a membrane-associated NADPH oxidase. Although definitive data are not yet available concerning the precise location and biochemical characterization of the O_2^- forming oxidase system in neutrophils, there appear to be at least two identifiable components. The first is a substrate-binding moiety with specificity for reduced nicotinamide adenine dinucleotide; the second component oxidizes the reduced form in the presence of oxygen, generating O_2^-. Several investigators have suggested that the oxidase system of the neutrophil is located at least in part on the external surface of the plasma membrane, accounting for the release of the substantial amounts of O_2^- into the external environment after cell stimulation (389,390). The role of neutrophil-derived oxygen radicals in microbicidal and tumoricidal activity as well as tissue damage at sites of inflammation has been the focus of extensive investigation in recent years (391,392).

4.2. Materials and Methods

4.2.1. Materials

Bio-Rex ion exchange membrane and activated charcoal were from Bio-Rad laboratories (Richmond, Canada). Ficoll-Paque was the product of Pharmacia Fine Chemicals (Uppsala, Sweden). 5,5,-dimethyl-1-pyrroline-1-oxide (DMPO), phorbol myristate acetate (PMA) and other reagents were purchased from Sigma Chemical Company (St Louis, MO U.S.A.). Lytic solution for preparation

of neutrophils contained 8.2 g of ammonium chloride, 0.84 g of sodium bicarbonate, and 0.0292 g of EDTA in 1 L of water.

4.2.2. Methods

4.2.2.1. Purification of DMPO

DMPO as supplied usually contains colored impurities which yield ESR signals, thus requiring further purification. An activated charcoal method is used for this purpose (393). Briefly, the mixture of 1 g of DMPO and 3 ml of distilled water was shaken vigorously with 2 g of decolorizing activated charcoal for 1 hour. The suspension was then filtered and the filtrate was checked for any contaminating nitroxides by ESR. The activated charcoal treatment was repeated until the filtrate was clear of impurities. All work with DMPO was conducted in subdued light conditions. The final concentration of DMPO was analyzed by the absorbance of a small aliquot diluted in 95% ethanol at 234 nm, E_{234}=7700M^{-1} cm^{-1}. The purified DMPO was stored in small aliquots frozen at -20° C in dark brown bottles.

4.2.2.2. Preparation of Human Neutrophils

Heparinized venous blood was freshly obtained from a healthy donor and centrifuged at 850 rpm for 10 minutes at room temperature. Then most of plasma was discarded and 1/2 inch of plasma, buffy coat and approximately 1/4 inch below the buffy coat were removed into another sterile tube with a pasteur pipet. These cells were diluted 1:1 (v:v) with Hanks Balanced Salt Solution and

by gently jetting, some foaming appeared on the surface of the mixture. This foam and a portion of 3 ml of diluted cell mixture were layered on cushions of 4 ml of Ficoll-Paque and centrifuged at 1500 rpm for 30 minutes. The centrifuge was brought up to speed very slowly so that the cell layer was not disturbed. After centrifugation, the buffer-Ficoll interphase contained lymphocytes and monocytes, and the pellets contained neutrophils (polymorphonuclear cells) and contaminating erythrocytes. The pellets were collected and mixed with approximately 5 volumes of freshly prepared lytic solution until the erythrocytes were lysed. The solution was again centrifuged at 1500 rpm for 10 minutes at room temperature. Repeated lysis was sometimes required. The cell pellets were then washed and resuspended in the appropriate media to constituted a neutrophil concentration of 5×10^6 cells/ml.

4.2.2.3. Electron Spin Resonance (ESR)

Trace metals present in buffer solutions can be a real problem in spin trapping experiments because metal ions catalyze air oxidation of cyclic nitrones leading to the formation of paramagnetic species. These metals were removed by passing all buffers through a Chelex 100 column or a chelating ion exchange membrane. The metal chelating agent, diethylenetriaminepentaacetic acid (DETAPAC) was also used in the experimental system.

For detecting oxygen radicals generated from a simple enzymatic reaction, 1 ml of reaction mixture containing 100 mM DMPO, 0.4 mM xanthine, 0.05 mM DETAPAC, and 50 mM phosphate buffer pH 7.8 was mixed with sufficient amount of xanthine oxidase. The reaction mixture was immediately drawn up

into the quartz flat cell, fitted into the cavity and the spectrum was recorded with a Bruker ESP 300 Spectrometer at 25° C.

For detecting oxygen radicals generated from a cellular system, 1 ml reaction mixture contained 5×10^6 neutrophils, 100 mM DMPO and 0.05 mM DETAPAC. The superoxide production was triggered by the addition of 100 ng of PMA in DMSO (0.14 M final concentration). Then the samples were transferred into the quartz flat cell and fitted into the cavity for detecting free radical signals. Typical ESR spectrometer settings for above experiments were: field set, 3477 G; modulation frequency, 100 KHz; microwave power, 10 mW; microwave frequency, 9.76 GHz; scan time, 4 minutes (8 minutes for neutrophils); cavity mode, TE_{102}.

4.3. Results

4.3.1. Spin Trapping of Free Radicals following the Reaction of Xanthine/Xanthine Oxidase

Figure 5a demonstrates a typical ESR spectrum obtained by the spin trapping of free radicals derived from the reaction of xanthine with xanthine oxidase. The spectrum consisted of a prominent DMPO-superoxide adduct, DMPO-OOH (peak 1) and a DMPO-hydroxyl adduct, DMPO-OH (peak 2). The hyperfine splitting constants for DMPO-OOH were A_N=14.2 G, A_b^H=11.4 G, A_d^H=1.25 G; for DMPO-OH were $A_N = A_b^H$ = 14.9 G compared with computed stick spectra. The DMPO-OH peaks shown in Figure 5a might result from spin trapping of hydroxyl radicals arising from the direct enzymatic reduction of H_2O_2

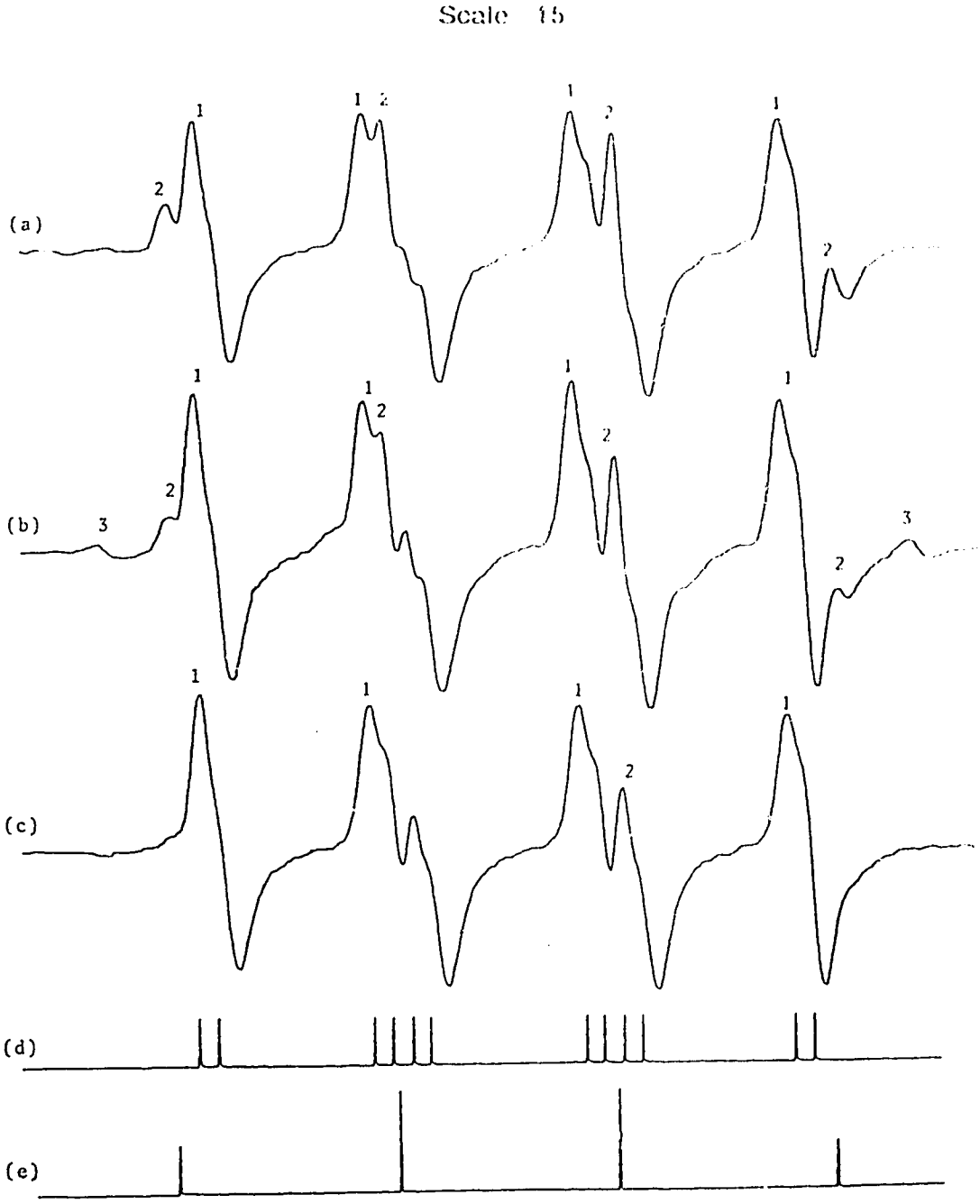


Figure 5: ESR spectra following the reaction of xanthine with xanthine oxidase in the presence of 0.1 M DMPO, 0.05 mM DETAPAC. (a), control spectrum; (b), 1% volume of DMSO added; (c), 400 units/ml of catalase added; (d), computed stick spectrum for DMPO-OOH; (e), computed stick spectrum for DMPO-OH.

by xanthine oxidase and/or from the decomposition of the DMPO-OOH to DMPO-OH (394,395). The spectrum (Figure 5a) agreed well with previously published data (394).

In order to determine whether ·OH was really formed in the xanthine/xanthine oxidase reaction, the experiment was performed in presence of 1% (v/v) of DMSO, a known hydroxyl radical scavenger. Since ·OH radicals are very reactive, once formed, they would react with DMSO to give a $\cdot CH_3$ radical which in turn would be spin trapped as a specific DMPO-CH_3 adduct. Figure 5b shows that after adding DMSO to the xanthine/xanthine oxidase system, a DMPO-CH_3 signal (peak 3) with hyperfine splitting constants of A_N =16.4 G and $A_b{}^H$=23.3 G appeared at the expense of DMPO-OH adduct (peak 2), suggesting the existence of hydroxyl radical in the reaction system (rest of DMPO-CH_3 peaks were overlapping with DMPO-OOH and DMPO-OH peaks). The result of the addition of a hydrogen peroxide scavenging enzyme, catalase, in the reaction system is shown in Figure 5c. Catalase decreased the intensity of the DMPO-OH signal (peak 2) but not the DMPO-OOH signal (peak 1), suggesting that hydrogen peroxide may participate in the formation of hydroxyl radicals. One possible mechanism suggests that hydrogen peroxide was directly reduced by xanthine oxidase to form a hydroxyl radical (396). Another possibility is that hydrogen peroxide might be reduced by iron to form hydroxyl radicals (Fenton reaction), if iron ions in the reaction buffer were not completely removed. However, the ESR experiment performed by the addition of 5 mM H_2O_2 and 100 mM DMPO to the reaction buffer did not show any DMPO-OH signals, which excluded the second possibility.

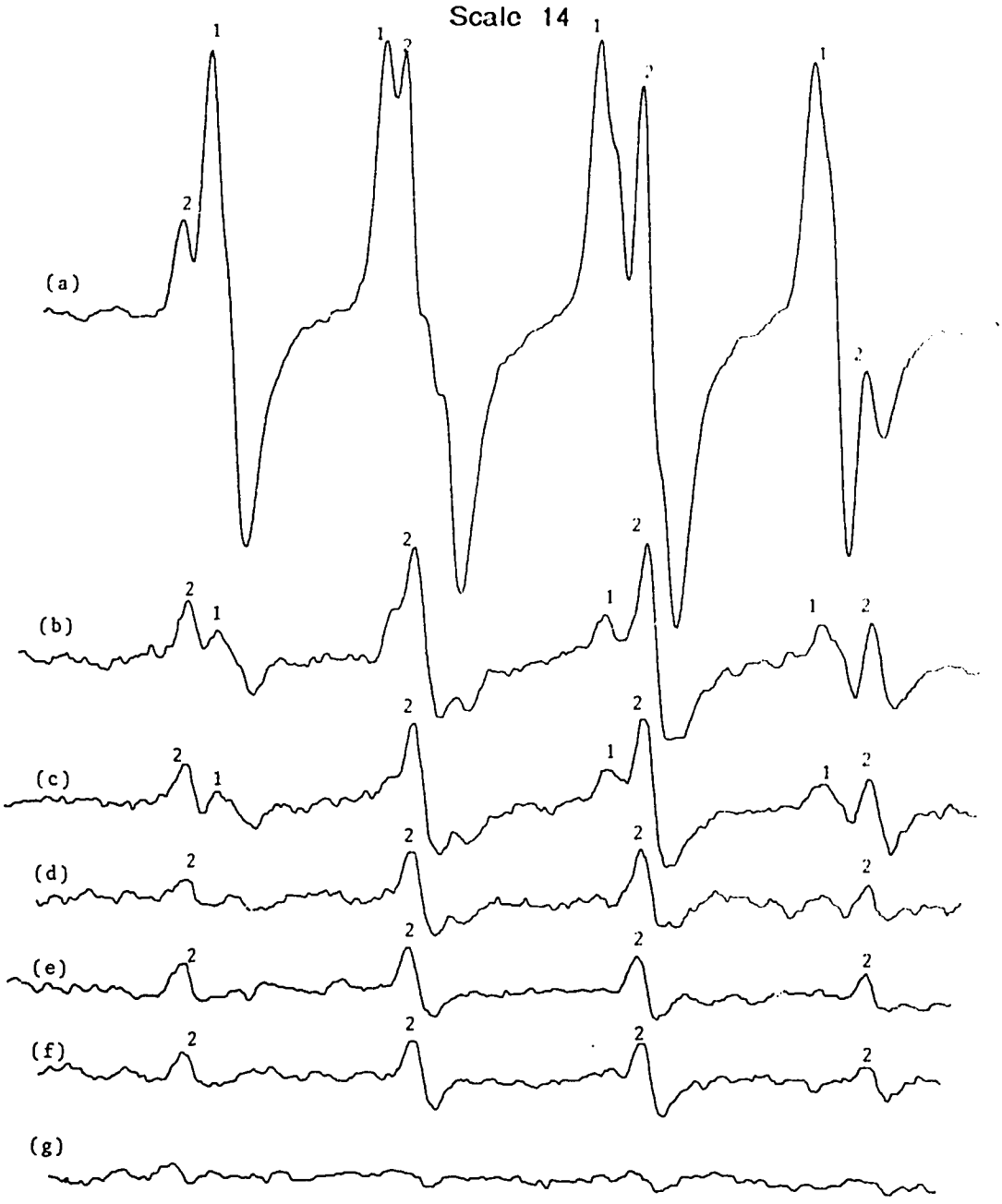


Figure 6: ESR spectra following the reaction of xanthine with xanthine oxidase in the presence of 0.1 M DMPO, 0.05 mM DETAPAC, and SOD. (a) no added SOD; (b) 5 units/ml of SOD; (c) 10 units/ml of SOD; (d) 40 units/ml of SOD; (e) 90 units/ml of SOD; (f) 240 units/ml of SOD; (g) 40 units/ml of SOD and 40 units/ml of catalase. Peak 1: DMPO-OOH adduct; peak 2: DMPO-OH adduct.

DMPO-OOH had distinctive hyperfine splitting constants that were easily recognizable, however, other peroxyl adducts of DMPO (e.g. benzyloxy radical adduct of DMPO) have a similar appearance (394). Therefore the convincing proof that the spectra observed was indeed due to superoxide radical was obtained by performing the experiments in the presence of SOD. Figure 6 shows that for all given concentrations of SOD, the enzyme effectively depleted $O_2^{\cdot}$ while only partially deplete ·OH signals following the reaction of xanthine with xanthine oxidase. Hence at high concentrations of SOD, no DMPO-OOH signal was observed but a small DMPO-OH signal remained to be detected no matter how high a dose of SOD had been applied, suggesting that this portion of DMPO-OH signal was not due to the decomposition of DMPO-OOH. A combination of SOD and catalase completely inhibited both DMPO-OOH and DMPO-OH suggesting that the portion of SOD uninhibitable DMPO-OH signal mainly arose from the direct reduction of H_2O_2 by xanthine oxidase because catalase was able to prevent it by removing hydrogen peroxide.

4.3.2. Spin Trapping of Free Radicals following Neutrophil Stimulation with Phorbol Myristate Acetate

The ESR spectrum of a sample containing resting unstimulated neutrophils and DMPO is shown in Figure 7a. The absence of signals suggests that no free radicals were produced by the unstimulated neutrophils or the product was rapidly consumed by the neutrophils.

In contrast, three different signals could be identified in the ESR spectrum resulting from neutrophils stimulated by PMA (Figure 7b). Peak 1 was the DMPO-superoxide adduct (DMPO-OOH) which had its characteristic parameters

Scale 16

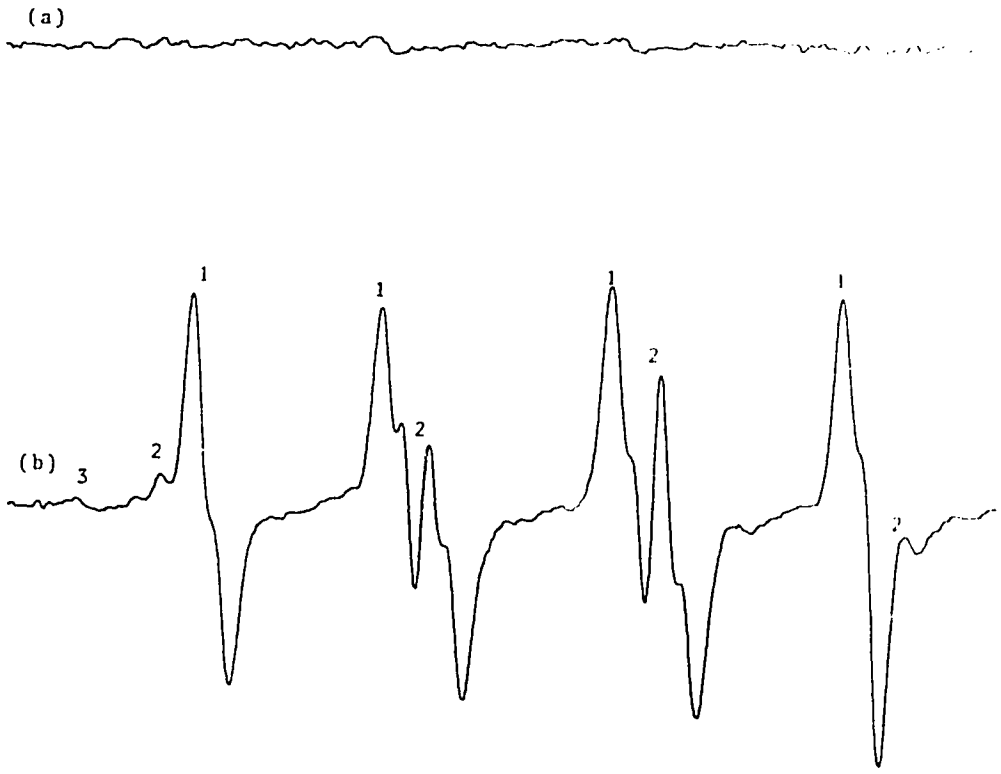


Figure 7: ESR spectra resulting from neutrophils (5×10^6/ml) in the presence of 0.1 M DMPO, 0.05 mM DETAPAC. (a), spectrum of resting unstimulated neutrophils; (b), spectrum of PMA (100ng/ml) stimulated neutrophils. Peak 1, DMPO-OOH adduct; peak 2, DMPO-OH adduct; peak 3, DMPO-CH_3 adduct.

and spectral suppression by SOD. Peak 2 was the DMPO-hydroxyl adduct (DMPO-OH) which could result from neutrophil hydroxyl radical production or the degradation of DMPO-OOH. Peak 3 was the DMPO-methyl radical adduct (DMPO-CH_3). The methyl radical in the reaction system could be the product of the reaction of the hydroxyl radical with the DMSO that was used as solvent for PMA.

The spectra obtained following the stimulation of neutrophils with PMA in the presence of SOD and catalase are shown in Figure 8. At a concentration of 250 IU/ml, SOD completely inhibited all free radical spin adduct signals while catalase had no significant effect on the ESR spectrum following the stimulation of neutrophils.

To determine whether the neutrophil generation of oxygen free radicals was a progressive process, experiments were carried out by incubating the neutrophils with PMA for a sequential time, then adding DMPO to trap the generated free radicals. The obtained ESR spectra thus represent the net result of production and decay of oxygen free radicals in the system. The results shown in Figure 9 indicate that the sizes of the DMPO-OH and DMPO-OOH peaks grew and reached a plateau during the first 5 minute stimulation of neutrophils with PMA, representing a "burst" of oxygen free radicals. For incubation periods longer than 5 minutes, only a gradual decrease in DMPO spin adduct signal was observed. ESR spectra in Figure 9 clearly indicate that the production of oxygen free radicals by stimulated neutrophils, observed as strong spin adduct signals, would last as long as 80 minutes suggesting that neutrophils might act as a sustained oxygen free radical source in response to the stimuli under pathogenic conditions.

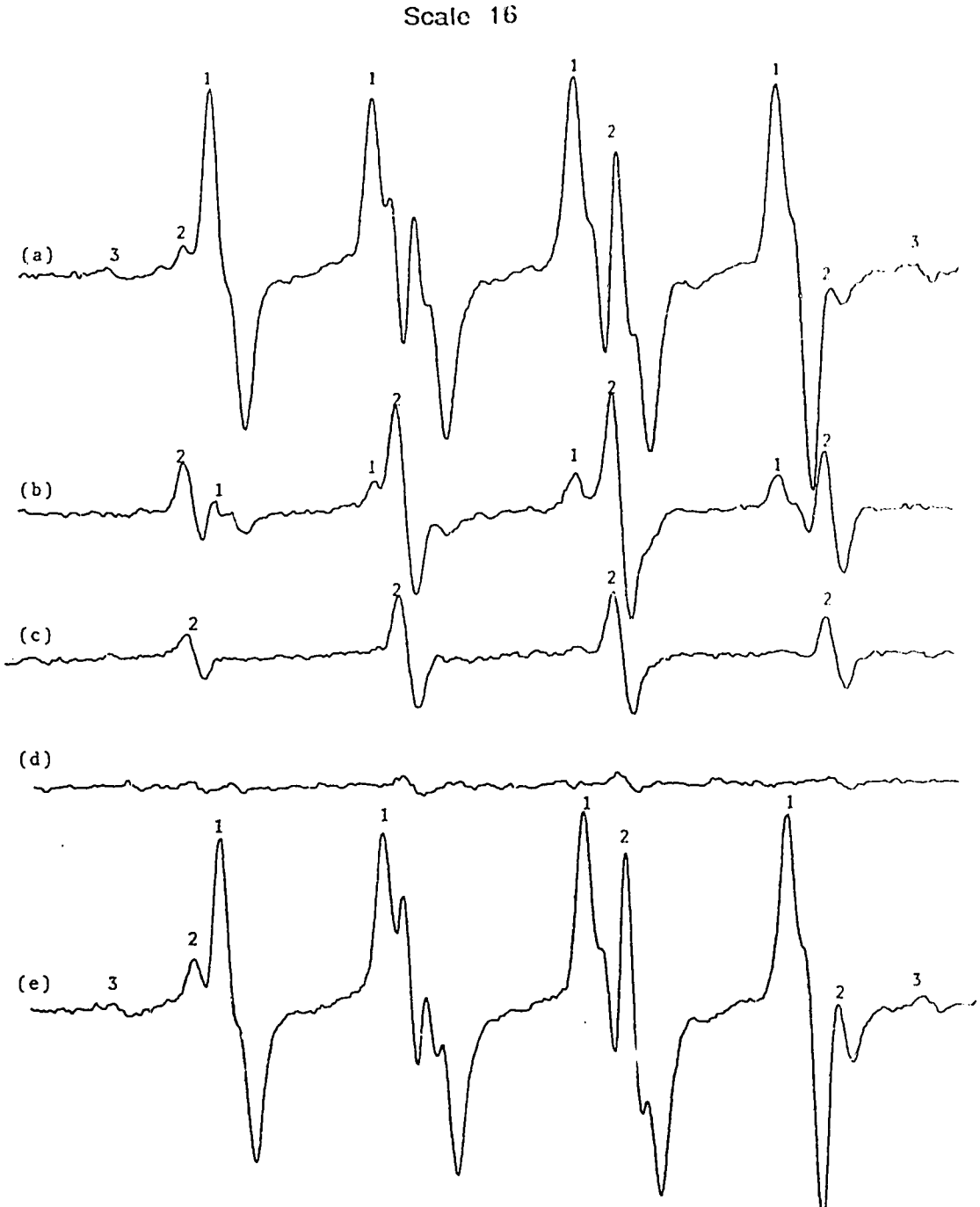


Figure 8: ESR spectra resulting from neutrophils (5×10^6/ml) stimulated with PMA (100 ng/ml) in the presence of 0.1 M DMPO, 0.05 mM DETAPAC, and scavengers. (a) no SOD; (b) 5 units/ml of SOD; (c) 25 units/ml of SOD; (d) 250 units/ml of SOD; (e) 400 units/ml of catalase. Peak 1: DMPO-OOH adduct; Peak 2: DMPO-OH adduct ; Peak 3: DMPO-CH_3 adduct.

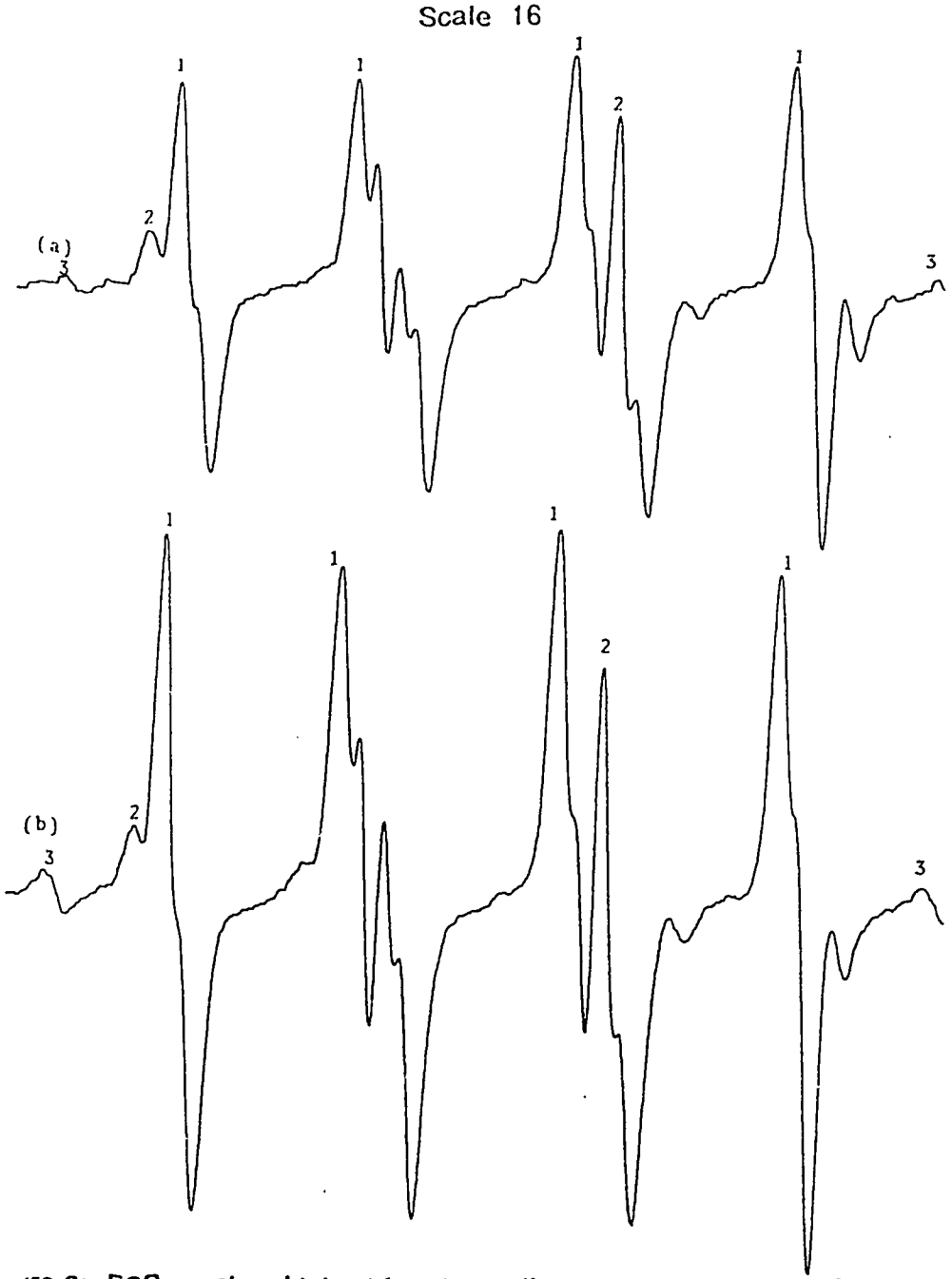


Figure 9: ESR spectra obtained from incubation of neutrophils (5×10^6/ml) with PMA (100 ng/ml) at 25°C for a sequential time, followed by adding 0.1 M DMPO to trap formed free radicals. Spectrum (a), no incubation; (b), 5 min incubation; (c), 10 min incubation; (d), 20 min incubation; (e), 30 min incubation; (f), 80 min incubation.

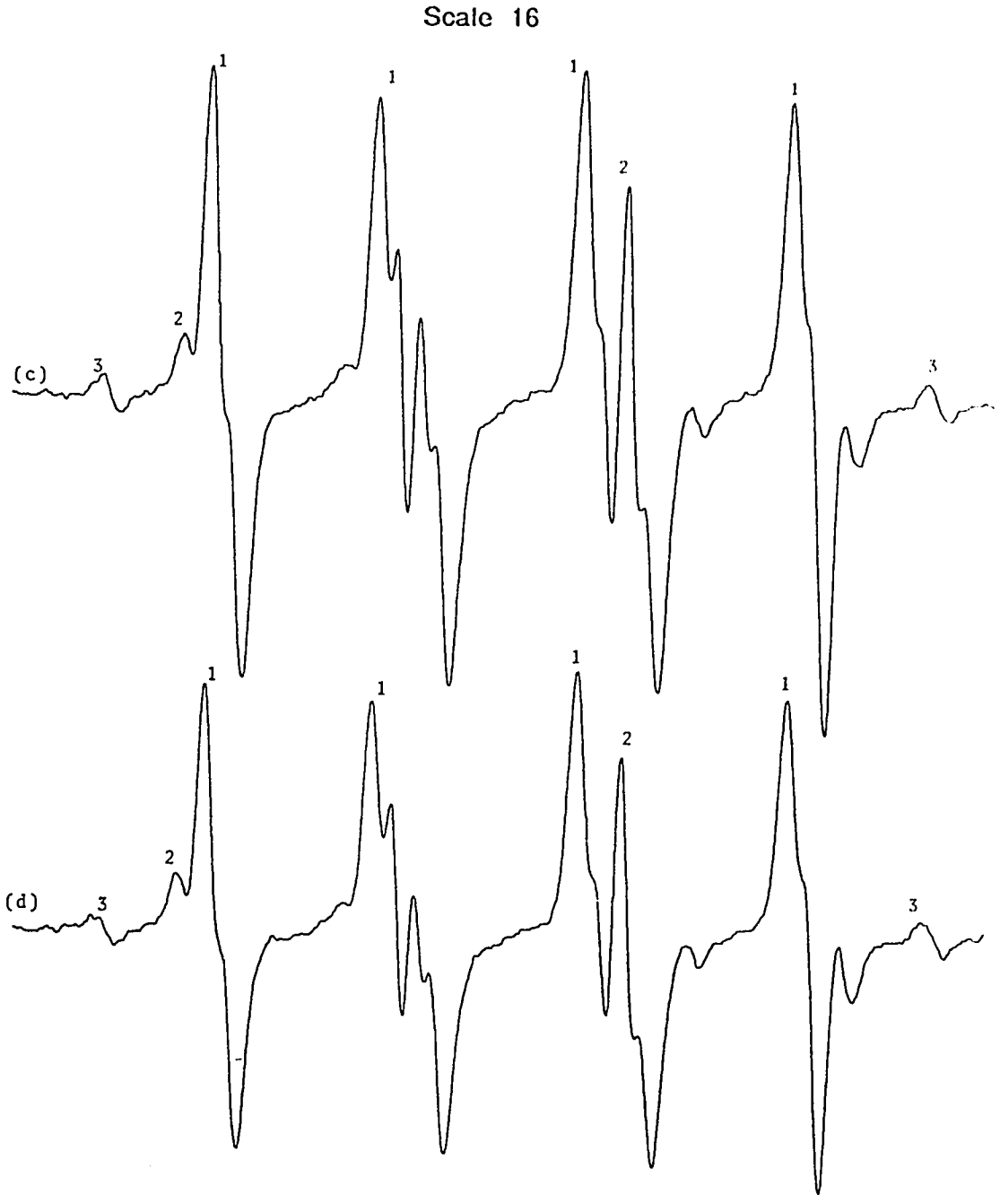


Figure 9: Continued.

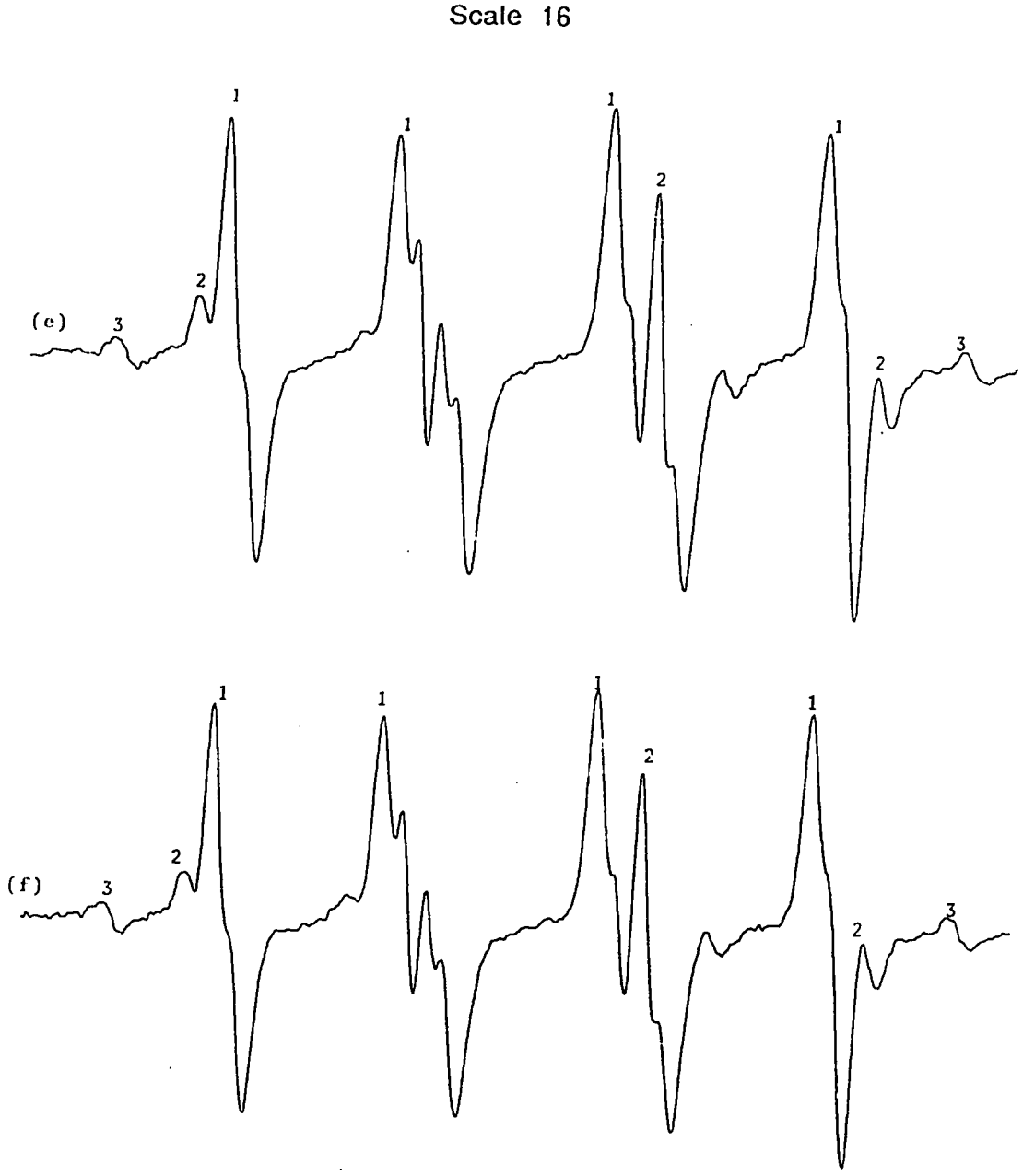


Figure 9: Continued.

4.4 Discussion

The superoxide radical is generated in many biological reactions. Enzyme systems that generate O_2^- during normal catalytic action include xanthine oxidase, aldehyde oxidase, and dihydroorotic dehydrogenase. The reaction of xanthine oxidase with xanthine has been used most frequently in vitro to generate O_2^- at a steady and controlled rate. It has been shown that xanthine oxidase is capable of reducing oxygen in both a one-electron step to form a superoxide radical and a two-electron step simultaneously to form hydrogen peroxide which is not a free radical under physiological conditions (397). Recently it has been reported by P. Kuppusamy and J.L. Zweier that xanthine oxidase can also reduce hydrogen peroxide to form hydroxyl radicals (396). They demonstrated that higher concentrations of O_2 favored the generation of superoxide radicals by xanthine oxidase while high concentrations of H_2O_2 favored the generation of hydroxyl radicals. Therefore it appears that O_2 and H_2O_2 compete for one-electron reduction by the enzyme. They suggested that the FAD prosthetic group of xanthine oxidase was essential for both the reduction of O_2 to O_2^- and the reduction of H_2O_2 to ·OH based on the experiment that the deflavo enzyme did not produce free radicals. The exact nature of free radical species derived from the reaction of xanthine oxidase needs to be clarified as more and more reports suggest that xanthine oxidase is an important source of free radicals in pathogenic conditions (361).

The rate of O_2^- production can be determined by measuring the rate of SOD-inhibitable cytochrome c reduction at 550 nm. However, the cytochrome c assay or other routine spectrophotometric assay is unable to tell whether the xanthine oxidase system also produces more toxic hydroxyl radicals and the exact

reaction pattern of xanthine oxidase. The advantages of the ESR spin trapping technique in the analysis of the xanthine/xanthine oxidase reaction are (a) since each adduct of DMPO and free radical has its characteristic parameters, this technique is a direct assay of superoxide and hydroxyl radicals, providing unequivocal detection and identification of these species, (b) the assay is very sensitive and the trapped spin adduct is relatively stable, and (c) the analysis of reaction mechanism becomes simpler because the propagation of a free radical chain reaction can be eliminated by the spin trapping agent. We have found both DMPO-superoxide (DMPO-OOH) and DMPO-hydroxyl radical (DMPO-OH) signals in the ESR spectra following the reaction of xanthine oxidase. The generation of superoxide radicals by the xanthine oxidase was easy to verify because the DMPO-superoxide adduct has unique splitting constants and was completely inhibited by superoxide dismutase. The determination of the origin of the DMPO-hydroxyl adduct was complicated because it could be formed by either the binding reaction of DMPO with ·OH or the potential breakdown of DMPO-OOH to DMPO-OH.

The differentiation of whether DMPO-OH in xanthine/xanthine oxidase resulted from the formation of hydroxyl radicals or decomposition of DMPO-OOH was based on the following criteria. In the first experiment, the xanthine oxidase reaction was initiated in the presence of the hydroxyl radical scavenger DMSO. As a result the intensity of the DMPO-OH adduct signal decreased and a DMPO-CH_3 signal appeared (Figure 5b) suggesting the existence of hydroxyl radicals and the reaction of hydroxyl radical with DMSO in the system. The second experiment performed was the addition of the hydrogen peroxide scavenger catalase to the reaction system. The resulting ESR spectrum exhibited an inhibition of the DMPO-OH adduct but not the DMPO-OOH adduct signals

(Figure 5c) suggesting that during the xanthine/xanthine oxidase reaction at least part of the hydroxyl radicals were formed via the reduction of H_2O_2 by xanthine oxidase. Catalase scavenged the H_2O_2 and thereby decreased the DMPO-OH signal. The final and important observation was that SOD was able to totally deplete DMPO-OOH signals but not the total DMPO-OH signals. The combination of SOD and catalase, however, blocked all free radical signals derived from the reaction of xanthine oxidase (Figure 6). These results are in agreement with Kuppusamy and Zweiers' report (396) and provide strong evidence that following the xanthine oxidase reaction, the hydroxyl radical spin adduct recorded in the ESR spectra consisted of two fractions. Those SOD-inhibitable hydroxyl radical spin adduct might arise from the breakdown of DMPO-OOH, and those SOD-resistant but catalase-inhibitable might result from direct spin trapping of hydroxyl radicals formed by reduction of H_2O_2 by xanthine oxidase.

The ESR spectra of PMA-stimulated neutrophils were composed of prominent of DMPO-OOH peaks and DMPO-OH peaks. We applied similar criteria as discussed above to identify and differentiate spin trapping adducts formed by stimulated neutrophils. For the purpose of verifying the direct formation of hydroxyl radicals by the neutrophils, dimethylsulfoxide (DMSO) as a solvent for PMA was added to the neutrophil stimulation system to a concentration of 0.14 M and DMPO to 0.1 M. Since the rates of hydroxyl radical reaction with DMPO and DMSO were similar (398), and the concentration of DMSO was higher, a large portion of ·OH, if it was formed by neutrophils, would be expected to react with DMSO and convert to the methyl radical. However, in the sequential ESR scanning spectra (Figure 9), no accumulation of DMPO-CH_3 along with the depletion of DMPO-OH was detected. This was inconsistent with neutrophil

hydroxyl radical formation. Another observation against direct production of hydroxyl radicals by stimulated neutrophils was that SOD was able to completely inhibit the DMPO adducts of $O_2^{\cdot-}$, $\cdot OH$ and $\cdot CH_3$ (Figure 8) while catalase had little or no effect on observed ESR spectra suggesting that DMPO-OH and DMPO-CH_3 were mediated by superoxide radicals rather than hydroxyl radicals. The notion from these observations was that upon stimulation, neutrophils progressively generated a large amount of superoxide radicals via the membrane-associated NADPH oxidase without evidence for direct generation of hydroxyl radical. The appearance of SOD-inhibitable DMPO-OH signal on the ESR spectra obtained from stimulated neutrophils would mainly be a consequence of the degradation of DMPO-OOH, independent of the mechanism of hydroxyl radical formation. The SOD-inhibitable DMPO-CH_3 in the ESR spectra might be due to a small amount of hydroxyl radicals yielded from the process of degradation of DMPO-OOH (394). These small amounts of hydroxyl radicals might have reacted with DMSO to form methyl radicals which were then spin trapped by DMPO as DMPO-CH_3 adducts.

It may be suggested that neutrophils might generate some hydroxyl radicals which escape detection by reacting with other components such as neutrophils themselves. However, considering the high concentrations of DMPO and DMSO (total 0.24 M) in the system and the high reaction rate between hydroxyl radicals and DMPO or DMSO ($3.4 \times 10^9\ M^{-1}\ s^{-1}$), it could be expected that most hydroxyl radicals, if they are generated, would be trapped by DMPO and DMSO (especially in sequential scanning) as SOD-uninhibitable DMPO-OH and DMPO-CH_3 adducts. The absence of these specific peaks in the ESR spectra of our experiments confirms that the consumption of hydroxyl radicals by other components, if any, would be negligible.

5. Permeability of Superoxide Radical in Lipid Bilayers and Biological Membranes

5.1. Introduction

Since oxygen free radicals are thought to play an important role in direct cellular injury, superoxide dismutase has been considered to be an important enzyme to strengthen cellular defense. However, because of the compartmentalized production of O_2^- and the localized distribution of SOD in cells (399), successful supplement of exogenous SOD to eliminate O_2^- in situ requires thorough knowledge of the permeation of cell membranes to oxygen free radicals.

The present reports of the permeation of lipid bilayers to O_2^- seems controversial. Takahashi et al. reported that the lipid bilayers were not permeable to O_2^- generated inside lipid vesicles by the illumination of flavin mononucleotide (400), while Rumyantseva et al. found that enzymatically produced O_2^- could cross a lipid bilayer to cause ferricyanide-ferrocyanide transformation inside lipid vesicles (401). The evidence of permeation of biological membranes to O_2^- is not sufficient, one available example is erythrocyte membrane (402). By using xanthine oxidase-loaded lipid vesicles and chemically defined reaction, Lynch and Fridovich reported that erythrocyte membrane was permeable to O_2^-.

In order to clarify the permeability of O_2^-, a precise method for detecting and differentiating free radical species appears indispensable. In our laboratory, we have recently applied electron spin resonance (ESR) and spin trapping

techniques, a more direct and sensitive method, to study the permeability of O_2^-. We have loaded phosphatidylcholine lipid vesicles and resealed erythrocyte ghosts with SOD according to established methods (403,404). Following the incubation of SOD vesicles or SOD ghosts with an external source of O_2^-, the permeation of membranes to O_2^- was determined by measuring retained external concentration of O_2^- using both electron spin resonance (ESR) and chemically defined reaction. The results show that SOD trapped inside lipid vesicles did not scavenge external O_2^- suggesting that lipid bilayers are not permeable to O_2^-. In contrast, SOD-loaded ghosts are able to scavenge external O_2^- as effectively as free SOD supporting that the erythrocyte membranes are permeable to O_2^-.

5.2. Materials and Methods

5.2.1. Materials

Yeast superoxide dismutase was from Carlsberg Biotechnology Ltd. (Copenhagen, Denmark). Polycarbonate membrane filters were obtained from Nuclepore Corporation (U.S.A.). Sephadex G-100 was a product of Pharmacia Fine Chemicals (Sweden). Ferricytochrome c, milk xanthine oxidase, xanthine, egg phosphatidylcholine, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), and other reagents were purchased from Sigma Chemical Company (St. Louis, MO U.S.A.).

5.2.2. Methods

5.2.2.1. Preparation of SOD Lipid Vesicles

200 mg of egg phosphatidylcholine (egg PC) was dried under nitrogen. 5 mg of SOD in 3 ml of phosphate buffer, pH 7.4, was mixed and then vortexed with egg PC to form multilamellar vesicles (MLV). One polycarbonate membrane (25 mm diameter, 0.1 μm pore size) was placed at the bottom of the extrusion apparatus. After pouring the MLV into the chamber, the apparatus was connected to a N_2 cylinder and a pressure of 250-500 psi was gradually applied. The extruded liquid from the outlet of the apparatus was collected and reloaded into the chamber. The extrusion cycle was repeated 5 times. Final extruded mixture of SOD vesicles and free SOD was separated by Sephadex G-100 chromatography.

5.2.2.2. Preparation of Sealed SOD Ghosts

Freshly drawn human blood was washed four times in isotonic buffer (0.9% KCl), the buffy coat was carefully removed after each centrifugation. The washed red cells (50% hematocrit) were lysed in 30 volumes of a hypotonic hemolysis buffer (4 mM $MgSO_4$, 3.8 mM CH_3COOH, pH 4) with stirring. After lysis for 5 minutes, the cell suspension was centrifuged at 13,000 rpm (23,000 g) for 10 minutes and the supernatant was removed as completely as possible. The collected ghosts (pellets) were washed with washing buffer (4 mM $MgSO_4$, 1.2 mM CH_3COONa, 2 mM NaH_2PO_4/Na_2HPO_4, pH 7) until the ghosts had a grayish white color. SOD-loaded ghosts were prepared by mixing SOD with the

ghosts in the washing buffer, the tonicity was restored to 100 mM by adding a small volume of reverse buffer (2 M KCl, 25 mM Tris/HCl, pH 7.2). The above experimental procedure was performed at 0° C. The SOD ghosts were fully resealed by incubation at 37° C for 45-60 minutes and final product was washed thoroughly in isotonic buffer.

5.2.2.3. Phospholipid Phosphorus Assay

Lipid Extraction: SOD vesicles were mixed with methanol and chloroform (1 : 5 : 5). After centrifuging the mixture at 5000 g for 10 minutes, the supernatant was collected, then the above process was repeated. The pooled supernatant was made 1 : 5 :10 (water : methanol : chloroform) by the addition of appropriate volumes of chloroform and water, and then centrifuged at 5000 g for 10 minutes. The lower organic phase was collected and evaporated under nitrogen.

Organic Phosphorus Assay: 0.5 ml of water, 0.25 ml of 16 N H_2SO_4 and 3 glass chips were added to each dried lipid extract. The samples were then placed in a digestion apparatus and heated until dense white fumes appeared. After adding 2 drops of 30% H_2O_2 to each sample, the samples were heated again for at least 1 minute after the dense fumes appeared. The samples were allowed to cool and the following was added: 4 ml of water, 1 ml of acid molydate (5 g ammonium molydate in 100 ml of 4 N H_2SO_4) and 1 ml of ELON reagent (5 g of Kodak ELON and 15 g of sodium bisulfite in 500 ml of water). After 15 minutes of reaction, the concentration of organic phosphorus was determined by reading the absorbance at 660 nm using potassium phosphate as a standard.

5.3. Results

5.3.1. Characteristics of SOD Lipid Vesicles

SOD vesicles prepared by the extrusion cycle method were separated from untrapped native SOD by Sephadex G-100 chromatography. Figure 10 shows the profile of organic phosphorus content and SOD activity in fractions collected from a Sephadex G-100 column (1.5 × 50 cm). It clearly indicates that lipid vesicles eluted at fractions 10-20 while the free SOD activity peak was located at fractions 75-85. In the absence of detergent, lipid vesicle fractions did not show any inhibitory effect on superoxide radicals produced by xanthine/xanthine oxidase. However, lysing lipid vesicles with 1% deoxycholate (DOC), entrapped SOD was released and lipid vesicle fractions exhibited typical SOD activity which was coincident with the phosphorus peak (Figure 10). DOC itself and empty lipid vesicles had no effect on the ferricytochrome c reduction assay used in this experiment. The conclusions of these results are (1) SOD was effectively trapped inside lipid vesicles, supported by the observation that DOC-lysed SOD vesicles exhibited typical enzyme activity; (2) superoxide radicals were not able to penetrate into the lipid vesicles, therefore intravesicular SOD did not inhibit extravesicular $O_2^{\cdot-}$–dependent cytochrome c reduction. The SOD vesicle fractions from Sephadex G-100 were collected for further permeability experiments.

5.3.2. Permeability of Lipid Bilayers to Superoxide Radicals

In order to determine whether $O_2^{\cdot-}$ passing through the lipid bilayer, if any, was a time-dependent process, intact and DOC-lysed SOD vesicles were

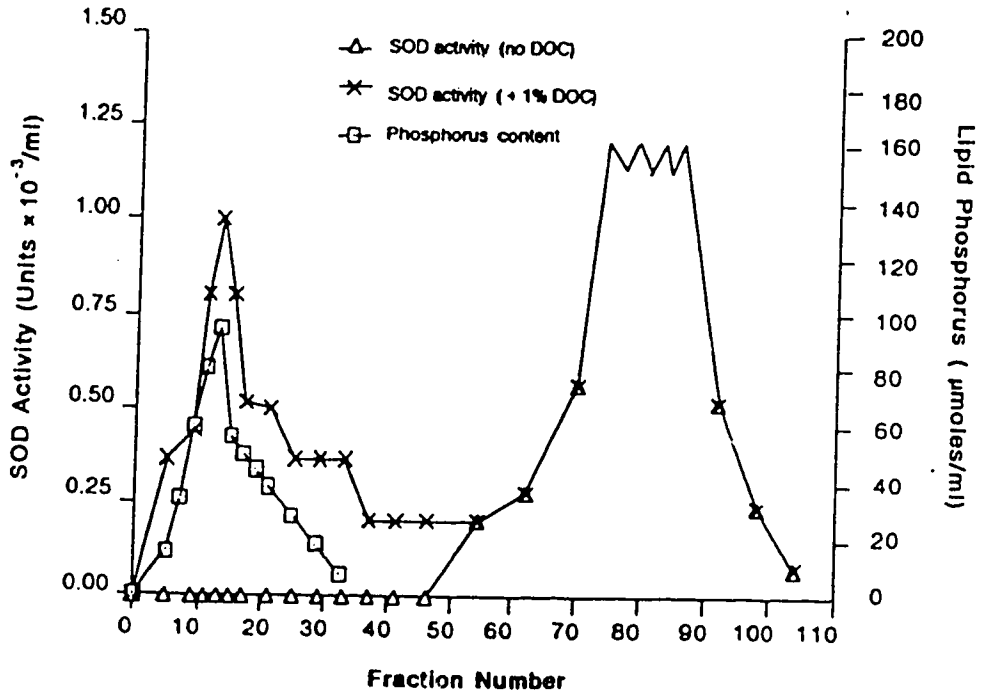


Figure 10: Sephadex G-100 chromatography of SOD lipid vesicles. The column had a 50 cm by 1.5 cm dimention and each fraction contained 300 µl of eluent. SOD activity and lipid phosphorus content were determined as described under Materials and Methods. Extravesicular SOD was eluted in fractions #65 to #95 with a peak at fraction #80 (2000 units/ml). Lipid vesicels eluted in the fractions#5 to #30 and showed no SOD activity. In the presence of 1% deoxy cholate (DOC), the vesical fractions exhibited SOD activity with a peak coincident with the phosphorus peak.

incubated with xanthine/xanthine oxidase. The samples were taken from the mixture during the incubation course to measure the retained superoxide radical concentration which was represented by O_2^-–mediated cytochrome c reduction. As shown in Figure 11, superoxide radical concentrations in the control system without SOD vesicles and the reaction system including SOD vesicles exhibited the same decline trend characterized by a gradual decrease of ΔA from 0.025 to 0.012 at 550 nm during the 15-minute incubation. Since a decrease of O_2^- concentration occurred in both the control and SOD vesicle systems, this effect could be attributed to the autodegradation of superoxide radicals rather than the scavenging of superoxide radicals by intravesicular SOD. The incubation of SOD vesicles with the O_2^- source did not result in a decrease of O_2^- concentration compared with the control. This result excluded the possibility that superoxide radicals permeating the lipid membrane was a time-dependent process. In contrast, the addition of DOC-lysed SOD vesicles in the xanthine oxidase system dramatically reduced O_2^- level. Figure 11 demonstrates that DOC-lysed SOD vesicles blocked O_2^--dependent cytochrome c reduction within 4 minutes. These results, shown in Figure 10 and 11, clearly indicate that permeability of the lipid membrane to superoxide radicals is negligible and intravesicular SOD had no effect on extravesicular O_2^- because of the existence of the lipid bilayer barrier.

Spin trapping and electron spin resonance offered additional evidence for these effects. Figure 12 shows typical spectra generated by xanthine/xanthine oxidase. The initial scan spectrum (a) was obtained 4 minutes after initiation of the reaction consisting of a combination of DMPO-OOH (DMPO-superoxide adduct) and DMPO-OH (DMPO-hydroxyl adduct). This indicates that both O_2^-

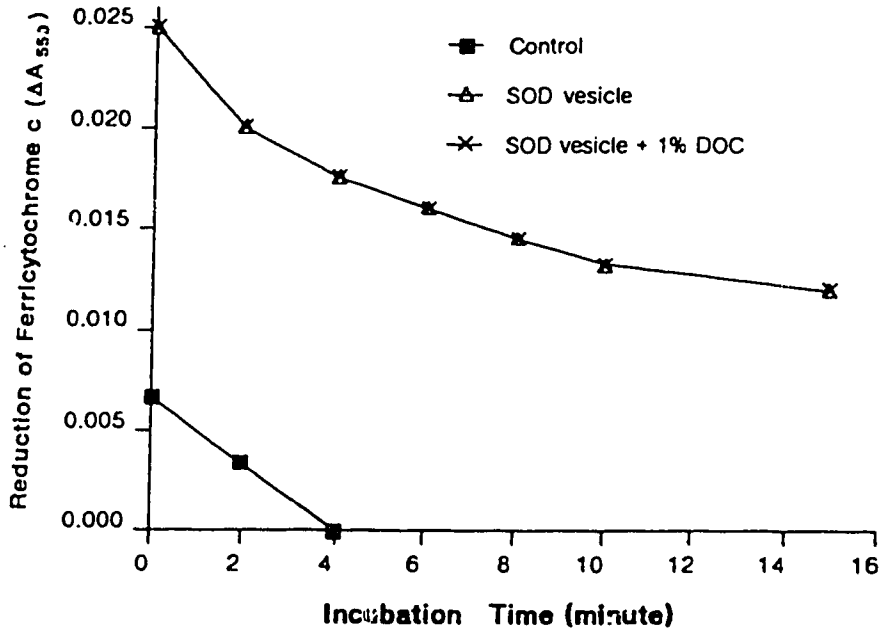


Figure 11: Accessibility of SOD enclosed in lipid vesicles to extravesicular O_2^-. The incubation medium contained 0.05 M potassium phosphate, 50 μM xanthine, 0.1 mM DETAPAC, 6nM xanthine oxidase and SOD vesicles (or DOC lysed SOD vesicles). The samples were taken at regular intervals to examine the remaining extravesicular O_2^- by the addition of 0.01mM ferricytochrome c. The DOC lysed SOD vesicles dramatically inhibited O_2^- -dependent ferricytochrome c reduction, while the same amount of intact SOD vesicles had no effect.

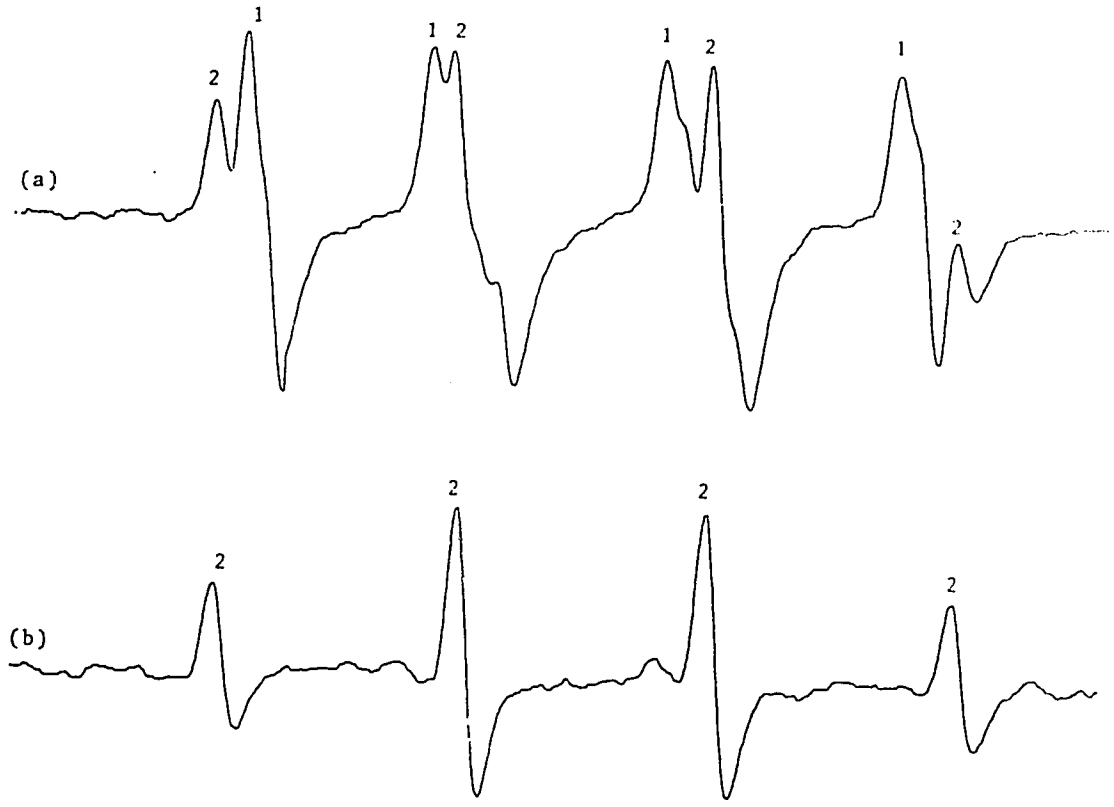


Figure 12: ESR spectra obtained following the reaction of xanthine with xanthine oxidase in the presence of 0.1 M DMPO and 0.05 mM DETAPAC. Initial 4-minute scan was recorded immediately after the reaction commenced (spectrum a); second 4-minute scan was recorded 4 minutes after the reaction commenced (spectrum b). Peak 1, DMPO-OOH adduct; peak 2, DMPO-OH adduct.

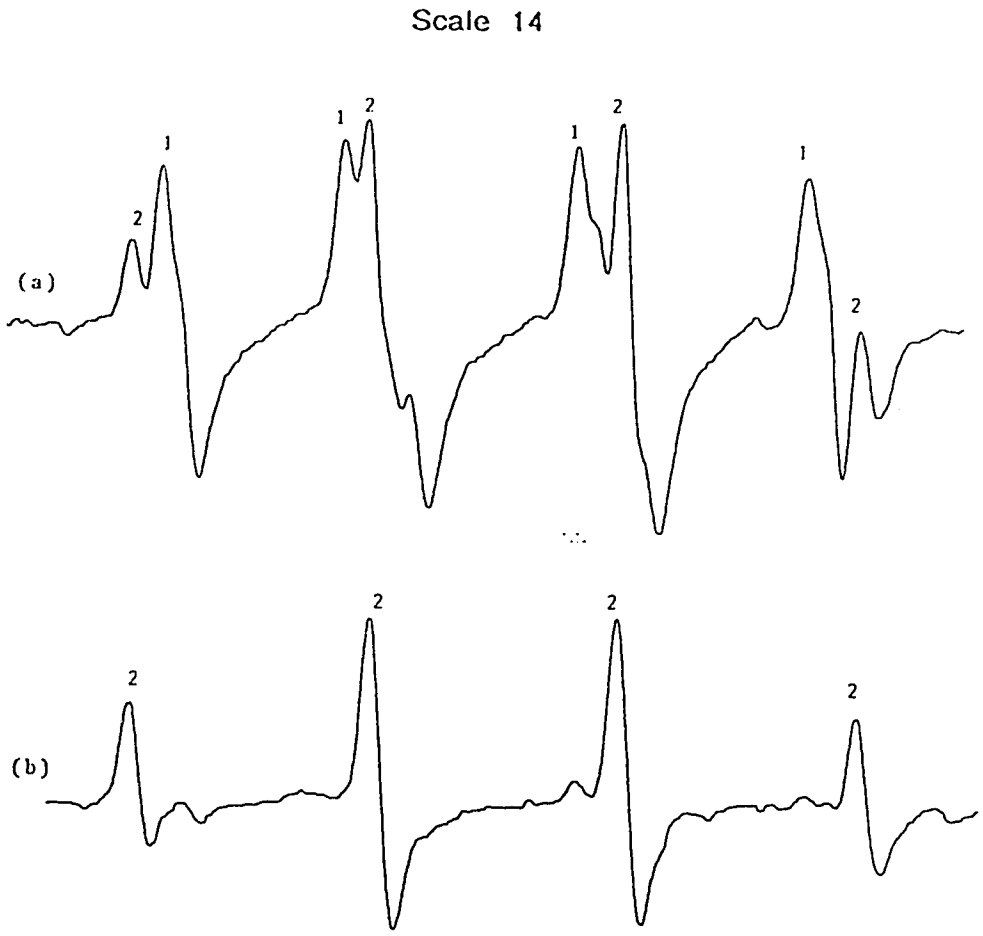


Figure 13: ESR spectra obtained following the reaction of xanthine with xanthine oxidase in the presence of SOD vesicles (2μmoles lipid phosphorus/ml). Initial 4-minute scan was recorded immediately after the reaction commenced (spectrum a); second 4-minute scan was recorded 4 minutes after the reaction commenced (spectrum b). Peak 1, DMPO-OOH adduct; peak 2, DMPO-OH adduct.

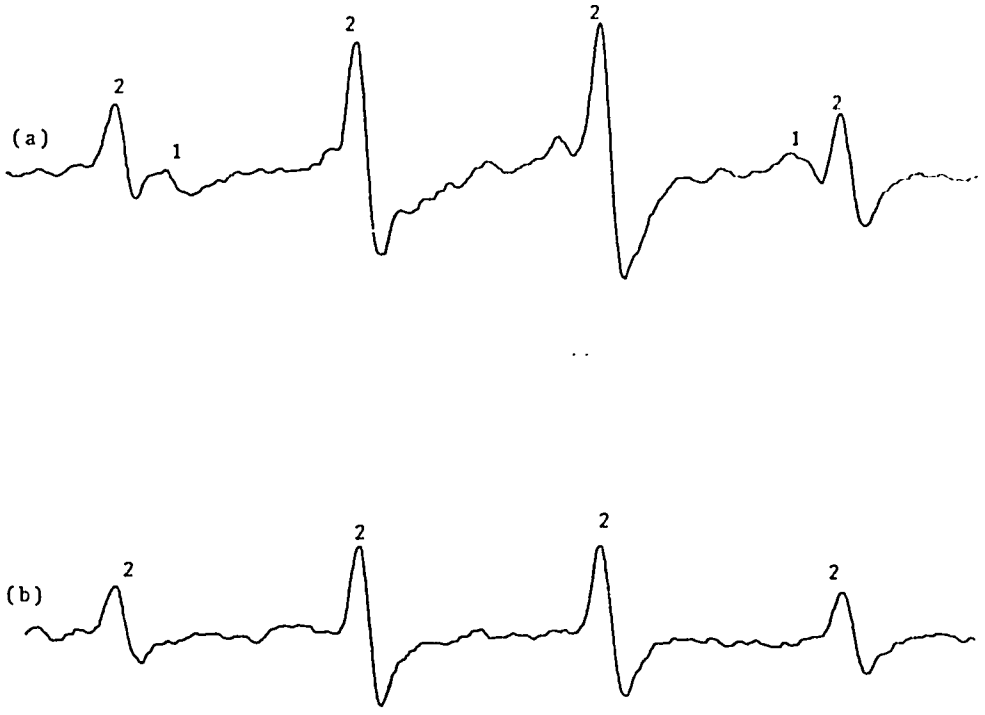


Figure 14: ESR spectra obtained following the reaction of xanthine with xanthine oxidase in the presence of 0.1 DOC-lysed SOD vesicles (2μmoles lipid phosphorus/ml). Initial 4-minute scan was recorded immediately after the reaction commenced (spectrum a); second 4-minute scan was recorded 4 minutes after the reaction commenced (spectrum b). Peak 1, DMPO-OOH adduct; peak 2, DMPO-OH adduct.

and ·OH are formed (for details see section 4.4 in this thesis). By the end of the second scan which was 8 minutes after the reaction was initiated, the dominant species was the DMPO-hydroxyl adduct. The DMPO-superoxide adduct peaks became smaller, probably due to decomposition of DMPO-OOH to DMPO-OH or other products (DMPO-OOH and DMPO-OH were designated peaks 1 and 2 respectively). The spectra in Figure 13 were obtained following the reaction of xanthine and xanthine oxidase in the presence of SOD vesicles. Adding intact SOD vesicles to the reaction system yielded an ESR spectra similar to that obtained without SOD vesicles indicating that no influx of O_2^- through the lipid bilayer had occurred. Adding DOC-lysed SOD vesicles to the reaction system, the SOD released from lipid vesicles effectively scavenged the superoxide radicals, resulting in depletion of DMPO-OOH peaks (Figure 14). These ESR data are in agreement with the cytochrome c reduction assay experiments confirming that superoxide radicals are unable to cross the lipid bilayer.

5.3.3. Characteristics of SOD Ghosts

SOD-loaded ghosts prepared as described under "Material and Methods" were "intact" and impermeable to SOD molecules. The leakage of SOD from the ghosts was determined by incubating SOD ghosts at 25° C for 35 minutes, then measuring the SOD activity in the supernatant after the sedimentation of ghosts. As shown in Table 7, no SOD activity (no inhibition of reduction of cytochrome c) was found in the supernatant following the incubation. There was also no non-specific binding of SOD to the erythrocyte membrane, which was demonstrated by mixing empty erythrocyte ghosts with SOD, followed by thorough washing. No SOD activity was found in the empty ghosts.

Table 7
Determination of Leakage of SOD Ghosts

Time (minutes)	Cytochrome c Reduction (ΔA 550 nm)
0	0.0247
15	0.0243
25	0.0247
35	0.0246

SOD ghosts were incubated at 25° C, then after sedimentation of SOD ghosts, SOD activity in supernatant was determined by cytochrome c reduction assay.

5.3.4. Permeability of Biological Membranes to Superoxide Radicals

Mixing SOD ghosts or empty ghosts with xanthine and cytochrome c, and then initiating the production of superoxide radicals by the addition of xanthine oxidase, it was found that empty erythrocyte ghosts did not influence $O_2^{\cdot-}$-dependent cytochrome c reduction while SOD ghosts inhibited $O_2^{\cdot-}$ production in a dose-dependent pattern. The results presented in Figure 15 shows that adding 2×10^6, 4×10^6, and 6×10^6 SOD ghosts to the reaction system decreased ferricytochrome c reduction by 29%, 42%, and 53% respectively, implying that superoxide radicals were able to penetrate "intact" erythrocyte membranes and be scavenged by entrapped SOD. Hypotonic relysed SOD ghosts had the similar effect on inhibition of ferricytochrome c reduction.

ESR experiments also supported the conclusion that biological membranes were permeable to superoxide radicals. In Figure 16, spectrum (a) represents the reaction of xanthine with xanthine oxidase in the presence of empty erythrocyte ghosts. Strong DMPO-OOH and DMPO-OH peaks appeared. The spectrum (b) was recorded under the same experimental condition as spectrum (a) except that empty ghosts were replaced with SOD ghosts. It clearly demonstrates that both DMPO-OOH and DMPO-OH peaks were inhibited by the intact SOD ghosts. Relysed SOD ghosts had a similar effect on inhibiting DMPO-OOH and DMPO-OH peaks. These results suggest that the superoxide radical is able to diffuse through the biological membrane and interact with entrapped SOD.

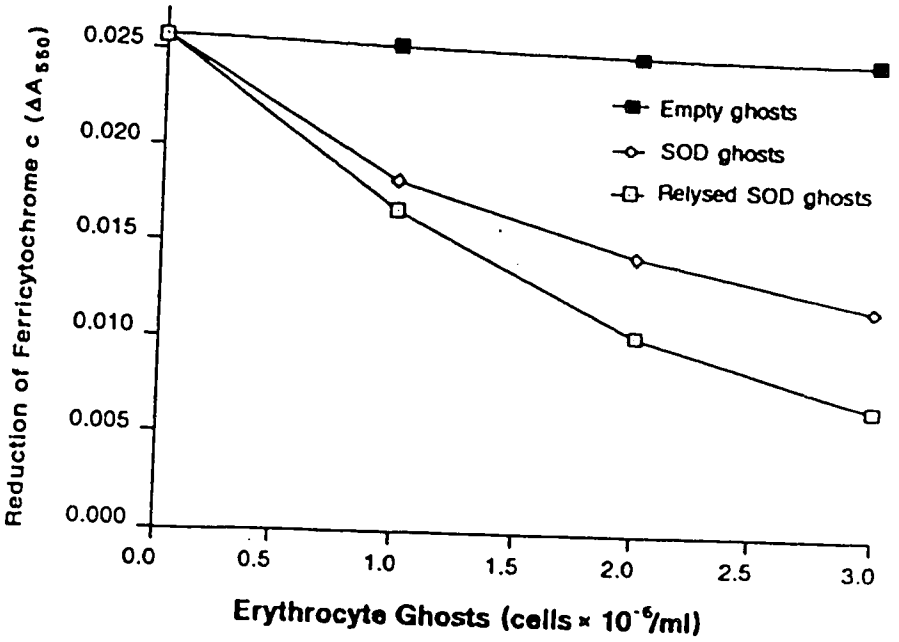


Figure 15: Accessibility of SOD enclosed in biological membrane to extracellular O_2^-. The reaction medium contained 0.05 mM potassium phosphate, pH 7.8, 50 μM xanthine, 0.1 mM DETAPAC, 0.01mM ferricytochrome c, and SOD ghosts (or hypotonic relysed SOD ghosts). The reaction was started by the addition of 6 nM xanthine oxidase. The SOD ghosts effectively inhibited O_2^--dependent ferricytochrome c reduction in the medium, while empty ghosts had no effect.

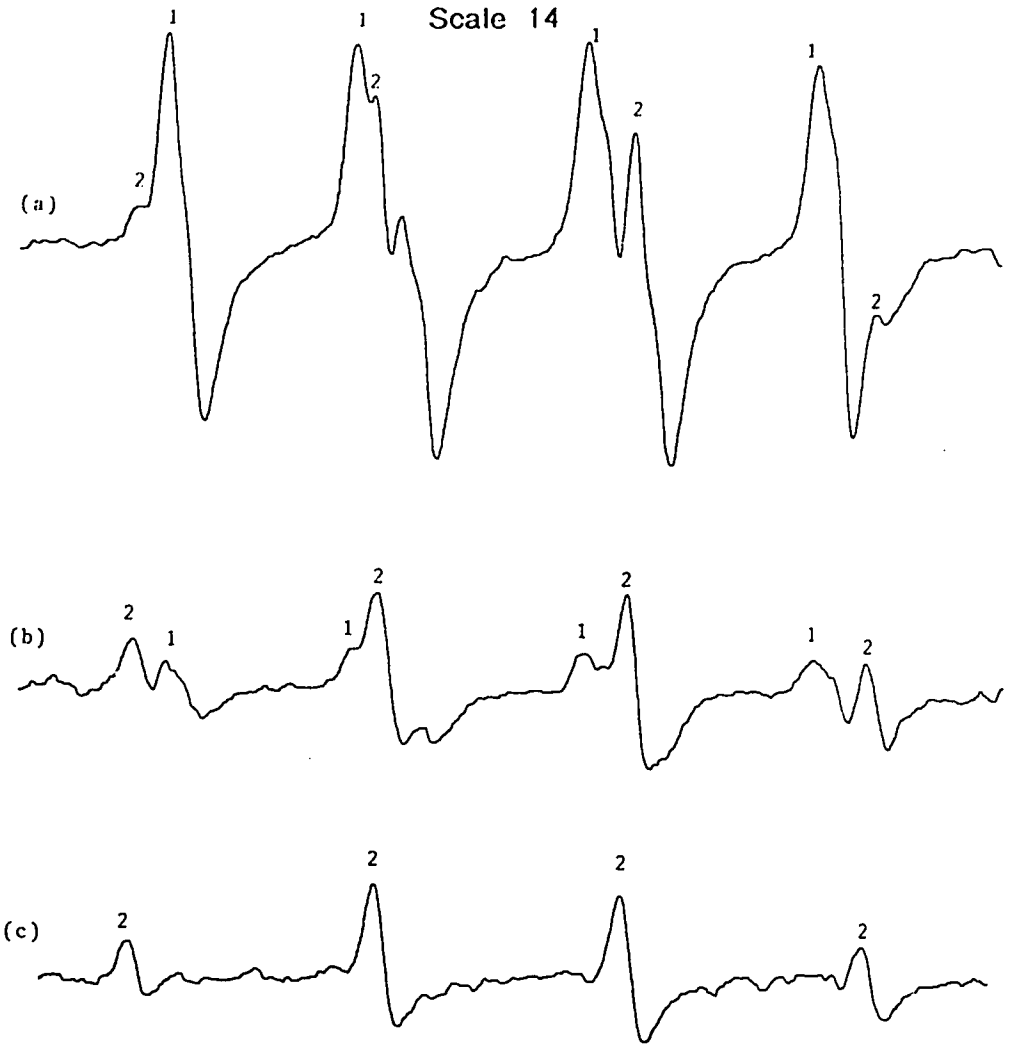


Figure 16: ESR spectra obtained following the reaction of xanthine with xanthine oxidase in the presence of empty erythrocyte ghosts (spectrum a); intact SOD-loaded ghosts (2×10^6 cells/ml) (spectrum b); hypotonic lysed SOD ghosts (2×10^6 cells/ml) (spectrum c). Peak 1, DMPO-OOH adduct; peak 2, DMPO-OH adduct.

5.4. Discussion

Superoxide dismutase has captured the interest of many biologists and clinicians for the development of an effective therapy against oxygen toxicity and reperfusion injury. However, the complexity of successfully using SOD for this purpose is great. The enzyme has to be delivered or targeted promptly or it will be cleared rapidly from the circulation (405). Some investigators have tried to protect native SOD from a destruction mechanism by encapsulating SOD within a lipid bilayer membrane so that SOD would remain in the circulation for a longer time (406,407). The product, the so-called SOD liposome, has advantages in enhancing SOD circulation life. However, one of the complications of the SOD liposome is that the enzyme once encapsulated no longer contacts the free radicals directly, the non-polar lipid bilayer separates SOD and oxygen free radicals. Therefore it is necessary to ascertain whether entrapped SOD will effectively scavenge external O_2^- or whether the SOD has to be released from the liposome for scavenging superoxide radicals. The answer to this question depends on the study of the permeability of lipid membranes to superoxide radicals. In our experiments, we have sealed SOD into intact lipid vesicles and incubated SOD-loaded vesicles with an O_2^--generating system. Using both cytochrome c reduction and spin trapping and ESR assays, we were able to show that entrapped SOD could not catalyze the dismutation of superoxide radicals generated outside the vesicles while detergent-lysed SOD vesicles scavenge O_2^- effectively. Two conclusions may be made from these results: (a) the lipid bilayer is likely impermeable to the superoxide anion and (b) SOD liposomes although improving the circulation time of SOD are likely ineffective until the enzyme is released.

In our laboratory, we have used SOD-loaded erythrocyte ghosts as a model for the study of the role of the biological membrane in superoxide radical permeation. The ability of SOD ghosts to scavenge external O_2^-, demonstrated by both depleted spin trapping adduct signals on ESR spectra and suppressed cytochrome c reduction reaction, confirms that superoxide radicals can diffuse freely across the erythrocyte membrane. Since most enzymatic free radical scavengers are membrane impermeable and most oxygen free radicals are locally produced, the finding that the biological membrane is permeable to superoxide radicals is of particular relevance to these new ideas: (a) SOD-enriched erythrocytes may be used as physiological scavengers of O_2^- in blood; (b) a specific carrier system may be designed so that enzymatic scavengers may pass through the membrane to attenuate oxygen free radicals in situ; (c) simultaneous administration of an additional agent that blocks free radicals from diffusing through one membrane to another may limit free radical damage and improve the therapeutic effect of free radical scavengers.

6. SOD Toxicity and Role of Iron

6.1. Introduction

6.1.1. Is a High Dose of SOD Toxic?

The protection of the myocardium by superoxide dismutase against reperfusion injury has been widely investigated and has also been a subject of intense debate. While some studies have shown that SOD limited the susceptibility of the myocardium to reperfusion-induced arrhythmias both in vitro and in vivo (408,409,410), effectively protecting it against global ischemia and limiting infarct size in regional ischemia (411,412), others failed to establish any protection (313,413). The main factors involved in this controversy have been reviewed in a previous section (for more details see 2.6.3.3. in this thesis). Recently the cardioprotective role of SOD has been further challenged by a series of reports that while SOD was protective at low doses, it may exacerbate the damage of reperfused heart at high doses. In the late 1980's, D. J. Hearse's laboratory first published their dose-response studies on the ability of SOD, catalase, and allopurinol to reduce the incidence of reperfusion-induced arrhythmias in the isolated rat heart with transient coronary artery occlusion and reperfusion. Over the wide range of doses they studied (SOD; 8×10^3, 2×10^4, 4×10^4, 6×10^4, 8×10^4, 1.2×10^5 or 1.6×10^5 IU/L), SOD exhibited an asymmetric U-shaped profile with loss of protective efficacy at higher and lower doses, while administration of catalase reduced ventricular fibrillation incidence in a linear dose-dependent manner. At that stage, the basis for this dose-response characteristic, the loss of protection of SOD, remained unkown

(410.414). More recently, J. M. McCord's laboratory reported their limited dose-response curves for SOD in two widely employed models, the hypoxic/ reoxygenated isolated heart (Langendorff rat heart) and the in vivo model of regional coronary artery occlusion and reperfusion, also showed reduced efficacy and toxicity at higher doses of SOD (415). Superoxide dismutase at 7×10^3, 2.1×10^4, 6×10^4, and **1.5×10^5 IU/L** suppressed creatine kinase release in Langendorff rat hearts by 61%, 63%, 72% and **30%** respectively. SOD at 1.5×10^3, 3×10^3, 1.5×10^4, **1.5×10^5 IU/L** suppressed lactate dehydrogenase release in Langendorff rabbit hearts by 32%, 48%, 54% and **12%** respectively. In rabbit hearts subjected to coronary artery ligation and reperfusion in vivo, SOD at 6×10^3, 1.5×10^4 and 4.5×10^4 IU/L reduced infarct size by 10%, 30%, and 19% respectively, however **1.5×10^5 IU/L SOD increased infarct size by 28% (SOD was toxic!)**. They concluded that the SOD dose-response curves in these different models all showed relatively sharp maxima with SOD losing its ability to protect at higher concentrations which was in agreement with the earlier reports of D. J. Hearse's laboratory.

The concept that too much SOD might be detrimental was supported by other published observations. Elroy-Stein et al. (416) showed that the overproduction of CuZnSOD in transfected cells resulted in an enhancement of lipid peroxidation. Ceballos et al. (417) found an increase in the glutathione peroxidase activity in mouse L cells and NS20Y neuroblastoma cells transfected with human SOD, which was an adaptive mechanism to the elevated levels of hydrogen peroxide induced by the high levels of SOD. Kedziora and Bartosz (418) suggested that the abnormalities observed in Down's syndrome were partially due to an imbalance in the reactive oxygen species caused by an excess of SOD. Therefore based on these observations, the dose of SOD was

clearly critical and the low therapeutic index of this drug should be taken into consideration in future trials.

6.1.2. Hypothesis of Mechanism for the Toxicity of SOD

The underlying mechanism for the toxicity of SOD has not been understood. We list below some proposed interpretations of the loss of protective activity at higher SOD doses although none of these interpretations have been either proven or excluded.

(a) Nonspecific effect of the enzyme protein

Enzyme (SOD) protein or some contaminants might have a toxic effect on tissue. This possible mechanism appears to have been ruled out by recent experiments using H_2O_2-inactivated SOD as a control system. J. M. McCord et al. have suggested that both the protective effect of SOD at lower doses and its lack of efficacy seen at higher doses were due to SOD activity rather than protein concentration or, for example, endotoxin contamination (415).

(b) Nonspecific peroxidase activity of SOD

CuZnSOD, in the presence of H_2O_2, can catalyze the peroxidation of a variety of molecules, including ferrocytochrome c and linoleic acid (419). This weak peroxidase activity might explain the toxic effect seen only at higher CuZnSOD doses. However MnSOD, which lacked such peroxidase activity (419), also displayed significant exacerbation of tissue damage at higher doses (420). Therefore, it is unlikely that toxicity of the CuZnSOD at higher doses is due merely to its peroxidase activity.

(c) Enhancement of lipid peroxidation by SOD

J. M. McCord et al. (415) suggested that the hydroperoxyl radical ($HO_2\cdot$), the protonated lipophilic form of O_2^-, might be important for the right balance of the termination of lipid peroxidation as follows: $LOO\cdot + HO_2\cdot \longrightarrow LOOH + O_2$. By this mechanism, overscavenging of superoxide radicals by an increased amount of SOD would eliminate an important termination step of lipid peroxidation, and thus exacerbate the damage. However, no evidence has been available to clarify this mechanism and confirm that the $HO_2\cdot$ can terminate lipid peroxidation reactions.

(d) Activation of EDRF by excess of SOD

Superoxide anion is important for the inactivation of the endothelial-derived vascular relaxation factor (EDRF) (421,422), thus overscavenging of superoxide may lead to an increased activity of EDRF, resulting in extravasation and edema. To our knowledge this hypothesis has not been explored.

(e) Overwhelming hydroxyl radical mechanism

In our laboratory, we have speculated that elevated levels of SOD may induce an increase of the hydrogen peroxide concentration via speeding up superoxide dismutation reaction. This enhancement of hydrogen peroxide possibly overwhelms endogenous catalase activity and promotes the production of highly damaging hydroxyl radicals via the Fenton reaction. The SOD toxicity, therefore, could be attributed to this "explosion" of hydroxyl radicals. The exact status of catalase in most studied systems is unknown, although in a comment in the discussion, McCord (420) suggests that catalase does not reverse the detrimental effect of a high dose of SOD. The transition metals (specifically iron)

may play an important role in the possible mechanism of SOD toxicity by participating in the Fenton reaction.

6.1.3. The Role of Iron in Fenton Reaction

It has been known that O_2^- and H_2O_2 are formed in all aerobic cells. These two species of activated oxygen react with each other to produce hydroxyl radicals with a much higher oxidizing potential and a greater biological hazard. This reduction of H_2O_2 by O_2^- is known as the Haber-Weiss reaction:

$$O_2^- + H_2O_2 \longrightarrow O_2 + OH^- + \cdot OH \qquad (a)$$

However, the rate constant for the Haber-Weiss reaction is low compared to that for competing reactions such as the spontaneous dismutation of O_2^-. Certain transition metals (specifically iron) will catalyze the decomposition of H_2O_2 to form $\cdot OH$, thus a plausible mechanism can be written as follows:

$$O_2^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2 \qquad (b)$$

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (c)$$

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + \cdot OH \qquad (d)$$

$$O_2^- + H_2O_2 \longrightarrow O_2 + OH^- + \cdot OH \qquad (e)$$

The superoxide radical reduces ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) (reaction b), which subsequently decomposes H_2O_2 (formed by dismutation of O_2^-, reaction c) to $\cdot OH$ and OH^-. The combination of reactions is now commonly called an iron-catalyzed Haber-Weiss reaction or superoxide-driven Fenton reaction. By including the participation of iron in oxygen radical reactions, the important point to note is that iron is capable of exacerbating any oxidative stress.

6.1.4. Research Aims

In order to verify the hypothesis that an excess of SOD could induce an enhancement of hydroxyl radicals which are able to cause damage in various cellular components, the present study is focused on mimicking the action of SOD on the reperfused heart in a test tube by mixing SOD with the xanthine/xanthine oxidase system, since the enzyme/substrate system has been considered a source of oxygen free radicals during the reperfusion phase. This is not an entirely hypothetical model of ischemia. Using spin trapping and ESR techniques as a direct measurement of oxygen radicals, we have undertaken a dose-response study of SOD on the enzymatic source of oxygen free radicals in the absence or presence of iron. The results supported our hypothesis that in the presence of iron, SOD promotes more production of hydroxyl radicals as compared to the control system. This Fenton-type hydroxyl radical production depended on both SOD and iron concentration. Further studies with ferric ion (stored form) and ferrous ion (catalytic form) have shown that an explosion of hydroxyl radicals could be attained at a micromolar range of ferrous iron concentration while more ferric iron was needed to display the same extent of hydroxyl radical production.

6.2. Materials and Methods

6.2.1. Materials

Yeast superoxide dismutase was from Carlsberg Biotechnology Ltd (Copenhagen N. Denmark). DMPO was purchased from Sigma Chemical

Company and used after further purification by activated charcoal. Ferric chloride and ferrous chloride were obtained from Fisher Scientific Company (New Jersey, U.S.A.). All other reagents were purchased from Sigma Chemical Company (St Louis, MO U.S.A.). All the buffers were passed through a chelating ion exchange membrane (Bio-Rad Laboratory, Canada) to remove trace metals.

6.2.2. Methods

Fe^{2+} solutions were prepared by mixing $FeCl_2$ and the chelator ADP in degassed H_2O purged with nitrogen (1 Fe^{2+} : 10 ADP). Fe^{3+} solutions were prepared by simply dissolving $FeCl_3$ in water. All iron solutions were prepared fresh and used immediately. Under these circumstances, precipitation of Fe^{3+} as $Fe(OH)_3$ was not a problem.

Electron Spin Resonance spectra were obtained at room temperature in a flat cell placed in the cavity of a Bruker ESP 300 Spectrometer. Measurements were done with field set of 3477 G, a modulation frequency of 100 KHz, and a microwave frequency of 9.76 GHz. SOD dose-response reactions were carried out in 0.05 M HEPES buffer, pH 7.4 containing 0.4 mM xanthine, 100 mM DMPO, 0.05 mM DETAPAC and varied concentrations of iron or SOD. The reactions were initiated by the addition of xanthine oxidase. The intensity of the DMPO-oxygen radical adduct was determined by double integration of their respective peaks on ESR spectra.

6.3. Results

6.3.1. Dependence of SOD Toxicity on Iron

6.3.1.1. Effect of Ferrous Ion

Xanthine/xanthine oxidase generates superoxide radicals, hydroxyl radicals and hydrogen peroxide (for more details see section 4.4.). Since hydroxyl radicals are spin trapped by DMPO at a rate constant of 3.4×10^9 M^{-1} s^{-1} to form a relatively stable DMPO-OH adduct (423), any further formation of hydroxyl radicals caused by the addition of SOD or iron in the xanthine oxidase system can be determined by measuring the increase of DMPO-OH signal. The co-operative role of SOD and iron in relation to the enhancement of hydroxyl radicals was shown in the following experiments which were performed under identical conditions except differing concentrations of Fe^{2+}.

ESR spectra in Figure 17a and 17b show that under the identical experimental conditions, addition of 2 μM ferrous ion did not cause a change of the hydroxyl radical signal in the xanthine/xanthine oxidase system. Addition of SOD at an activity of 4×10^4 IU/L completely inhibited superoxide radical signals in the control system but not the hydroxyl radical peaks which may arise from the enzymatic reaction of xanthine oxidase (Figure 17c). However, when Fe^{2+} and SOD at the above concentrations were combined (Figure 17d), the intensity of hydroxyl radical peaks were 10-fold higher than those in the absence of iron (Figure 17c) indicating that SOD has the potential to promote the Fenton-type reaction to form more ·OH even at very low concentration of Fe^{2+} ions.

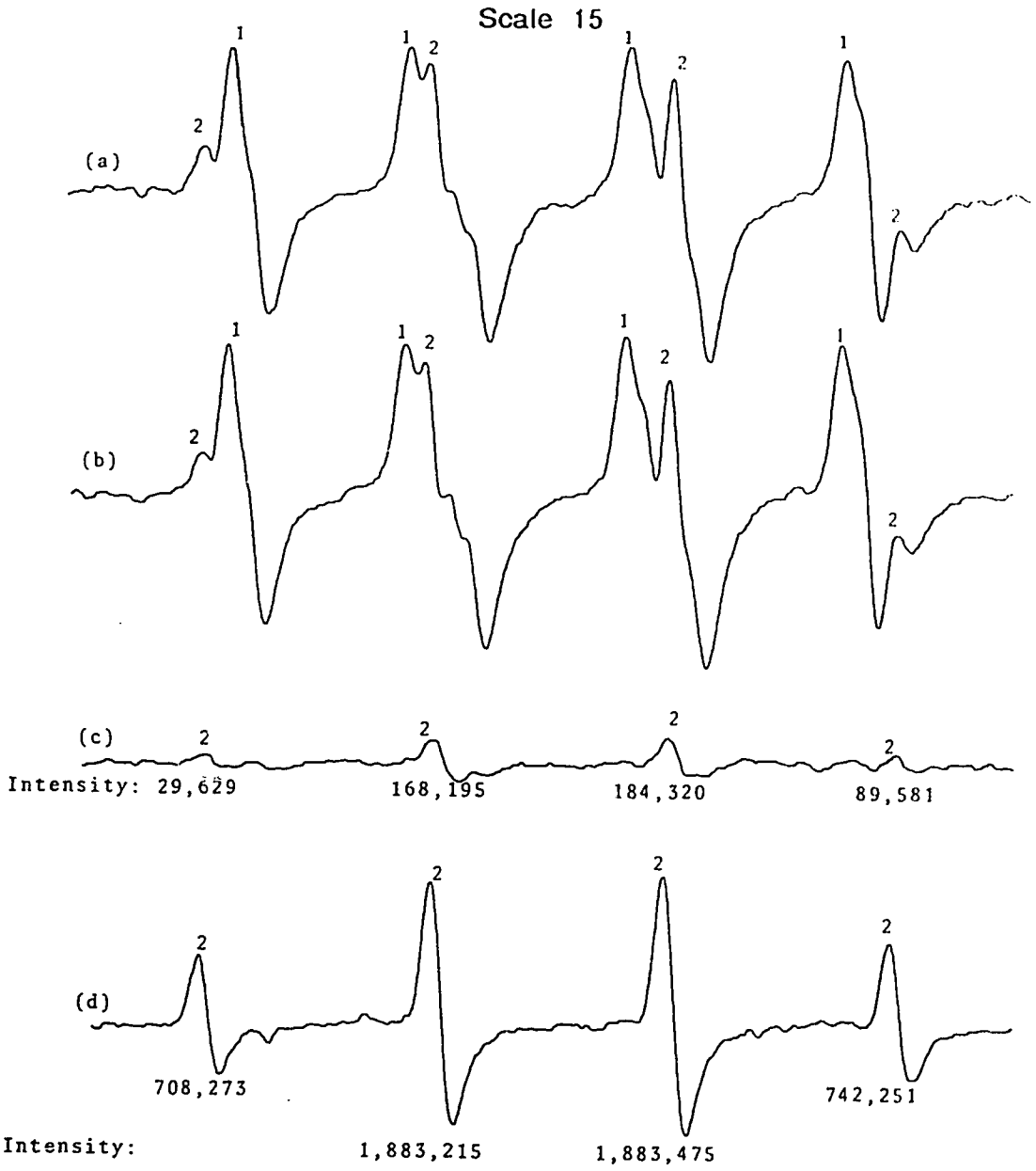


Figure 17: Effect of 2 μM of ferrous ion on the formation of hydroxyl radicals in the presence of SOD. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of (a) phosphate control; (b) 2 μM Fe^{2+}; (c) 40 units/ml SOD; (d) 2 μM Fe^{2+} and 40 units/ml SOD.

Additional studies of the participation of Fe^{2+} and SOD in the enhancement of hydroxyl radicals are shown in Figures 18 and 19. Comparison of Figure 18a with 18b indicates that an increase of Fe^{2+} from 2 μM to 10 μM without SOD only slightly stimulated the formation of hydroxyl radical signals (peak 2) at the expense of superoxide radical signals (peak 1). Superoxide radicals under these conditions may have provided a limited amount of H_2O_2 via its spontaneous dismutation reaction to facilitate the formation of ·OH. In contrast, increasing Fe^{2+} to 10 μM, in the presence of SOD, resulted in a remarkable increase of the total amount of hydroxyl radicals. As shown in Figure 18d, the intensity of the main DMPO-OH peak obtained from the reaction mixture containing both SOD and 10 μM Fe^{2+} was 2,509,442 which was 15 times the corresponding value of the reaction mixture without iron (Figure 18c). These observations point to the possibility of potential SOD toxicity because hydroxyl radicals are much more toxic than superoxide radicals.

When the concentration of Fe^{2+} reached 20 μM, the combination of SOD and Fe^{2+} caused an explosive production of hydroxyl radical. Figure 19d demonstrates that the intensity of hydroxyl radical spin adduct of the sample containing SOD and 20 μM Fe^{2+} increased nearly 30-fold as compared to that of the sample containing SOD only (Figure 19c) , and was even greater than the initial hydroxyl radical signals derived from the reaction of xanthine oxidase (Figure 19a and 19b). However, the ESR spectrum shown in Figure 19b demonstrates that 20 μM Fe^{2+} itself only marginally raised the DMPO-OH signal probably due to the increase of decomposition of DMPO-OOH to DMPO-OH. These observations (Figures 17,18,19) support our hypothesis that in the presence of Fe^{2+} , SOD actually facilitates an ongoing Fenton-type reaction to

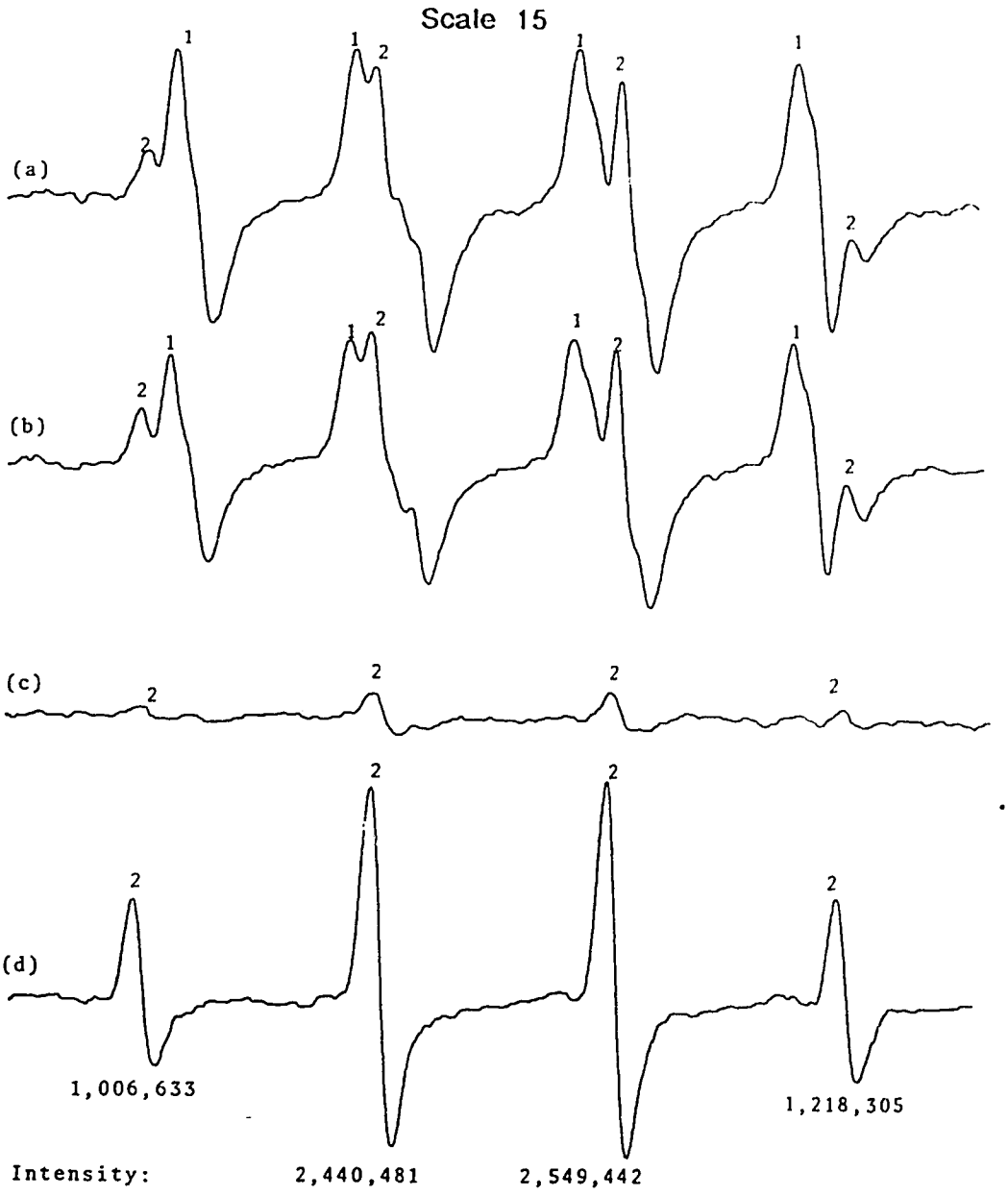


Figure 18: Effect of 10 μM of ferrous ion on the formation of hydroxyl radicals in the presence of SOD. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of (a) phosphate control; (b) 10 μM Fe^{2+}; (c) 40 units/ml SOD; (d) 10 μM Fe^{2+} and 40 units/ml SOD.

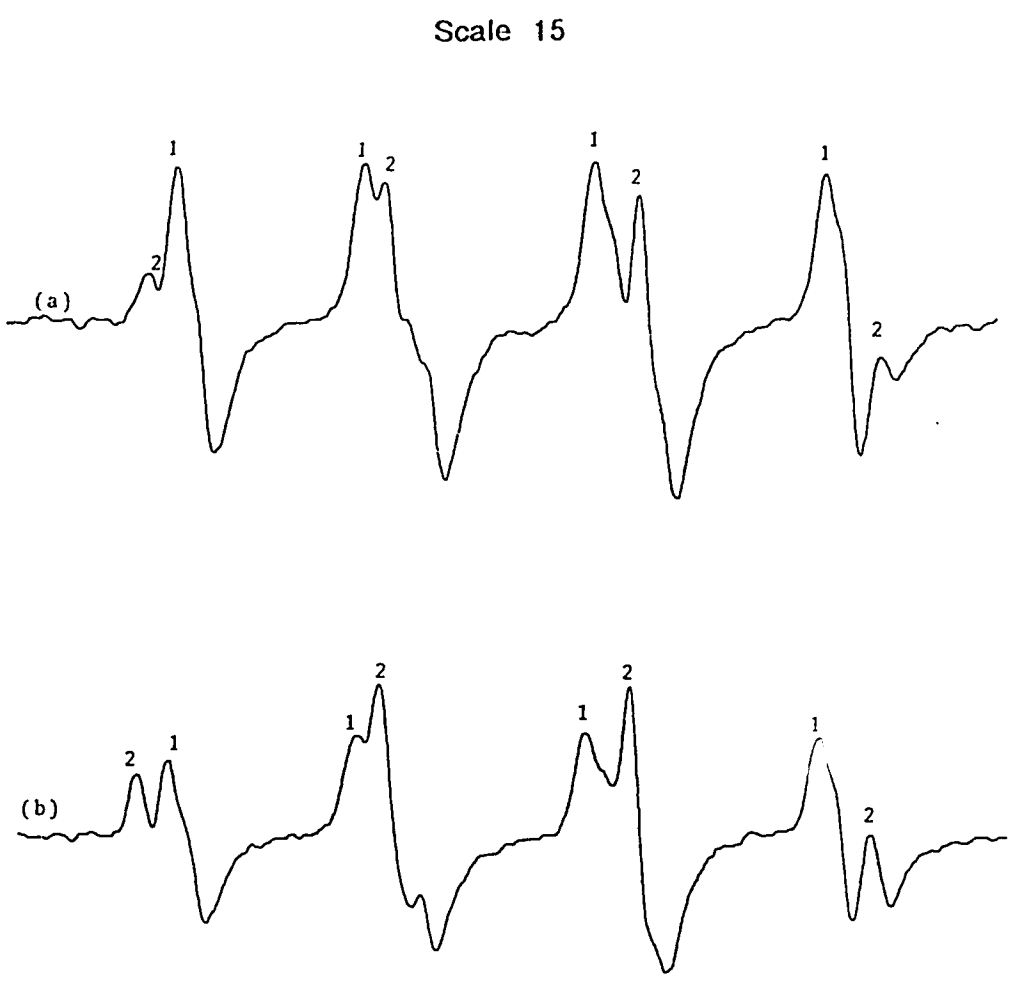


Figure 19: Effect of 20 μM of ferrous ion on the formation of hydroxyl radicals in the presence of SOD. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of (a) phosphate control; (b) 20 μM Fe^{2+}; (c) 40 units/ml SOD; (d) 20 μM Fe^{2+} and 40 units/ml SOD.

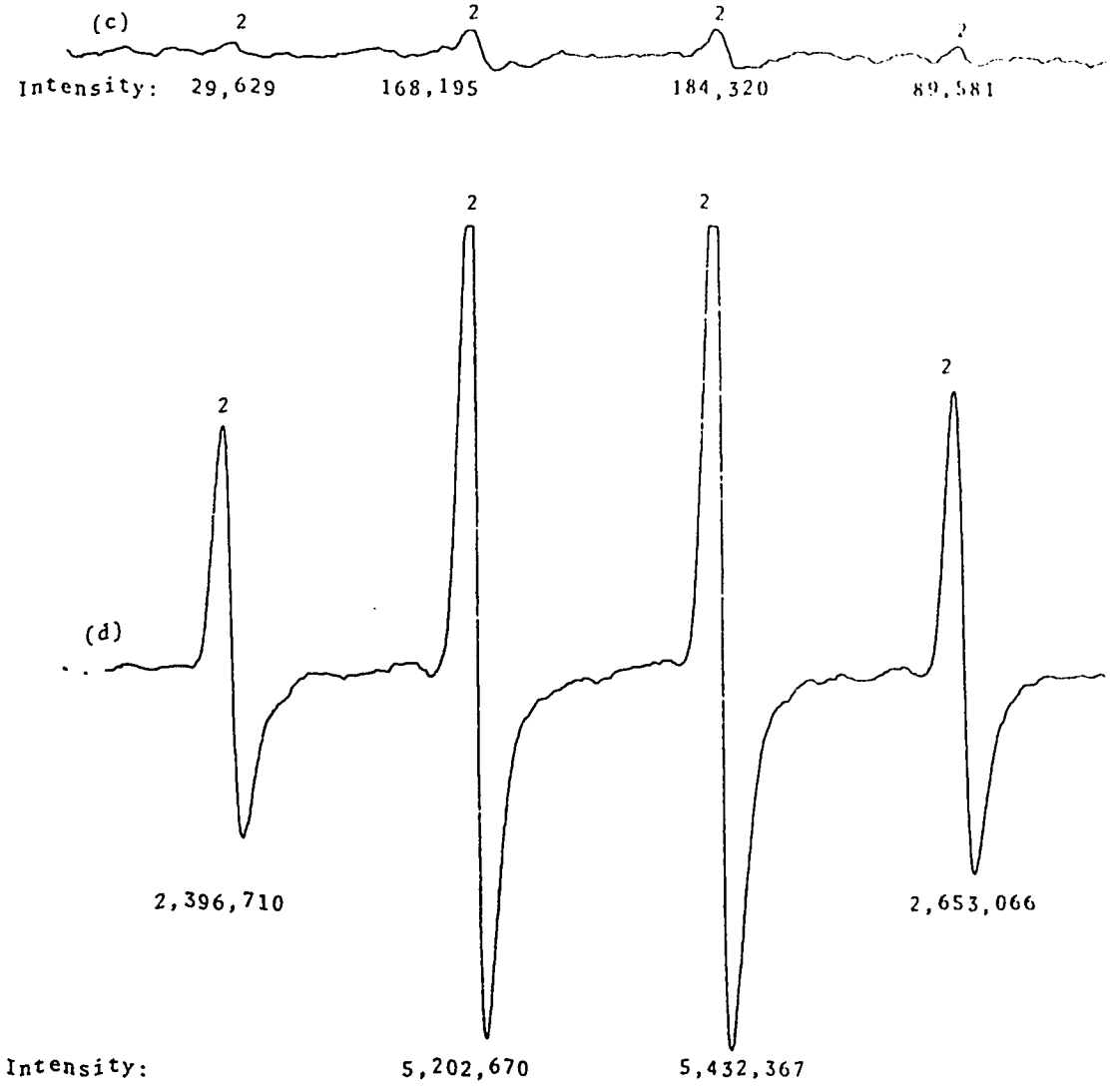


Figure 19: Effect of 20 μM of ferrous ion on the formation of hydroxyl radicals in the presence of SOD. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of (a) phosphate control; (b) 20 μM Fe^{2+}; (c) 40 units/ml SOD; (d) 20 μM Fe^{2+} and 40 units/ml SOD.

form more hydroxyl radicals by supplying more hydrogen peroxide. The consequences of this process might aggravate the oxygen stress damage.

A more complete study of the effect of Fe^{2+} concentration and SOD on the formation of hydroxyl radical has been carried out. The results are shown in Figure 20 by plotting the height of the third DMPO-OH adduct peak (the height of peaks were picked up and calculated by Bruker ESP 300 Spectrometer) against the Fe^{2+} concentration at a fixed SOD concentration. There appeared to be a sharp maximum of the hydroxyl radical spin adduct accumulation corresponding to the concentration of 20 μM Fe^{2+}. When the Fe^{2+} concentration was higher than 20 μM the net production of hydroxyl radicals was progressively lost, corresponding with the depressed production of oxygen free radicals by xanthine oxidase (Figure 21). The latter phenomenon (Figures 21a and 21c) may be attributed to the inhibition of xanthine oxidase by an excess of Fe^{2+} since the enzyme has been shown to be susceptible to metal ions during its catalytic cycle (424). The depressed net generation of oxygen free radicals by xanthine oxidase could in turn result in the decrease of DMPO-OH signals in Figures 21b and 21d, probably due to (a) the decrease in available superoxide radicals to be converted to hydrogen peroxide, leading to decreased hydroxyl radical production via the Fenton reaction; (b) the decrease of hydroxyl radicals directly derived from the reaction of xanthine oxidase.

ESR spectra shown in Figures 22a and 22b are obtained from replacing Fe^{2+}-ADP (ADP acted as a weak chelator) by Fe^{2+}-DETAPAC (1:5) in the xanthine oxidase system. Since DETAPAC is a strong iron chelator and Fe^{2+}-DETAPAC is not capable of inhibiting xanthine oxidase (425), the iron inhibition of xanthine

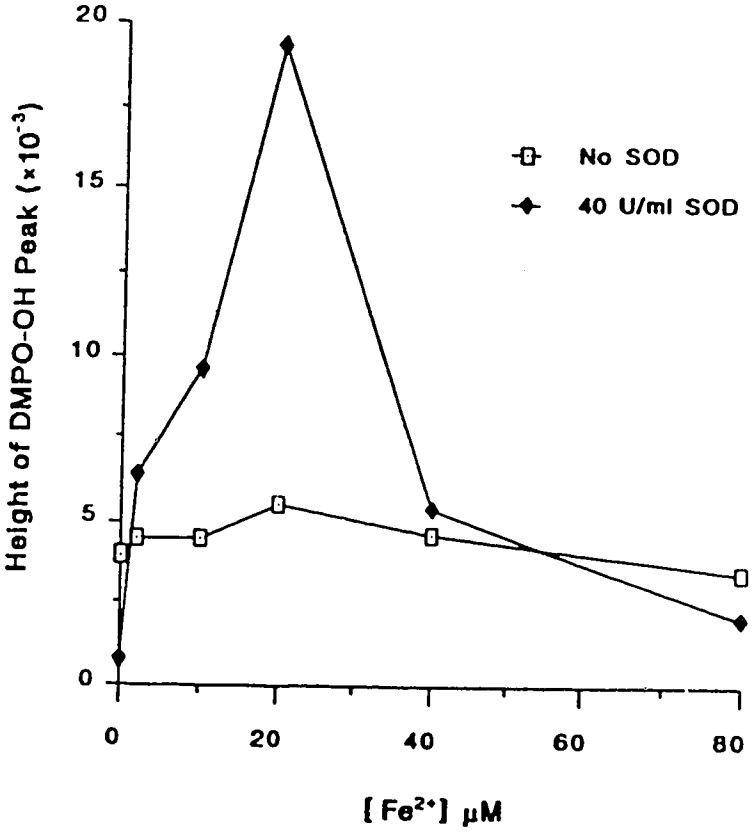


Figure 20: Dependence of the maximum level of accumulated hydroxyl radicals on the concentration of Fe^{2+}. The xanthine/xanthine oxidase reactions were carried out in the presence of (upper line) and absence of (bottom line) SOD. The varied amounts of Fe^{2+} were added in the system. In each case, the height of DMPO-OH peak was plotted against the Fe^{2+} concentration.

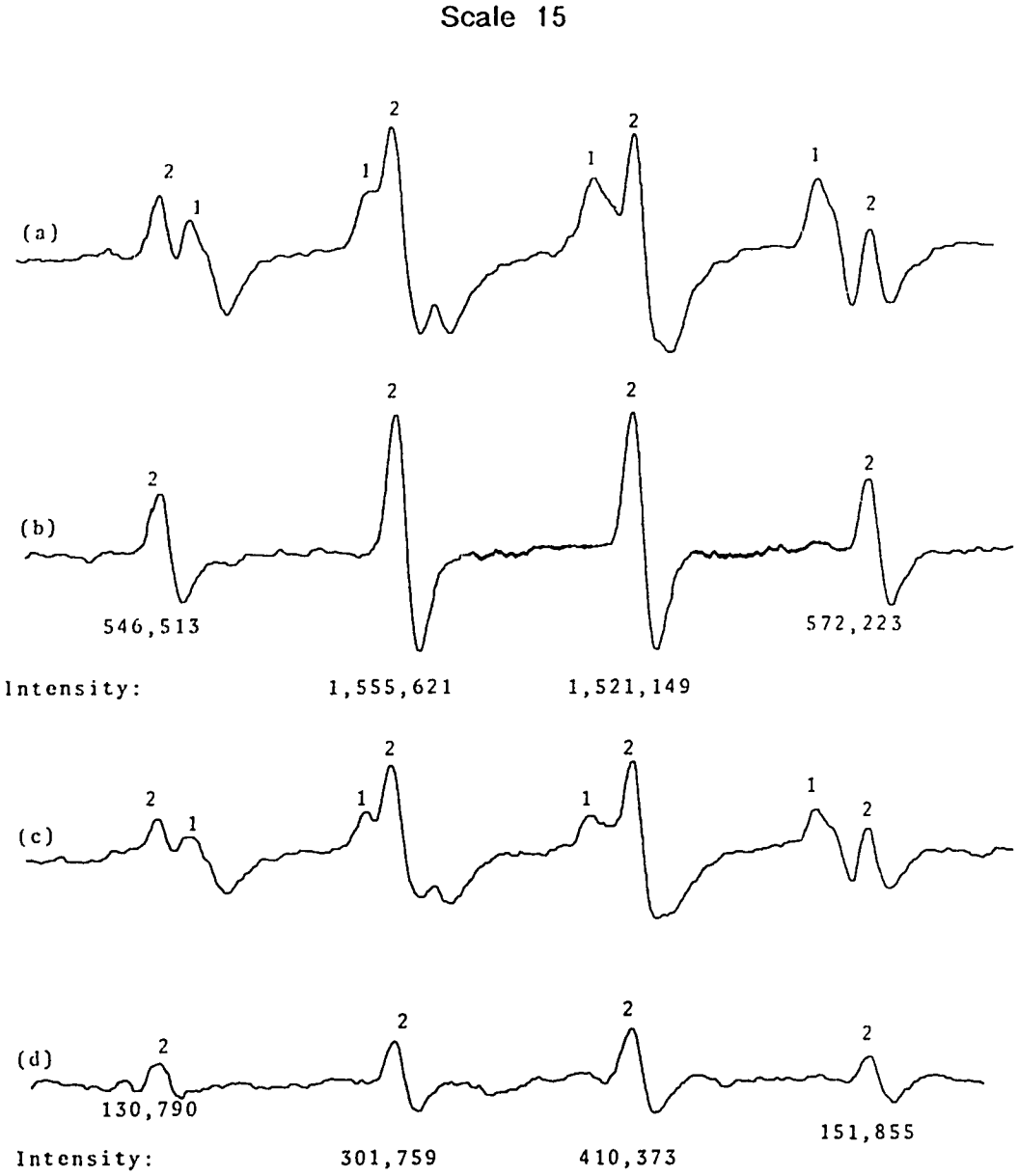


Figure 21: Effect of high concentrations of ferrous ion on the formation of hydroxyl radicals in the presence of SOD. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of (a) 40 μM Fe^{2+}; (b) 40 μM Fe^{2+} and 40 units/ml SOD; (c) 80 μM Fe^{2+}; (d) 80 μM Fe^{2+} and 40 units/ml SOD.

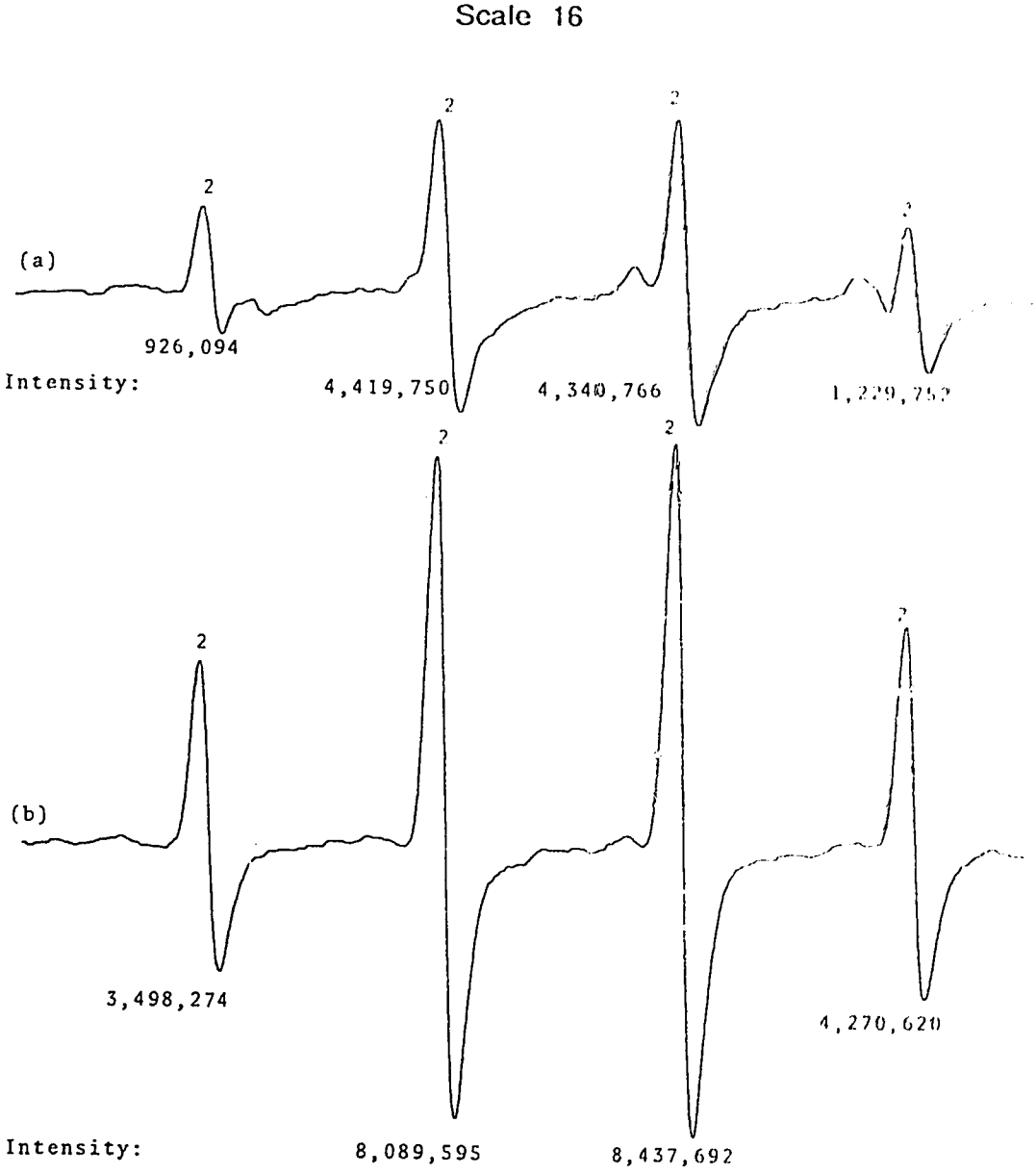


Figure 22: Effect of high concentrations of Fe^{2+}-DETAPAC on the formation of hydroxyl radicals in the presence of SOD. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of (a) 40 µM Fe^{2+}-DETAPAC; (b) 40 µM Fe^{2+}-DETAPAC and 40 units/ml SOD; (c) 80 µM Fe^{2+}-DETAPAC; (d) 80 µM Fe^{2+}-DETAPAC and 40 units/ml SOD.

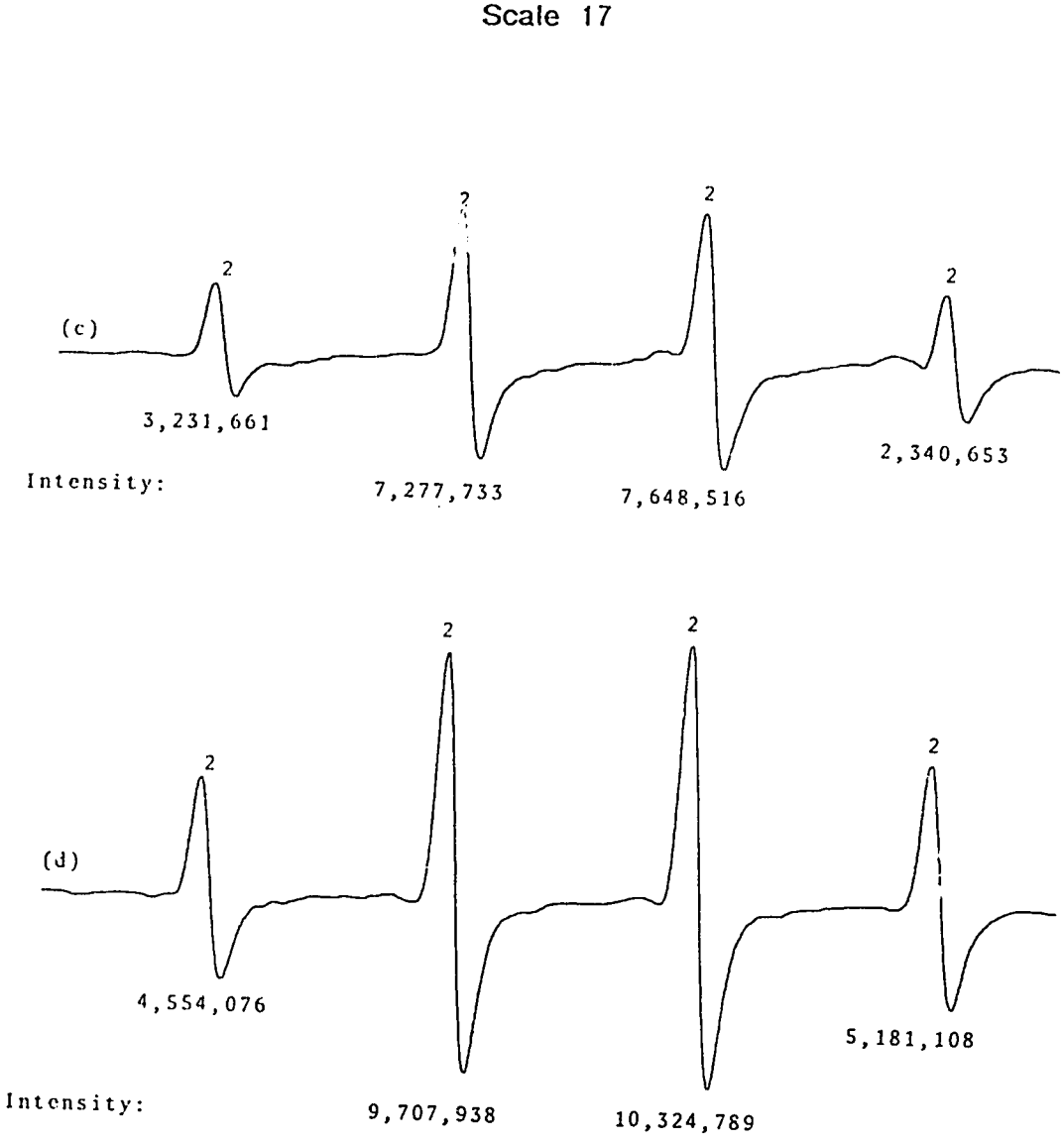


Figure 22: Continued.

oxidase thereby was removed. In addition, Fe^{2+}-DETAPAC would facilitate the decomposion of DMPO-OOH to DMPO-OH and DETAPAC would strengthen DMPO spin trapped adducts by an unknown mechanism, although several papers have reported that Fe^{2+}-DETAPAC is a more efficient Fenton reagent for the formation of hydroxyl radicals (426,427). Therefore as the concentration of Fe^{2+}-DETAPAC increased to 80 μM, corresponding larger DMPO-OH signals were observed (Figure 22). Superoxide dismutase still exhibited the ability to enhance hydroxyl radical formation at high Fe^{2+}-DETAPAC concentrations. Figure 22c and 22d demonstrate that SOD caused a progressive increase of ·OH proportional to the Fe^{2+}-DETAPAC concentration.

6.3.1.2. Effect of Ferric Ion

Ferric ion was also capable of catalyzing the formation of hydroxyl radicals. According to the superoxide-driven Fenton reaction scheme, Fe^{3+} added to the xanthine oxidase system must first be reduced to the ferrous form by superoxide radicals before reacting with H_2O_2. Thus, more Fe^{3+} was needed in the reaction system to exert the similar effect as Fe^{2+} on the formation of hydroxyl radicals. ESR spectra in Figure 23 represent a consequence of the reaction of xanthine with xanthine oxidase in the presence of 0.1 mM, 0.3 mM, 0.5 mM $FeCl_3$ respectively. At the low concentration of Fe^{3+} (0.1 mM), partial conversion of DMPO-OOH to DMPO-OH was found (Figure 23b) while at higher concentrations of Fe^{3+}, more DMPO-OH signals were formed with a disappearance of DMPO-OOH signals. This phenomenon could be attributed to Fe^{3+}-facilitated decomposition of DMPO-OOH to DMPO-OH and /or superoxide-mediated reduction of Fe^{3+} to Fe^{2+} which in turn reacts with H_2O_2 to generate hydroxyl radicals. Thus, the DMPO-OH increased at the expense of DMPO-OOH signals.

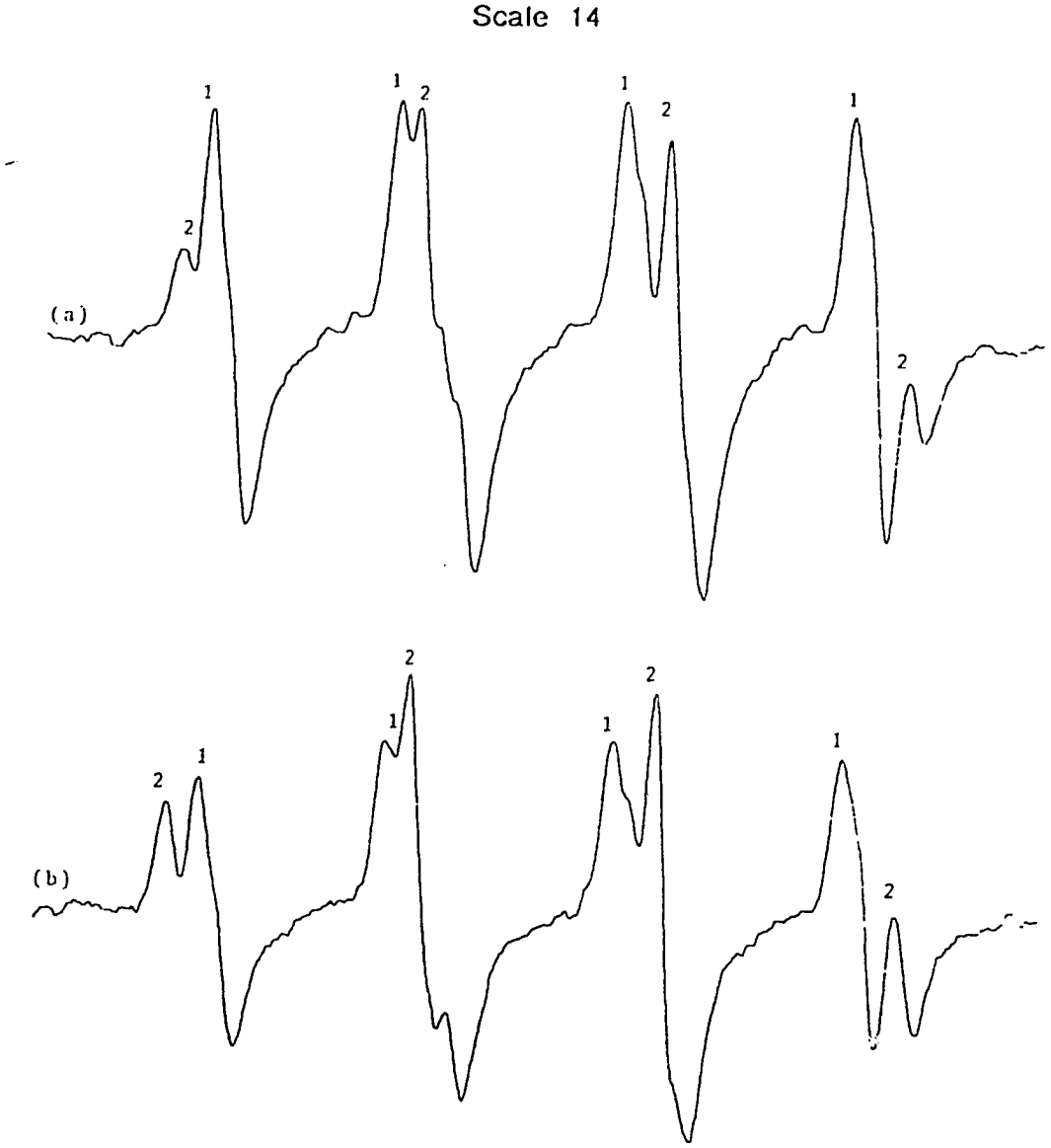


Figure 23: Effect of ferric ion on the formation of hydroxyl radicals in the absence of SOD. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of (a) phosphate control; (b) 0.1 mM Fe^{3+}; (c) 0.3 mM Fe^{3+}; (d) 0.5 mM Fe^{3+}.

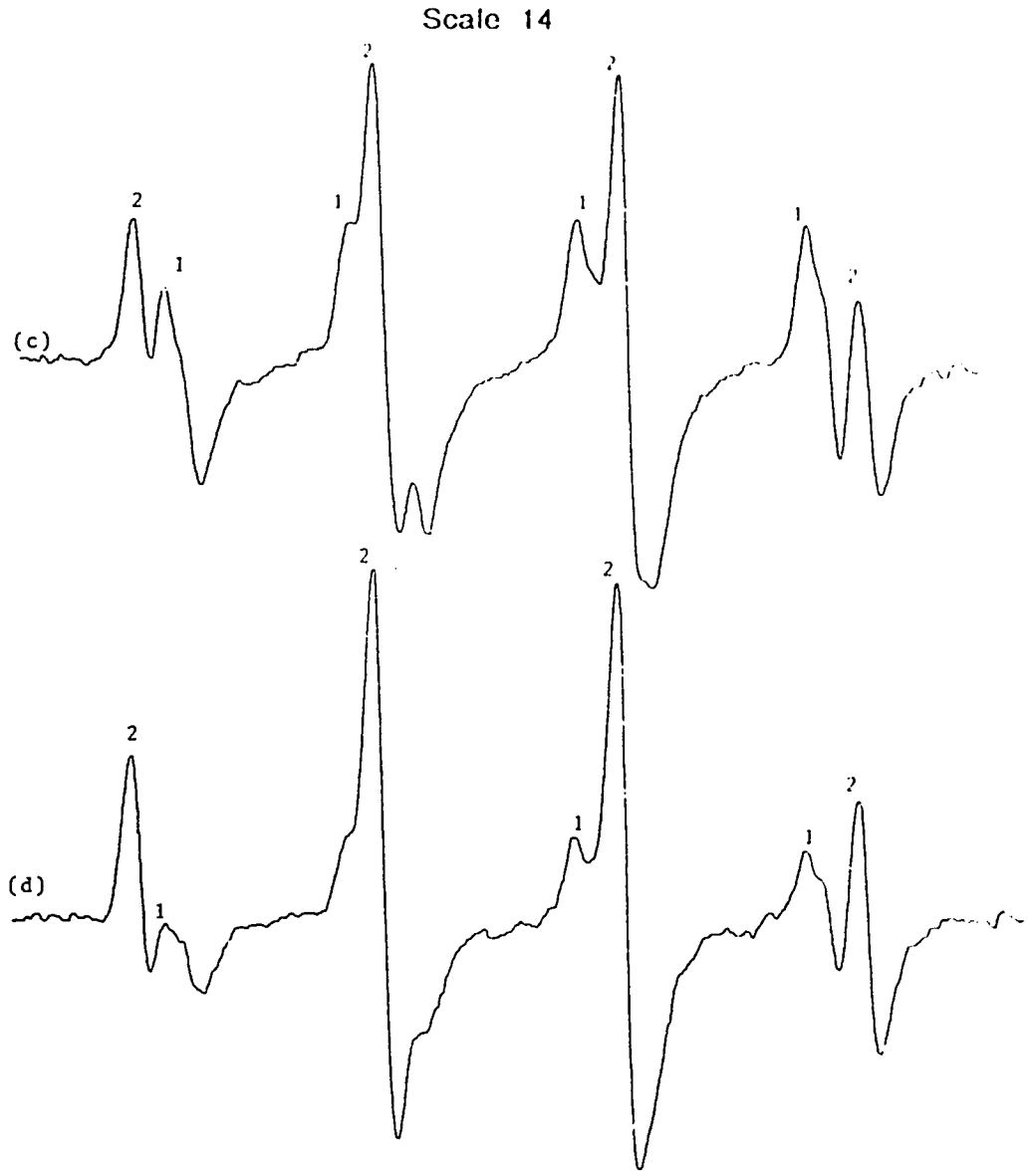


Figure 23: Continued.

ESR spectra shown in Figure 24 were obtained under the same experimental conditions as in Figure 23 except that SOD was added to the reaction system. At 0.1 mM concentration of Fe^{3+}, SOD depleted all DMPO-OOH signals and a portion of the DMPO-OH signals. However, at higher concentrations of Fe^{3+} (0.3 mM and 0.5 mM), SOD eventually accelerated the production of hydroxyl radicals resulting in much stronger DMPO-OH signals (Figures 24b and 24c) than those formed in the absence of SOD. The comparison of DMPO-OH peak intensities have also shown that the amount of hydroxyl radicals generated in the presence of SOD and 0.5 mM Fe^{3+} were 4 to 5-fold of those generated in the presence of SOD and 0.1 mM Fe^{3+}. These results suggest that the addition of SOD to the xanthine oxidase system in the presence of Fe^{3+} created a situation in which SOD and Fe^{3+} competed with one another for the superoxide. When the Fe^{3+} concentration was low (0.1 mM), iron was unable to compete effectively with SOD thus a large portion of the superoxide radicals would be scavenged by SOD rather than being used for reducing Fe^{3+} to Fe^{2+}. Consequently, the production of hydroxyl radicals was retarded since iron must be in its reduced form (Fe^{2+}) to catalyze the formation of hydroxyl radicals from hydrogen peroxide. When the proportions of SOD and Fe^{3+} in the system were such that Fe^{3+} successfully competed with SOD for available superoxide as at higher concentrations of Fe^{3+}, SOD would actually increase the rate of hydroxyl radical formation by increasing the production of hydrogen peroxide. If this competitive mechanism is true, optimal administration of SOD under such conditions as iron-overload might have to be taken into special consideration or it may impose a risk of toxicity.

A more extensive study of the effect of Fe^{3+} revealed a nearly similar reaction pattern as that of Fe^{2+} but with a concentration shift. The height of the DMPO-

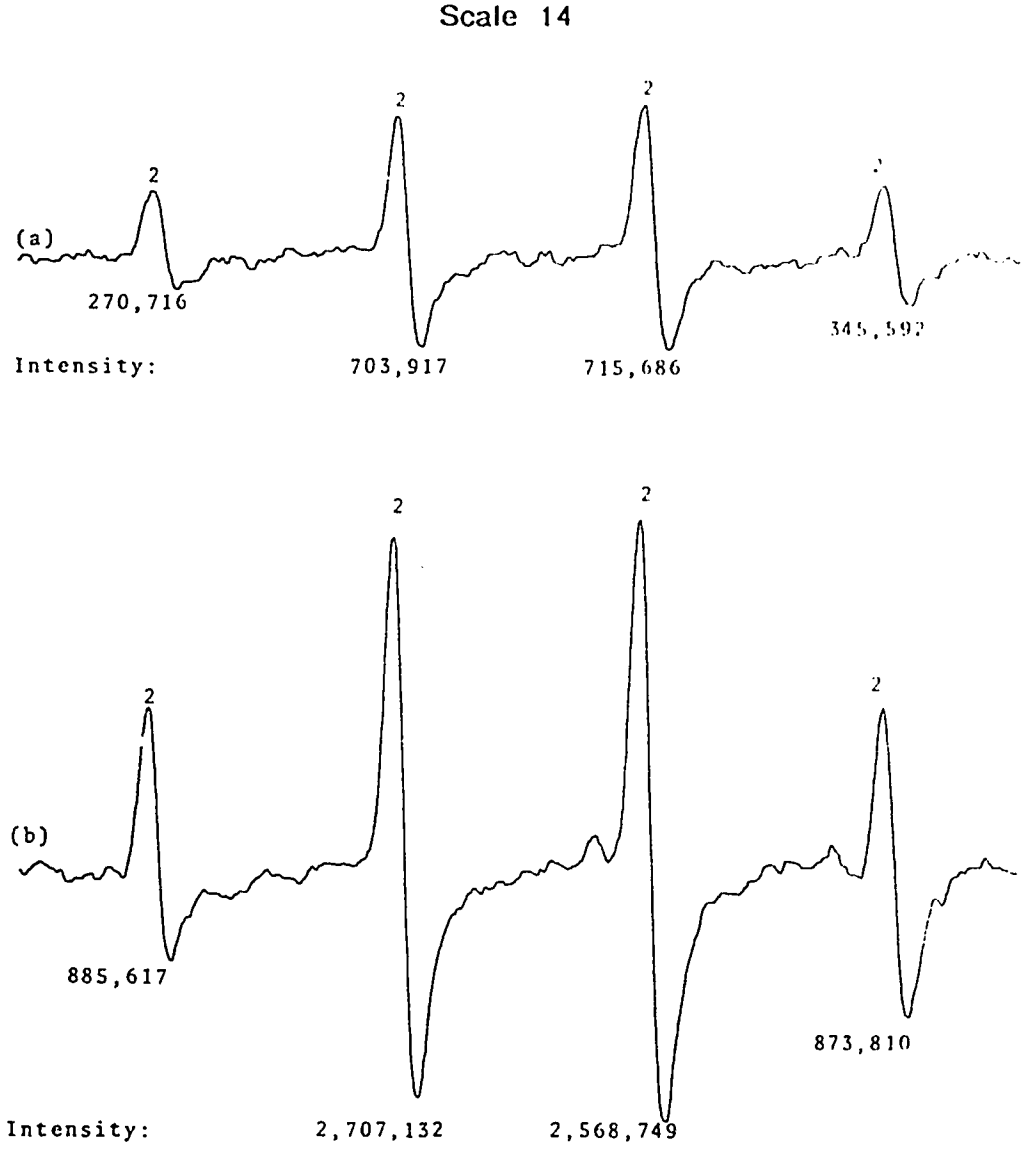


Figure 24: Effect of ferric ion on the formation of hydroxyl radicals in the presence of SOD. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of (a) 0.1 mM Fe^{3+} and 40 units/ml SOD; (b) 0.3 mM Fe^{3+} and 40 units/ml SOD; (c) 0.5 mM Fe^{3+} and 40 units/ml SOD.

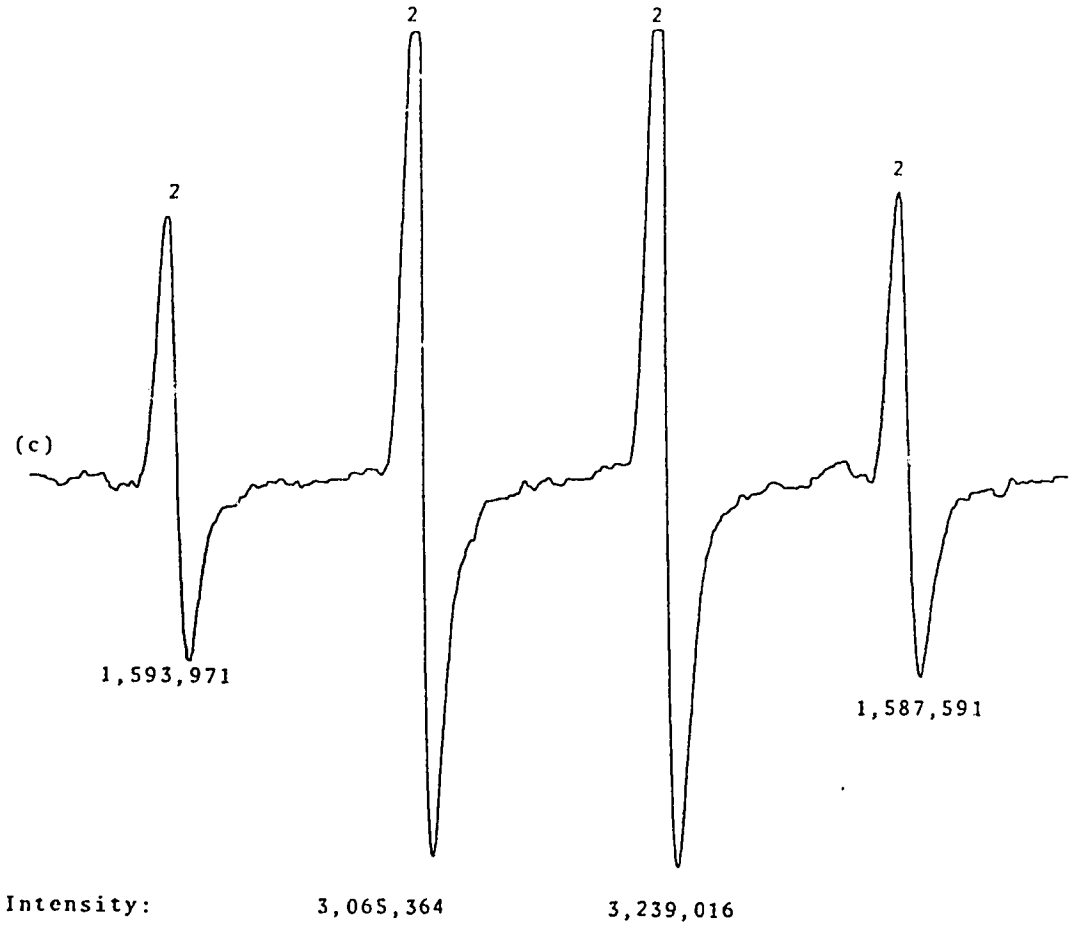


Figure 24: Continued.

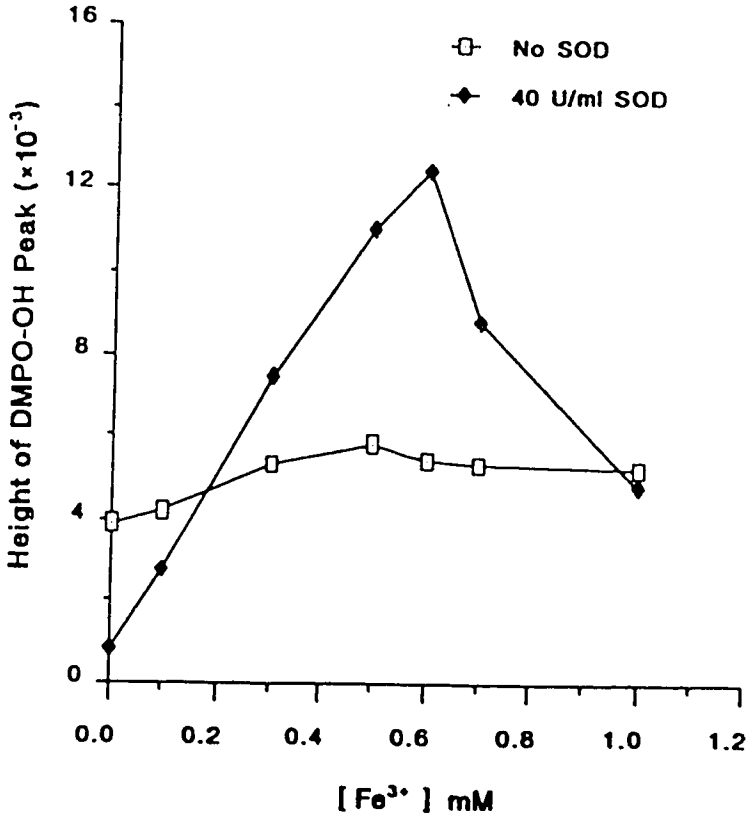


Figure 25: Dependence of the maximum level of accumulated hydroxyl radicals on the concentration of Fe^{3+}. The xanthine/xanthine oxidase reactions were carried out in the presence of (upper line) and absence of (bottom line) SOD. Varied amounts of Fe^{3+} were added to the system. In each case, the height of DMPO-OH peak was plotted against the Fe^{3+} concentration.

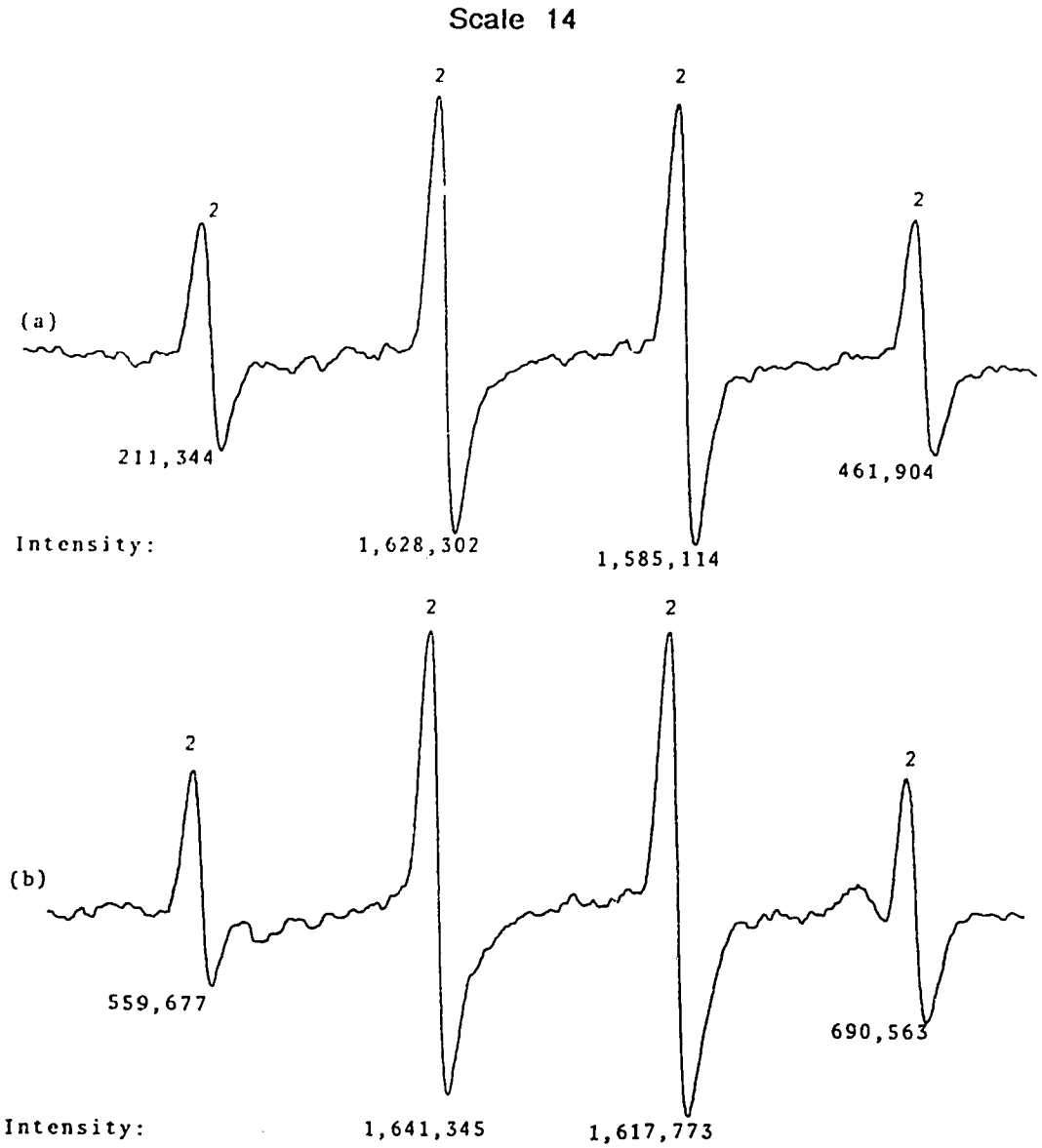


Figure 26: Effect of high concentration of ferric ion on the formation of hydroxyl radicals in the presence of SOD. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of (a) 0.7 mM Fe^{3+}; (b) 0.7 mM Fe^{3+} and 40 units/ml SOD.

OH peak (third peak) versus concentration of Fe^{3+} was plotted in Figure 25. From low to high concentrations of Fe^{3+}, in the absence of SOD, a slight net increase of hydroxyl radical spin adduct was observed. This may partly be due to the decomposition of the DMPO-OOH adduct facilitated by Fe^{3+} and partly due to increased Fenton reaction. As just discussed, SOD reduced DMPO-OH adduct signals when the concentration of Fe^{3+} was low and promoted the formation of the signals as the amount of Fe^{3+} increased. When the concentration of Fe^{3+} reached 0.7 mM or above, the formation of hydroxyl radicals was retarded. A possible explanation is that xanthine oxidase may also be inhibited by high concentrations of Fe^{3+} as was suggested for the effect of Fe^{2+} (Figure 26). The short supply of superoxide radicals caused by the inactivation of xanthine oxidase might have limited the reduction of Fe^{3+}, which in turn limited the formation of hydroxyl radicals via the Fenton reaction.

6.3.2. Dose-Response of SOD in the Absence of Iron

To characterize the hydroxyl radical-inducing effect of SOD in the absence of iron, a series of doses (5×10^3, 1×10^4, 2×10^4, 4×10^4, 8×10^4, 3×10^5 IU/L) of SOD were added to the xanthine/xanthine oxidase system. The free radicals were spin trapped with DMPO and observed by ESR. As shown in Figure 27, SOD completely inhibited superoxide radical signals, but none of the doses of SOD used had a significant effect on the remaining hydroxyl radical signals, neither abolishing nor enhancing them. These remaining hydroxyl radicals could be derived from direct enzymatic reduction of H_2O_2 by xanthine oxidase in the absence of iron. It is unlikely that merely a large amount of SOD would trigger the Fenton-type reaction to increase the production of hydroxyl radicals.

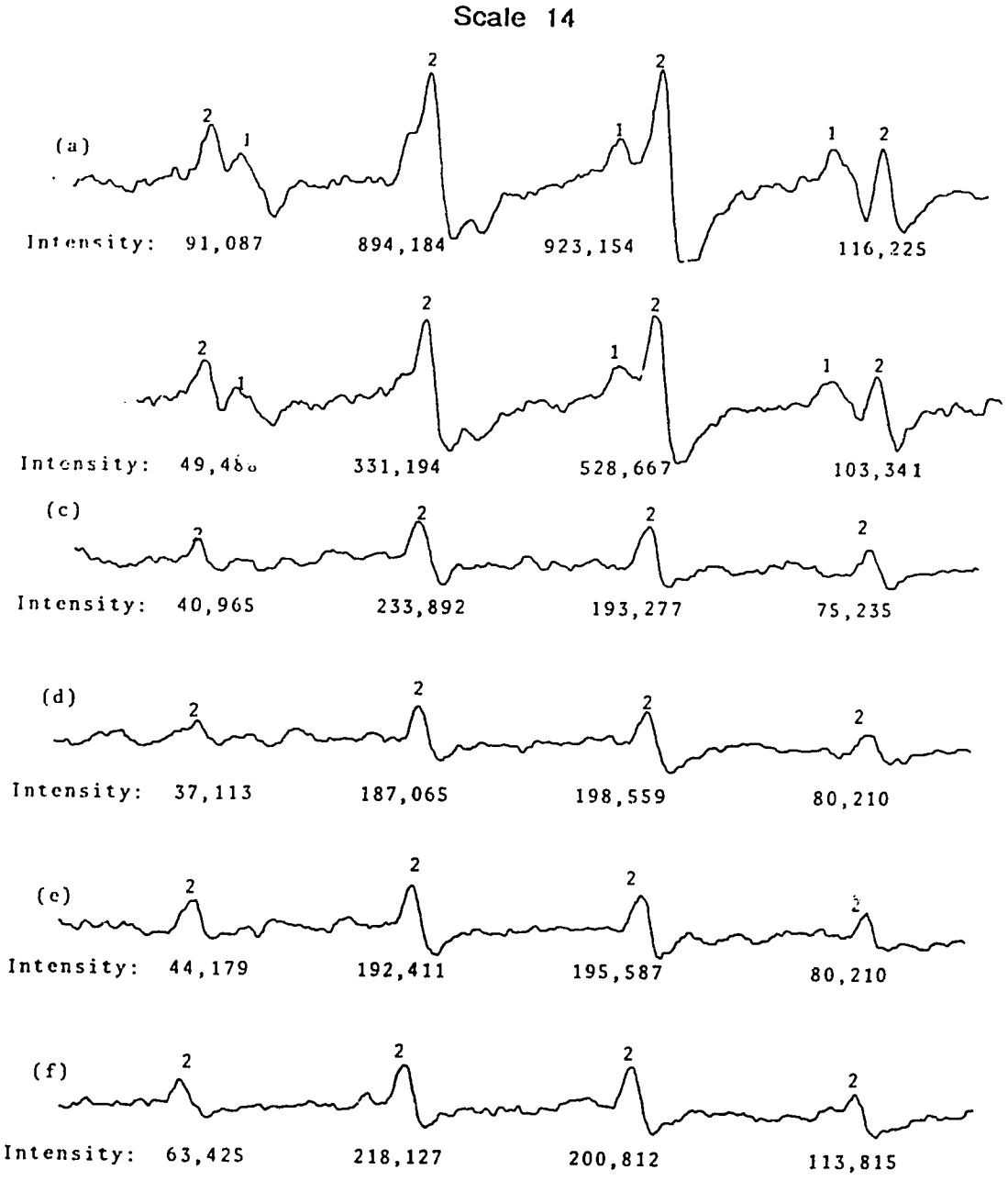


Figure 27: Dose-response characteristics of SOD on enzymatic source of oxygen free radical in the absence of iron. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of (a), 5 units/ml SOD; (b), 10 units/ml SOD; (c), 20 units/ml SOD; (d), 40 units/ml SOD; (e), 80 units/ml SOD; (f), 300 units/ml SOD.

6.3.3. Dose-Response of SOD in the Presence of Ferrous Ion

With the inclusion of iron in the reaction medium, SOD tended to increase the formation of hydroxyl radicals as discussed in section 6.3.1.. In order to study the dependence of the formation of hydroxyl radicals on the concentration of SOD initially added, six doses of SOD (5×10^3, 1×10^4, 2×10^4, 4×10^4, 8×10^4, 3×10^5 IU/L) were added to the xanthine oxidase system which contained 20 μM Fe^{2+} and 100 mM DMPO. After initiating the reaction by xanthine oxidase, free radical spin adducts were recorded in the ESR spectra shown in Figure 28. We found that in the presence of SOD and 20 μM Fe^{2+}, although the DMPO-superoxide adduct signal had disappeared, the intensity of the DMPO-hydroxyl adduct had increased as a function of the SOD dose. A DMPO-hydroxyl signal plateau appeared coincident with SOD activity of 3×10^5 IU/L. At SOD concentrations above 3×10^5 IU/L, however, no further significant enhancement of DMPO-OH signal was observed suggesting two possibilities, (a) all the ferrous ions had been oxidized to the ferric ions during the reduction of H_2O_2 , thus the ongoing Fenton reaction was sustained by the superoxide-driven recycling of iron from the oxidized (ferric) to the reduced (ferrous) form; (b) all the superoxide radicals produced by xanthine oxidase under this condition had been utilized, thus no more hydrogen peroxide would be formed from superoxide radicals to facilitate the ongoing hydroxyl radical formation even with a further increase of SOD .

To compare the above ESR data with those of the control system, we plotted the height of DMPO-OH peak (third peak) of Figure 27 (absence of Fe^{2+}) and Figure 28 (presence of 20 μM Fe^{2+}) against respective SOD concentrations in Figure 29. It clearly illustrates that in the absence of iron, the addition of SOD to

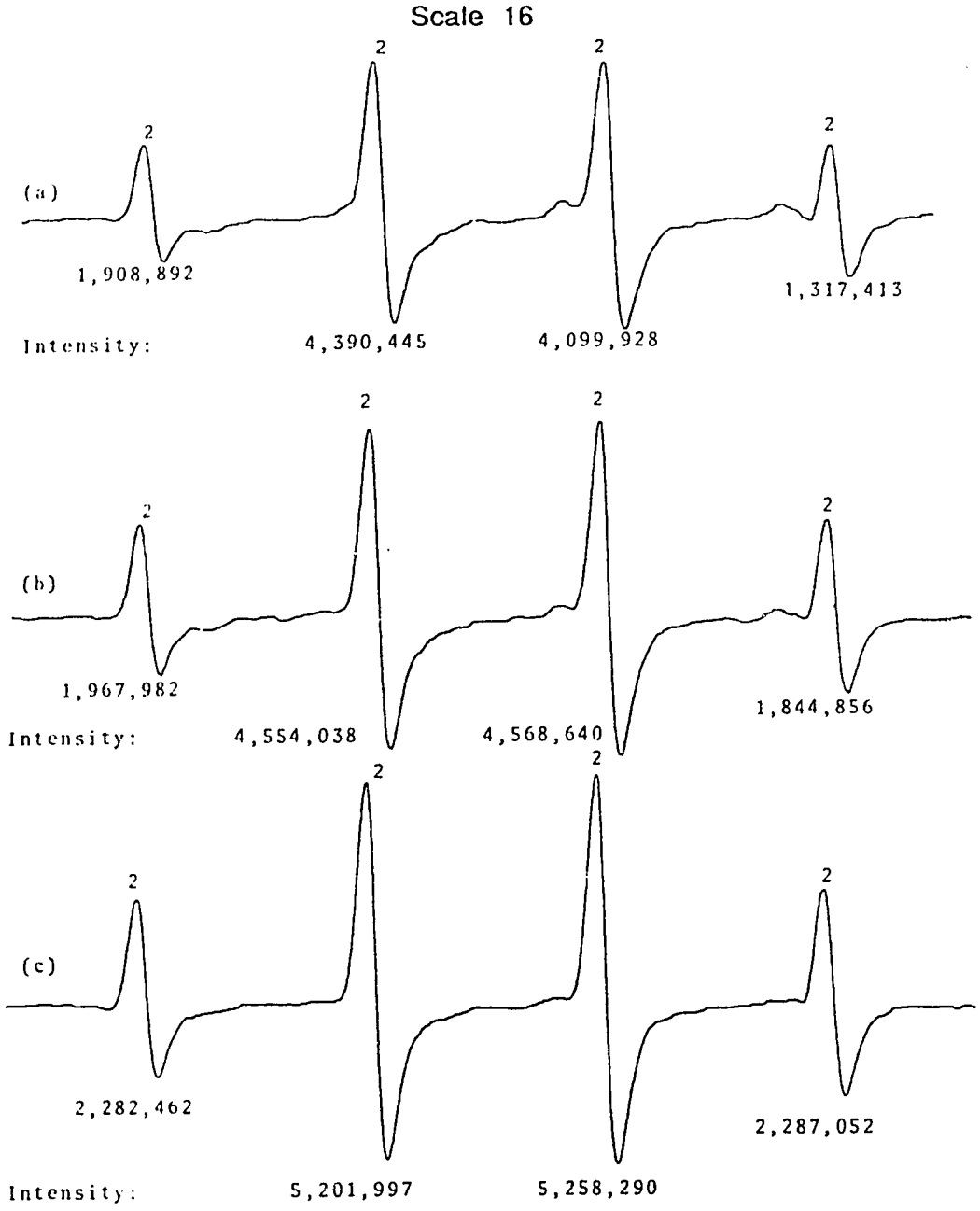


Figure 28: Dose-response characteristics of SOD on enhancing the formation of hydroxyl radical in the presence of iron. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of 20 μM Fe^{2+} and (a), 5 units/ml SOD; (b), 10 units/ml SOD; (c), 20 units/ml SOD; (d), 40 units/ml SOD; (e), 80 units/ml SOD; (f), 300 units/ml SOD.

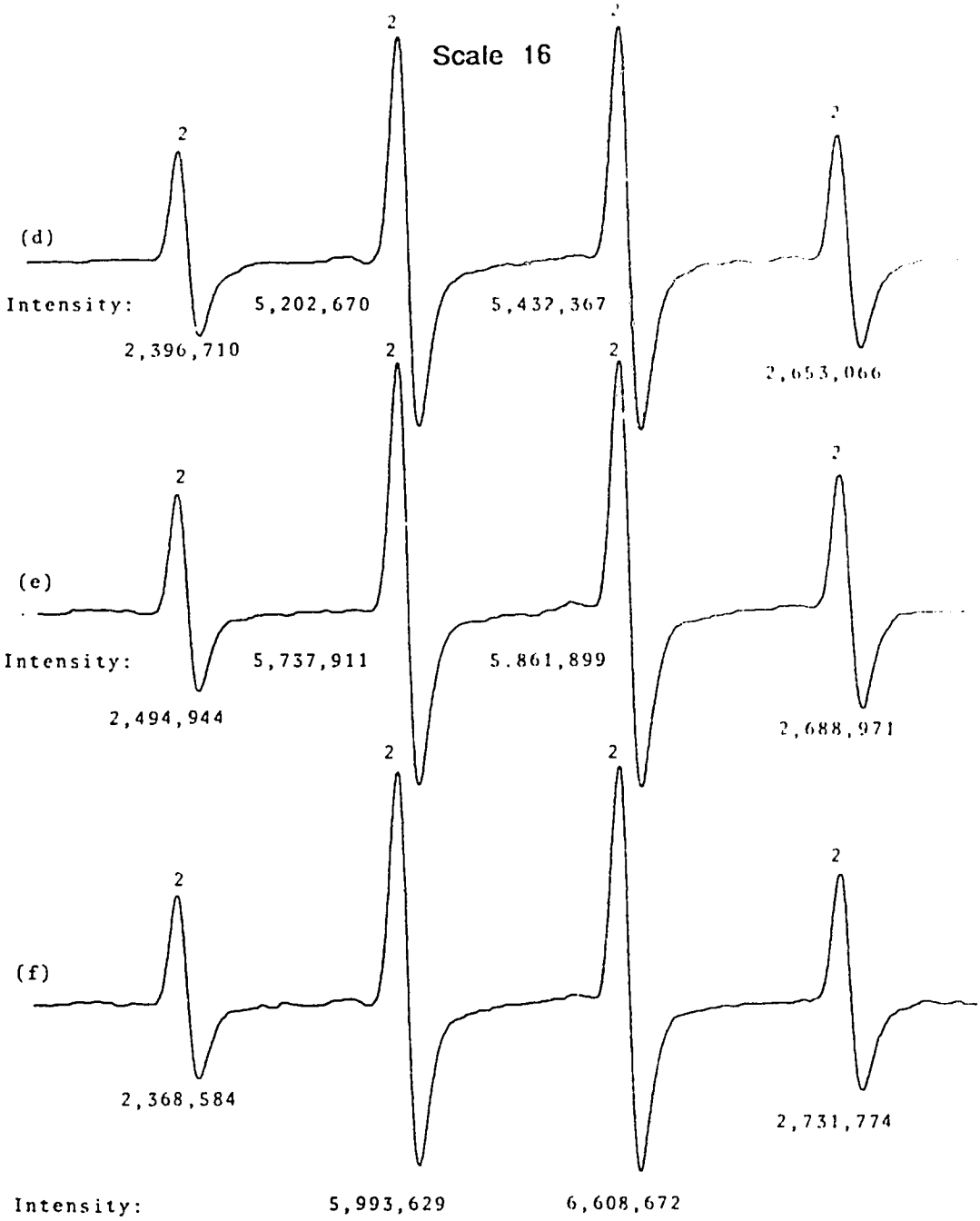


Figure 28: Dose-response characteristics of SOD on enhancing the formation of hydroxyl radical in the presence of iron. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of 20 μM Fe^{2+} and (a), 5 units/ml SOD; (b), 10 units/ml SOD; (c), 20 units/ml SOD; (d), 40 units/ml SOD; (e), 80 units/ml SOD; (f), 300 units/ml SOD.

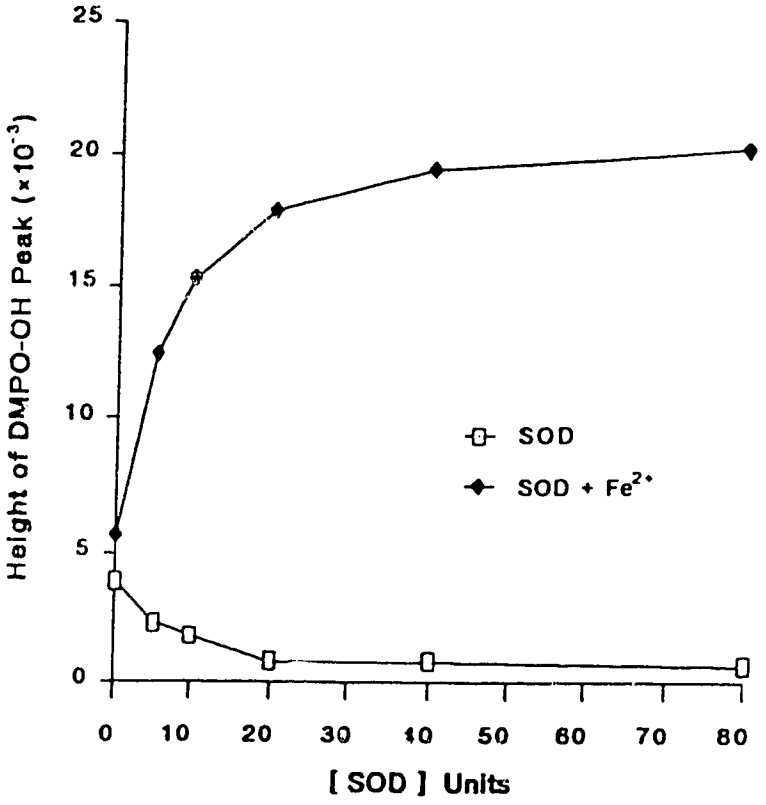


Figure 29: SOD dose-response characteristics for enhancing hydroxyl radical in the presence of 20 μM Fe^{2+} . The peak height of DMPO-OH adducts were derived from the ESR spectra following the reaction of xanthine with xanthine oxidase. The reactions were carried out in a 100 μl of mixture containing 100 mM DMPO, 0.4 mM xanthine, 6 nM xanthine oxidase, and varied concentrations of SOD in the absence (bottom line) and presence of 20 μM Fe^{2+} (upper line).

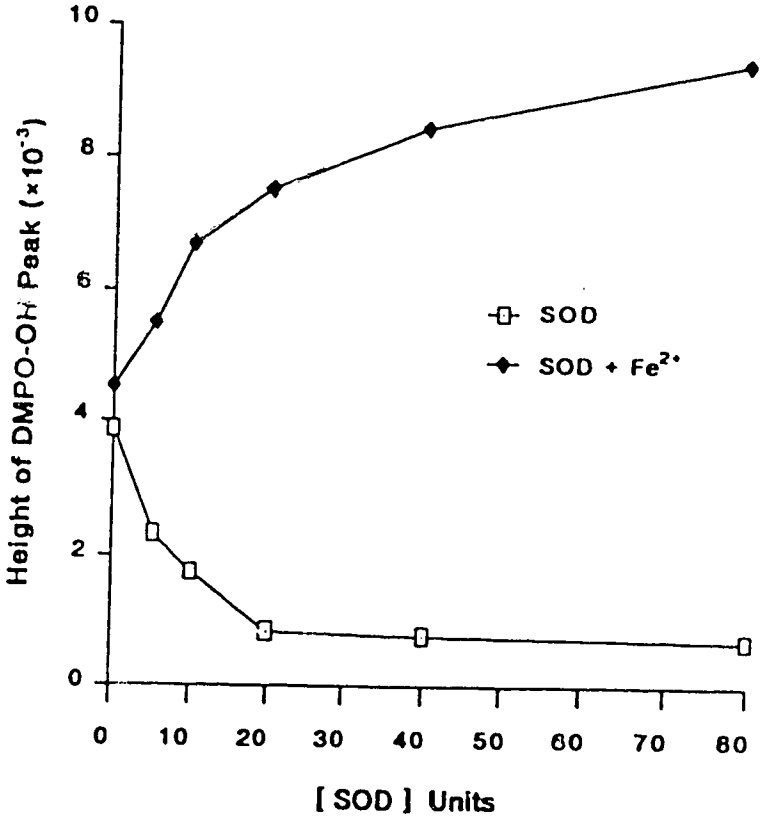


Figure 30: SOD dose-response characteristics for enhancing hydroxyl radical in the presence of 10 μM Fe^{2+} . The peak height of DMPO-OH adducts were derived from the ESR spectra following the reaction of xanthine with xanthine oxidase. The reactions were carried out in a 100 μl of mixture containing 100 mM DMPO, 0.4 mM xanthine, 6 nM xanthine oxidase, and varied concentrations of SOD in the absence (bottom line) and presence of 10 μM Fe^{2+} (upper line).

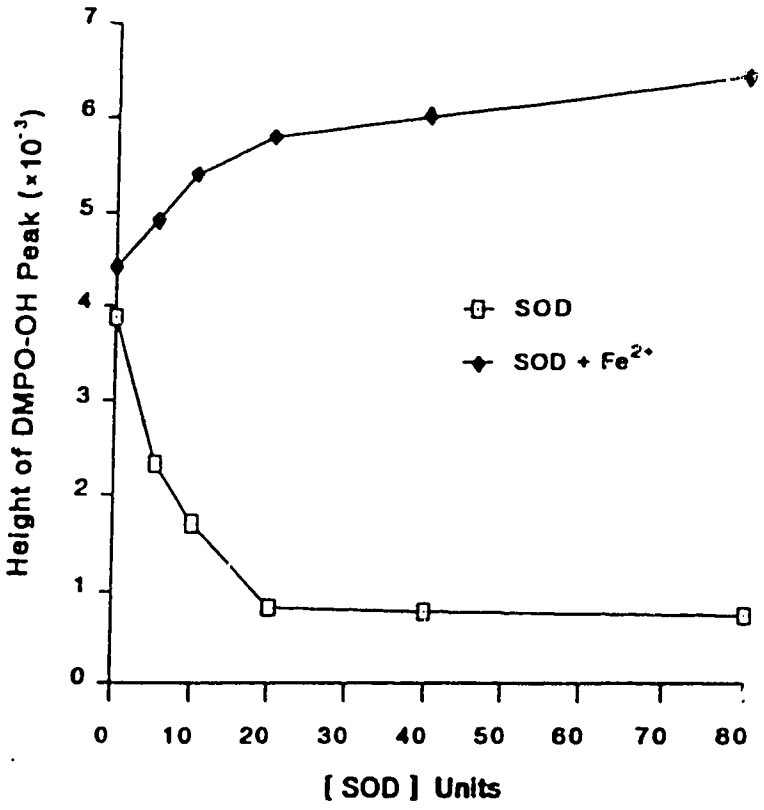


Figure 31: SOD dose-response characteristics for enhancing hydroxyl radical in the presence of 2 μM Fe^{2+} . The peak height of DMPO-OH adducts were derived from the ESR spectra following the reaction of xanthine with xanthine oxidase. The reactions were carried out in a 100 μl of mixture containing 100 mM DMPO, 0.4 mM xanthine, 6 nM xanthine oxidase, and varied concentrations of SOD in the absence (bottom line) and presence of 2 μM Fe^{2+} (upper line).

the xanthine/xanthine oxidase system initially inhibited a portion of the DMPO-OH peaks probably by decreasing the availability of DMPO-OOH to be decomposed to the DMPO-OH. The remaining hydroxyl radicals, however, were neither depleted nor enhanced by a further increase of SOD concentration. This suggested that the Haber-Weiss reaction was unable to proceed under the experimental conditions, although a high dose SOD may provide more hydrogen peroxide. In contrast, an increase of SOD concentration in the presence of 20 μM Fe^{2+} caused a dramatic enhancement of hydroxyl radicals. The maximum level was approximately 4-fold of that in the system without SOD. This is in agreement with the suggestion that high doses of SOD might become more toxic if an active form of iron was available at relatively low concentrations.

SOD enhanced the formation of hydroxyl radicals in a similar dose-response pattern at even lower concentrations of Fe^{2+}. The amount of hydroxyl radicals was directly proportional to the concentration of SOD until a maximum was reached. The profiles in Figures 30 and 31 demonstrate the SOD dose-response on the formation of hydroxyl radicals in the presence of 10 μM and 2 μM Fe^{2+} respectively. It appears that more toxic hydroxyl radicals could be induced by the increase of SOD concentrations even in at as low as 2 μM Fe^{2+}.

6.3.4. Dose-Response of SOD in the Presence of Ferric Ion

With the inclusion of ferric ions (stored form) in the reaction medium of a superoxide radical source, SOD again exhibited a dose-response on enhancement of the formation of hydroxyl radicals. Figure 32 shows that with the addition of SOD in the xanthine/xanthine oxidase system which contained

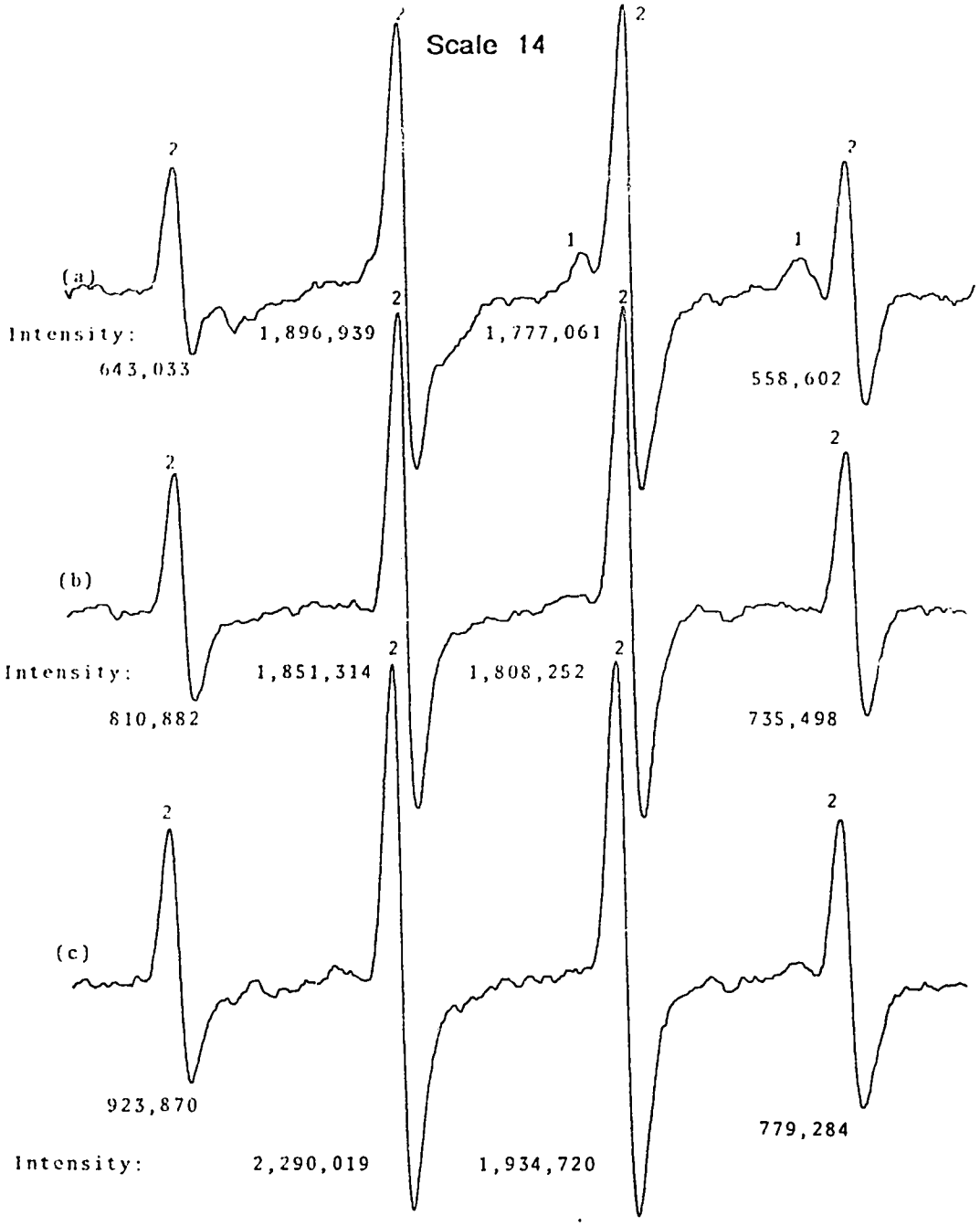


Figure 32: Dose-response characteristics of SOD on enhancing the formation of hydroxyl radical in the presence of ferric ion. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of 0.3 mM Fe^{3+} and (a), 5 units/ml SOD; (b), 10 units/ml SOD; (c), 20 units/ml SOD; (d), 40 units/ml SOD; (e), 80 units/ml SOD; (f), 300 units/ml SOD.

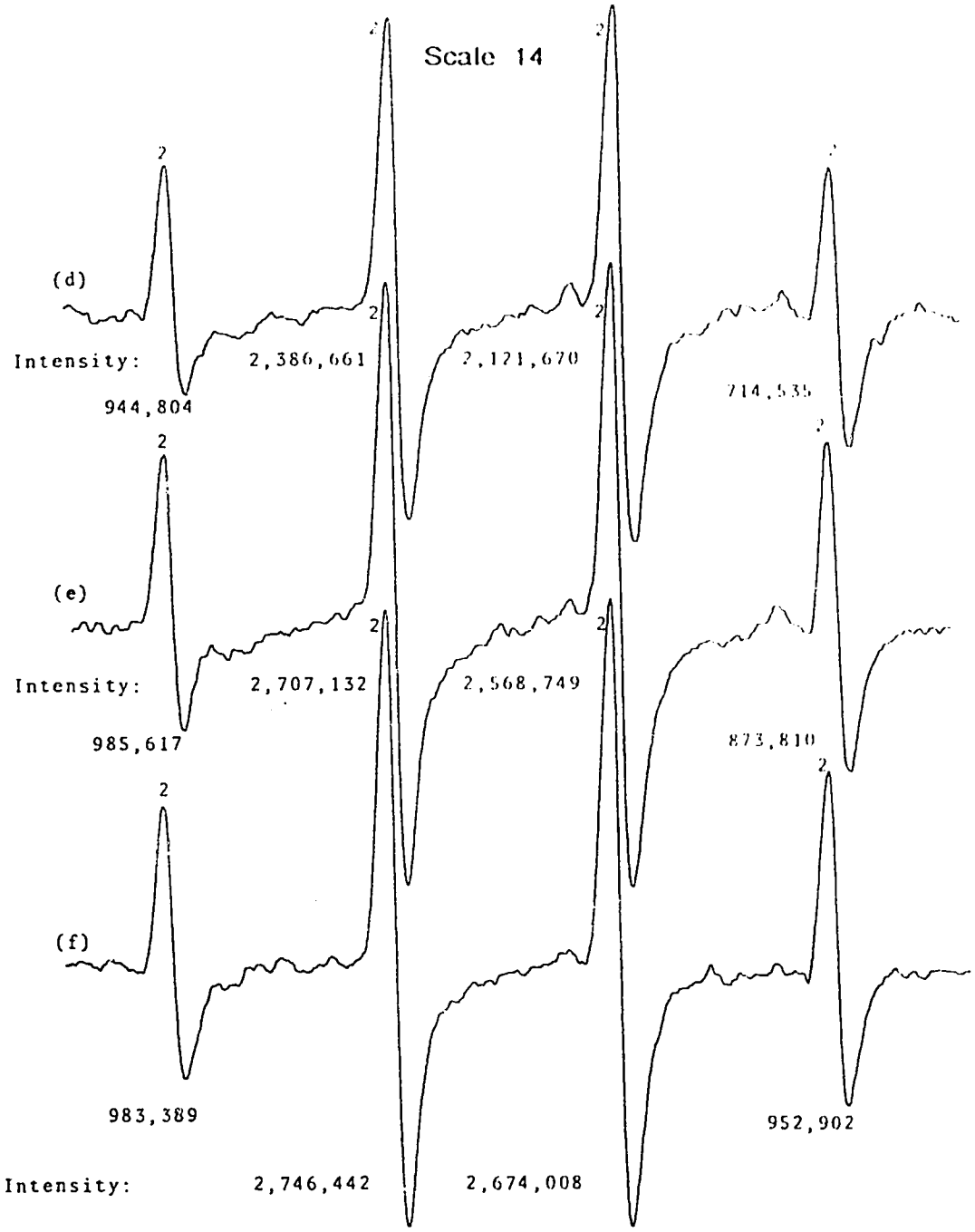


Figure 32: Dose-response characteristics of SOD on enhancing the formation of hydroxyl radical in the presence of ferric ion. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of 0.3 mM Fe^{3+} and (a), 5 units/ml SOD; (b), 10 units/ml SOD; (c), 20 units/ml SOD; (d), 40 units/ml SOD; (e), 80 units/ml SOD; (f), 300 units/ml SOD.

0.3 mM Fe^{3+}, the intensity of DMPO-OH peaks increased as the concentration of SOD increased. A more dramatic increase of DMPO-OH peak intensity corresponding to SOD concentration was observed in ESR spectra by increasing Fe^{3+} to 0.5 mM (Figure 33). In this situation, more ferric ions were available to be reduced to the ferrous ions (Fenton reaction catalyst), thus the increase in intensity of DMPO-OH signals caused by the increase of SOD was much greater than that in the control system or the system containing 0.3 mM Fe^{3+}.

6.3.5. Effect of Catalase on the Toxicity of SOD

If the underlying mechanism for SOD toxicity was due to the over-production of hydrogen peroxide which in turn was converted to toxic hydroxyl radicals via the Fenton reaction, addition of the H_2O_2 scavenger catalase would be expected to remove hydrogen peroxide and thereby attenuate SOD toxicity. In order to verify this idea, one series of experiments was performed under the same conditions used for determining the SOD toxicity but with catalase added. The ESR spectra shown in Figure 34 indicate that the huge amount of hydroxyl radical spin adducts induced by SOD (8×10^4 IU/L) and iron (20 μM ferrous ion or 0.5 mM ferric ion) were depleted by the addition of catalase (2×10^5 IU/L). This result strongly supports our proposal that SOD-mediated hydrogen peroxide increment may play a key role in the enhancement of hydroxyl radicals and toxicity of SOD. Based on these investigations, a new approach which may be a solution to overcome the toxicity of SOD in application was born in our laboratory: that is the SOD-CATALASE conjugate described in the following chapter.

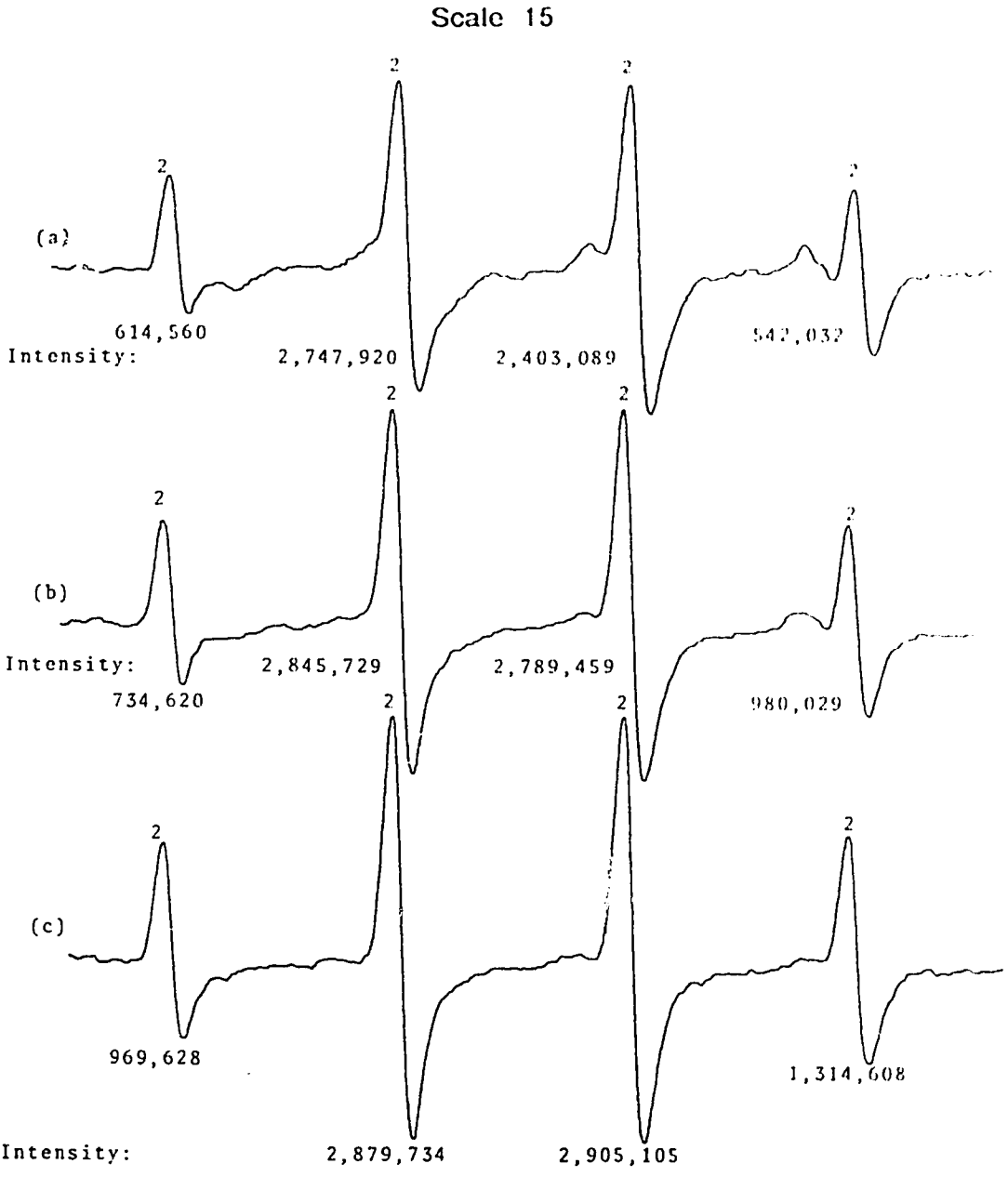


Figure 33: Dose-response characteristics of SOD on enhancing the formation of hydroxyl radical in the presence of ferric ion. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of 0.5 mM $Fe^{3+}$and (a), 5 units/ml SOD; (b), 10 units/ml SOD; (c), 20 units/ml SOD; (d), 40 units/ml SOD; (e), 80 units/mi SOD; (f), 300 units/ml SOD.

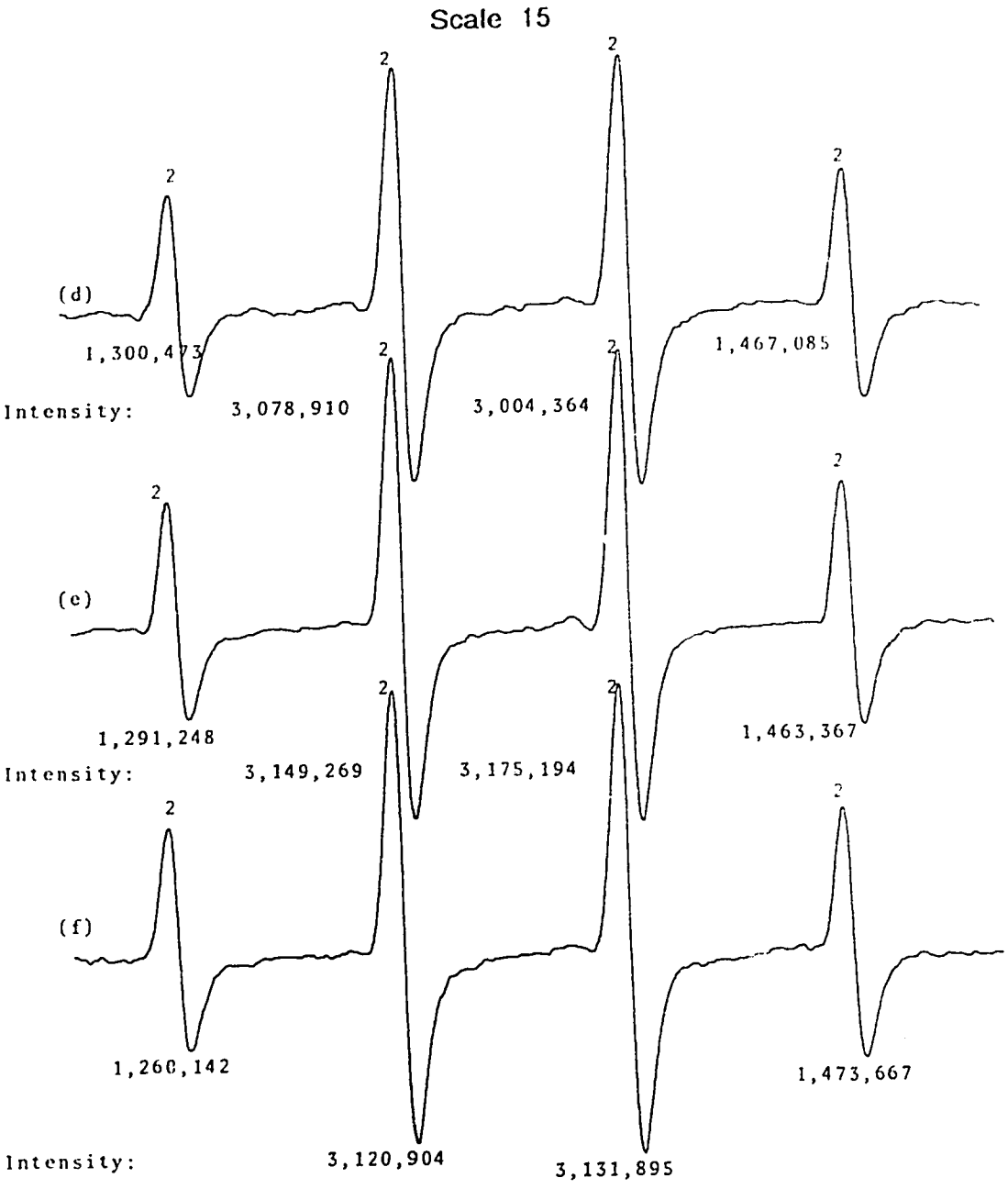


Figure 33: Dose-response characteristics of SOD on enhancing the formation of hydroxyl radical in the presence of ferric ion. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of 0.5 mM $Fe^{3+}$and (a), 5 units/ml SOD; (b), 10 units/ml SOD; (c), 20 units/ml SOD; (d), 40 units/ml SOD; (e), 80 units/ml SOD; (f), 300 units/ml SOD.

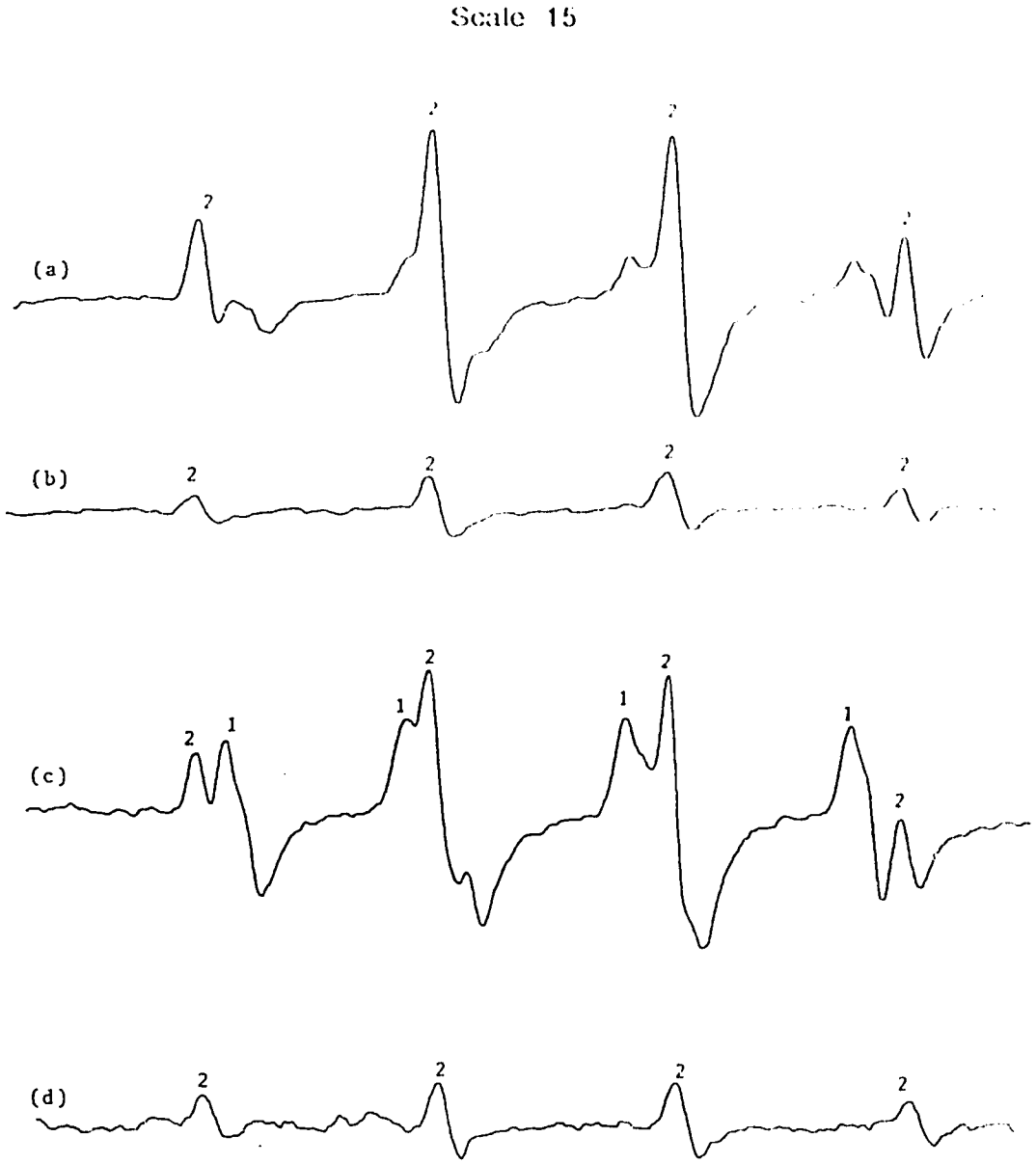


Figure 34: Effect of catalase on inhibiting hydroxyl radical induced by SOD and iron. ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of (a) 0.5 mM Fe^{3+}, (b) 0.5 mM Fe^{3+} plus free SOD (80 units/ml) and catalase (200 units/ml), (c) 20 μM Fe^{2+}, (d) 20 μM Fe^{2+} plus SOD (80 units/ml) and catalase (200 units/ml).

6.4. Discussion

The present study in vitro imply that the dose-dependent SOD toxicity may contribute to the reactive potential and toxicity of hydroxyl radicals in biological systems. A high dose of SOD may cause an accumulation of one of the Fenton reaction substrates, hydrogen peroxide, via an extensive dismutation of superoxide radicals. The accumulated hydrogen peroxide, in the presence of iron, may then be reduced forming a large amount of hydroxyl radicals. The availability and activity of iron would probably determine the toxicity of SOD in terms of inducing hydroxyl radical formation. Our investigation has revealed the following features: in the absence of iron, the effect of SOD on enhancing hydroxyl radicals was negligible; in the presence of iron, SOD promoted the production of hydroxyl radicals. The intensity of hydroxyl radicals induced by SOD greatly depended on the concentration of active form of iron. From these observationa, it appears that the administration of a metal chelating agent may reduce the risk of SOD toxicity. However studies in vitro have shown that iron chelated by agents such as EDTA, DETAPAC, and deferoxamine were still capable of catalyzing the Haber-Weiss reaction to form hydroxyl radicals (427,428). Therefore, investigation of a new method to remove the other Fenton reaction substrate, hydrogen peroxide, and thereby attenuating the SOD toxicity seemed encouraging.

Although we have proposed that an underlying mechanism of SOD toxicity may be due to the overproduction of hydroxyl radicals, our main evidence is based on experiments in vitro. In order to apply this mechanism to explain SOD toxicity in vivo, one question needing to be discussed is: is iron available for the Fenton reaction in vivo?

6.4.1. Physiological Sources of Iron

It seems unlikely that "free" iron exists in biological systems probably because of its reactivity. The iron taken up by the gut enters the plasma bound to the protein transferrin, which functions as a carrier molecule (429). Transferrin, a glycoprotein having two iron binding sites, then transports Fe^{2+} to the iron-storage protein or directly to iron-requiring biosynthetic pathways.

Ultimately, the majority of intracellular iron is deposited in ferritin. Ferritin can contain as many as 4500 molecules of iron per complex, although it is usually not saturated (430). A small amount of ferritin exists in plasma, but the amount can increase dramatically in serious pathological conditions (429). Another major pool of iron, in terms of concentration, is present in various heme proteins, mostly hemoglobin and myoglobin. Table 8 shows iron data for various tissues in humans and Table 9 gives the distribution of iron among various compounds in humans.

6.4.2. Availability of Fenton-Reactive Iron <u>in vivo</u>

When the idea that the toxicity of ·OH derived from the Fenton reaction was first being formulated, there was much debate about the availability of "catalytic" metal ions <u>in vivo</u> (431,432). The picture emerging now seems clear. Organisms are particularly careful about how iron is handled. Iron must be absorbed in the ferrous state (Fe^{2+}) and transported in the ferric state (Fe^{3+}) by transferrin and stored in the ferric state by ferritin. The idea that these iron-loaded proteins are efficient catalysts of hydroxyl radical formation from $O_2^{\cdot}$ and

Table 8: Iron Content of Human Tissues (µg/g of wet tissue)

Tissue	Iron content
Adrenals	38 ± 20
Aorta	56 ± 36
Brain	58 ± 17
Diaphragm	47 ± 23
Heart	55 ± 18
Duodenum	41 ± 18
Jejunum	38 ± 28
Ileum	27 ± 13
Kidney	76 ± 31
Liver	195 ± 113
Lung	319 ± 176
Muscle	42 ± 14
Ovary	59 ± 50
Pancreas	51 ± 42
Spleen	336 ± 210
Skin	15 ± 9
Testis	29 ± 14
Thyroid	62 ± 29

* **Adapted from A. Bezkorovainy, in Biochemistry of Nonheme Iron, edited by E. Frieden, Plenum Press, New York (1980).**

Table 9: Distribution of Iron among the Various Compounds in Normal Adult Male *

	Total compound in body (g)	Iron content (%)	Total iron in compound (g)
Hemoglobin	900	0.34	3.06
Myoglobin	120	0.34	0.4
Cytochrome c	0.8	0.43	0.004
Hemosiderin	1.2	30	0.36
Ferritin	2.0	20	0.4
Transferrin	14.0	0.05	0.007

*Adapted from A. Bezkorovainy, in Biochemistry of Nonheme Iron, edited by E. Frieden, Plenum Press, New York (1980).

H_2O_2 has now been disproved (433,434). For one thing it would be expected that little or none of any ·OH formed by iron attached to a protein could escape from the protein and be measurable outside it: the ·OH would attack the protein instead (435,436), in view of the very high reactivity of this free radical species. It is the iron which is detached from the protein by the reducing agents that is the real catalyst of the observed ·OH radical production (437). Importantly, oxidant stress can itself mobilize iron from ferritin or degrade heme protein to release iron for the Fenton reaction.

6.4.2.1. Release of Iron from Ferritin by Superoxide Radical

Iron attaches to ferritin as Fe^{2+}, which becomes oxidized to Fe^{3+} and deposited in the interior. Similarly, iron can be removed from ferritin as Fe^{2+} the action of a number of biological reducing agents, including cysteine, reduced flavins and ascorbate (429). The superoxide radical is capable of reducing ferritin-bound iron to the ferrous state, whereupon it is released (438,439). Experiments have shown that when ferritin was incubated with xanthine/xanthine oxidase slow but significant rates of iron release were observed (440), SOD completely blocked iron release, indicating the superoxide-driven mechanism. When paraquat and NADPH-cytochrome P_{450} reductase were used as the superoxide-generating system, more extensive release of iron from ferritin was observed than with xanthine oxidase and SOD did not completely inhibit the release of iron. This led to the realization that the paraquat radical itself was a very effective reductant of iron in ferritin (441,442). Stimulated polymorphonuclear leukocytes also caused mobilization of iron from ferritin, which depended on O_2^- since it could be prevented by the addition of SOD, but not by catalase or DMSO (438). This superoxide-driven release of iron

from ferritin may potentiate the formation of the hydroxyl radical in inflammatory states.

6.4.2.2. Release of Iron from Hemoglobin by Hydrogen Peroxide

The ability of intact hemoglobin to accelerate ·OH radical formation seems to be a matter of controversy because there is no clear evidence that those proteins react with H_2O_2 to form ·OH that can be detected outside the proteins. However, incubation of hemoglobin with a molar excess of H_2O_2 can cause degradation and release of iron ions. Gutteridge found that incubation of hemoglobin with H_2O_2 caused formation of ·OH, as measured by the deoxyribose assay (443,444), and also a release of iron from the protein. He showed that the ·OH formation was inhibited by desferrioxamine and transferrin, suggesting that it was mediated by the iron ions released from the protein by the H_2O_2 rather than by the hemoglobin protein itself (445). Hence Fe^{2+} or Fe^{3+} is available in vivo, what about H_2O_2?

6.4.3. Availability of Fenton-Reactive H_2O_2 in vivo

A biological system generating O_2^- will probably produce H_2O_2 (unless the O_2^- reacts immediately with another molecule) by the dismutation reaction, the rate of which will depend on the pH and on the concentration of O_2^-. Under oxidant stress, a large amount of superoxide radicals may be generated from various sources and SOD (exogenous and /or endogenous) may speed up the dismutation reaction to form H_2O_2. In addition, several enzymes including glycollate and urate oxidase produce hydrogen peroxide without the intermediary O_2^- radicals (446). The lens of the human eye contains micromolar

concentrations of H_2O_2 (447), and H_2O_2 vapor has been detected in expired human breath (448), although it is possible that some or all of this might originate from oral bacteria (449).

Overall, both "catalytic" iron (enhanced by oxidant stress) and hydrogen peroxide (enhanced by the presence of SOD) are available in vivo, and furthermore, in pathological conditions, certain changes can liberate "catalytic" metal ions from broken cells into the surrounding environment or facilitate metal release from attached protein (450). If "catalytic" iron and hydrogen peroxide come into contact, extremely reactive and toxic hydroxyl radicals will be produced to wreak additional cellular havoc. SOD at low doses is beneficial because it scavenges O_2^- and thereby attenuates the oxidant stress, however at high doses, SOD may result in the accumulation of H_2O_2 which probably overwhelms endogenous activities of scavengers and is rapidly reduced to ·OH by iron. From this point of view, the potential toxicity of SOD can be predicted. Thus, for the full assessment of the antioxidant properties of SOD, it is clearly important to pay considerable attention to both time and dose of administration as well as the potential condition with respect to Fe^{2+}. It should be stressed that our results, in agreement with the other observations reported in the literature, show that both SOD and catalase should be used simultaneously for a maximum protective effect (412,451).

7. SOD-CATALASE: a New Antioxidant

7.1. Introduction

SOD catalyzes the dismutation reaction of superoxide to form hydrogen peroxide and oxygen, thereby rescuing the tissue from oxygen free radical damage. Hydrogen peroxide, however, has the potential to be damaging in a living system because it is an oxidizing agent and can inactivate some enzymes usually by the oxidation of essential -SH groups. Hydrogen peroxide can penetrate the cell membrane rapidly (452), and once inside the cell, it can react with Fe^{2+} to give rise to hydroxyl radical. This may be the origin of most of its toxic effects. The amount of hydrogen peroxide in vivo, under normal conditions, is controlled by the enzymes such as catalase and glutathione peroxidase converting it to water and oxygen.

When a living organism suffers oxidant stress (e.g. during ischemia/ reperfusion), however, a large amount of superoxide radicals will be produced from various sources and an accumulation of H_2O_2 would be expected if these superoxide radicals rapidly undergo dismutation catalyzed by endogenous or/and exogenous SOD. As a result, the endogenous catalase may be overburdened and the toxic hydroxyl radical may be formed provided metal ions are present, which then may provoke a whole series of free radical reactions. Therefore it may be biologically advantageous to apply SOD and catalase simultaneously in order to eliminate superoxide and hydrogen peroxide at the same time.

Unfortunately, catalase, as a bioactive protein, is rapidly removed from the circulation after entering the organism, the half-life of catalase in vivo has been reported to be only 6 minutes (453). In addition, catalase is inhibited by superoxide radicals (454), and hence under the condition that superoxide radicals are abundant, the effect of native catalase is restricted. Consequently, a search for an optimal approach for applying SOD and catalase simultaneously and effectively is of great importance.

Our interest in this respect has centered upon using enzyme modification techniques to produce a new compound: SOD-CATALASE conjugate. In principle, catalase can be considered as a biologically active carrier for SOD. After cross-linking these two enzymes, the following advantages could be predicted: (a) improvement of the pharmacokinetics of both SOD and catalase, a high molecular weight of product will enable conjugated enzymes to extend their half-life in the circulation. (b) increase of stability of the conjugated enzyme and its resistance to inhibitors, SOD scavenges superoxide radicals thereby protecting the conjugated catalase from superoxide inhibition, and catalase scavenges hydrogen peroxide thereby preventing conjugated SOD from H_2O_2 inhibition. (c) decrease of the potential of forming hydroxyl radicals, since superoxide and hydrogen peroxide are less reactive than hydroxyl radicals and can diffuse away from the sites of formation, this may lead to the generation of hydroxyl radical in different parts of the cell whenever they meet a "spare" transition metal ion. By conjugating SOD and catalase, H_2O_2 formed from SOD catalyzed superoxide dismutation is more likely to be scavenged by vicinal catalase before it diffuses and reacts with a metal ion. In the section below, we will report our encouraging investigation of the SOD-CATALASE conjugate.

7.2. Materials and Methods

7.2.1. Materials

Yeast superoxide dismutase was the product of Carlsberg Biotechnology Ltd (Copenhagen, N. Denmark). Bovine liver catalase with specific activity of 9300 unit/mg was the product of Sigma Chemical Company. Ultrafiltration membranes were from Amicon Company (Danvers, MA U.S.A.). Sephadex G-150 and a gel filtration calibration kit were from Pharmacia Fine Chemicals (Uppsala, Sweden). Folin & Ciocalteu's phenol reagent and other reagents were purchased from Sigma Chemical Company (St. Louis, MO U.S.A.).

7.2.2. Methods

7.2.2.1. SOD Activity Assay

See section 3.2.2. in the thesis.

7.2.2.2. Catalase Activity Assay

We typically followed the Sigma Chemical Company procedure for measuring catalase activity (Sigma catalog 1990, p 276). The SOD-CATALASE conjugate preparations were promptly diluted with 0.05 M phosphate buffer, pH 7.0. The substrate solution was prepared by adding 0.1 ml of 30% H_2O_2 to 50 ml of 0.05 M phosphate buffer, pH 7.0. For optimal results, the A_{240} of the substrate solution should be adjusted between 0.550 to 0.520. For measuring catalase activity, 0.1 ml of the enzyme solution was added to 2.9 ml of the substrate

solution in a quartz cuvette (1 cm light path) at 25° C. The initial A_{240} would exceed 0.450 and then decrease. The time required for A_{240} to decrease from 0.450 to 0.400 corresponded to the decomposition of 3.45 μmoles of H_2O_2 in the 3 ml solution. One unit of catalase is defined as the amount of the enzyme which will decompose 1.0 mM of H_2O_2 per minute at pH 7.0 at 25° C, while the H_2O_2 concentration falls from 10.3 to 9.2 mM. Thus total catalase activity in 3 ml reaction mixture was calculated using the equation: 3.45/minutes required = Total Sigma Units.

7.2.2.3. Total Protein Assay

We typically followed the Lowry procedure for measuring protein concentration (367). The reagents used for the assay included a copper carbonate solution which was prepared by mixing 1 ml of 2.5% Na-K tartrate, 1 ml of 1.0% $CuSO_4 \cdot 5H_2O$, 25 ml of 4.0% Na_2CO_3 and 25 ml of distilled water. Folin reagent, 1 N, was usually prepared simply by diluting the commercial reagent 1:1 with water. To the protein sample in a final volume of 0.5 ml water, 0.5 ml of 1 N NaOH was added, then 5 ml of copper carbonate reagent was added with vortexing. The sample mixture was allowed to stand for 10 minutes at room temperature, and then 0.5 ml of 1 N Folin reagent was added to the mixture with vortexing. After 30 minutes incubation, the concentration of protein was determined by reading vs a reagent blank at 620 nm. BSA was used as the protein standard.

7.2.2.4. Preparation of SOD-CATALASE Conjugates

16 mg SOD and 5 mg catalase were dissolved in 2 ml of 0.2 M phosphate buffer, pH 7. 10 μl of fresh reagent grade 25% glutaraldehyde was then added to the mixture and reaction was allowed to proceed for 4 hours with stirring at 4° C. The cross-linking reaction was terminated by the addition of 5 mg glycine.

The preparation was dialyzed overnight against 2 liters of 0.02 M PBS, 0.15 M glycine and 0.13 M NaCl, and then against 0.05 M PBS, pH 7.4. The dialysate was loaded onto a Sephadex G-150 column (approximately 15 × 100 mm), which was calibrated using thyroglobulin, ferritin, catalase, aldolase, and bovine serum albumin. The eluted fractions were collected and assayed for protein, SOD activity and catalase activity. The pooled appropriate fractions were concentrated, if required, using an Amicon ultrafiltration cell with an XM 300A membrane. The activity ratio of SOD and catalase in SOD-CATALASE conjugates could be changed by using different proportions of SOD and catalase in the initial reaction mixture. We have prepared SOD-CATALASE conjugates with activity ratios of SOD to catalase ranging from 7:1 to 1:10.

7.2.2.5. Animal Experiment

Sprague-Dawley rats were anaesthetized with Inactin. One jugular vein and one femoral vein were cannulated for injecting and sampling. After SOD-CATALASE conjugates were injected, the blood samples were taken over a period of time. The SOD activity in rat plasma was measured by the cytochrome c assay, and catalase in plasma was measured by Sigma catalase activity assay procedure.

7.3. Results

7.3.1. Chromatography Profile of SOD-CATALASE Conjugates

Under the conditions of described method, the glutaraldehyde-mediated cross-linking reaction resulted in approximately 100% retention of the original SOD enzymatic activity and 80% of catalase activity. Figure 35 shows a representative Sephadex G-150 chromatography profile obtained from a conjugate with an activity ratio of SOD to catalase of 5:1. The total protein assay indicates that fractionation of SOD-CATALASE conjugates have an apparent molecular weight ranging from 1.5×10^5 to 4×10^5 daltons (fractions 25 to 37). The peak of the conjugate in terms of the activities of SOD and catalase coincided with an average molecular weight of 3.5×10^5 daltons (fractions 27 to 29). Native SOD has a molecular weight of 32,000 daltons and native catalase has a molecular weight of 232,000 daltons. The absence of a native SOD activity peak (fraction 49) and a native catalase activity peak (fraction 32) indicates that both the enzymes had participated in the cross-linking reaction. SOD activity and catalase activity were not entirely congruent. This may be explained by the formation of SOD-SOD conjugates as well as SOD-CATALASE conjugates during the cross-linking reaction.

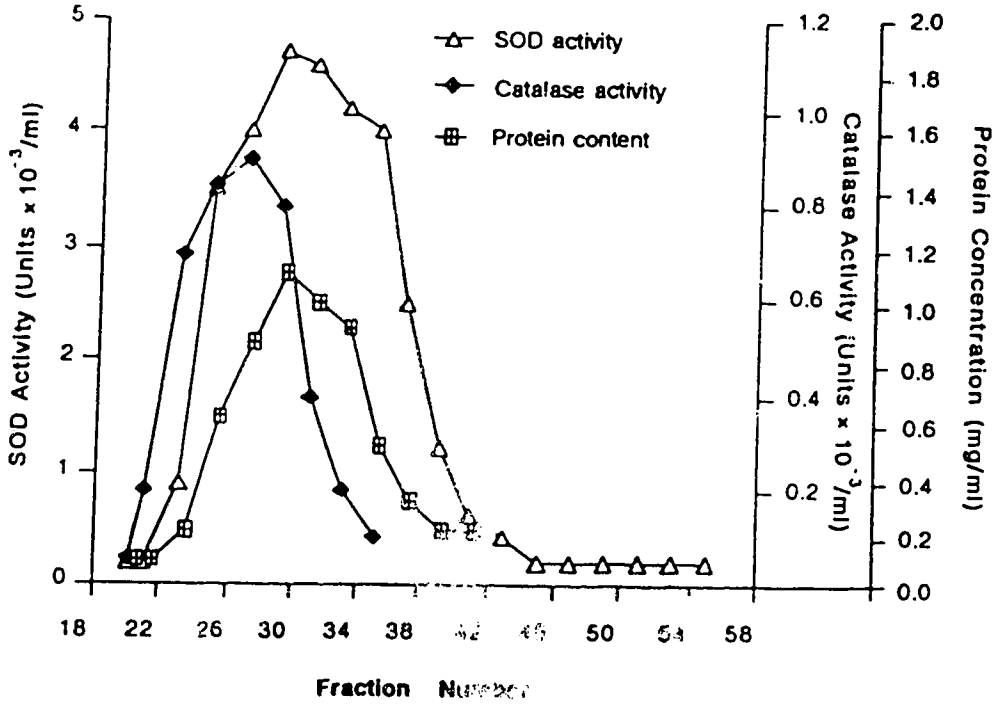


Figure 35: Molecular sieve chromatography of SOD-CATALASE conjugate. The Sephadex G-150 column was standardized using thyroglobulin, ferritin, catalase, aldolase and bovine serum albumin. Each fraction contained 3 ml of eluant. Enzymatic activities and protein concentration were measured as described under Materials and Methods.

7.3.2. Plasma Clearance of SOD-CATALASE Conjugates

Figure 36 shows the clearance of SOD and catalase enzymatic activities from the plasma of rats. Animals were injected intravenously with pooled conjugates of molecular weights between 2×10^5 and 4.5×10^5 daltons. By contrast, native SOD and catalase were cleared with a half-life of 6 minutes in plasma, conjugated SOD and catalase tremendously prolonged both enzymes' half-life to about 4 hours. This plasma clearance of SOD-CATALASE conjugates provided new possibilities for using these two enzymes in biology and medicine effectively.

7.3.3. Resistance of Conjugates to Inhibitors

Conjugating SOD with catalase greatly enhanced SOD resistance to hydrogen peroxide inhibition, which has been demonstrated in section 3.3.3..

Superoxide radicals have been known to inhibit catalase mainly by converting catalase to the ferro-oxy state (catalase-Fe^{2+}-O_2) or ferryl state (catalase-Fe^{4+}), neither of which was active in the catalatic reaction pathway (454). We have incubated catalase with a flux of O_2^- generated <u>in situ</u> by the xanthine/xanthine oxidase system. The reaction mixture contained 10 mM H_2O_2, 50 μM xanthine 0.1 mM EDTA, and 50 mM sodium carbonate buffer at pH 10. The 0.9 nM (2 units/ml) catalase and 5 nM xanthine oxidase were added to the mixture as required. In order to determine the effect of the reaction of xanthine oxidase on the steady-state of hydrogen peroxide, the control experiment was carried out by incubation of H_2O_2 with xanthine/xanthine oxidase in the absence of SOD and catalase. As shown in Figure 37 (line 1), the absence of a change

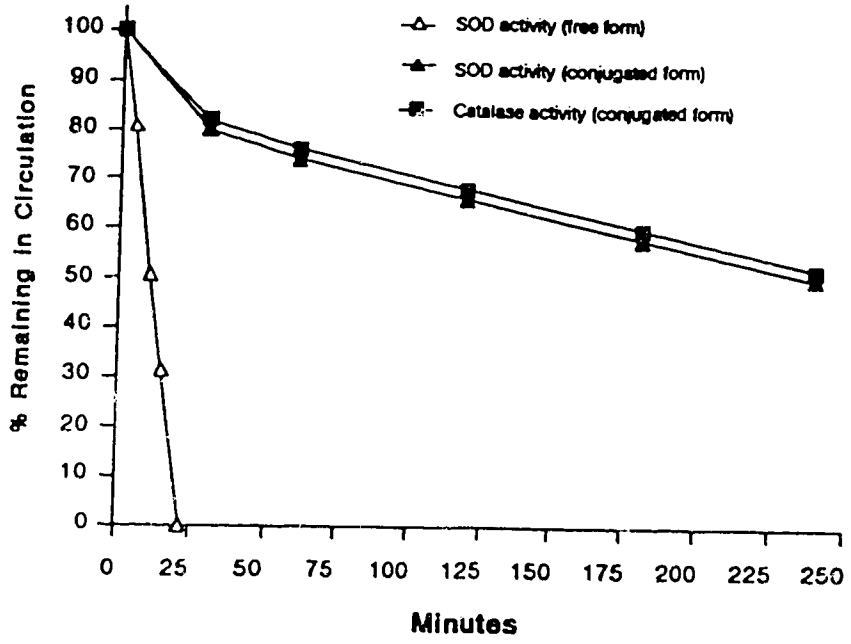


Figure 36: Plasma clearance of SOD-CATALASE conjugate in rats. Following intravenous injection of the conjugates, the blood samples were taken at regular intervals and the enzymatic activities in plasma were measured as described under Materials and Methods. The enzymatic activities/ml plasma obtained at specified times were expressed as a percentage of the value obtained right after injection.

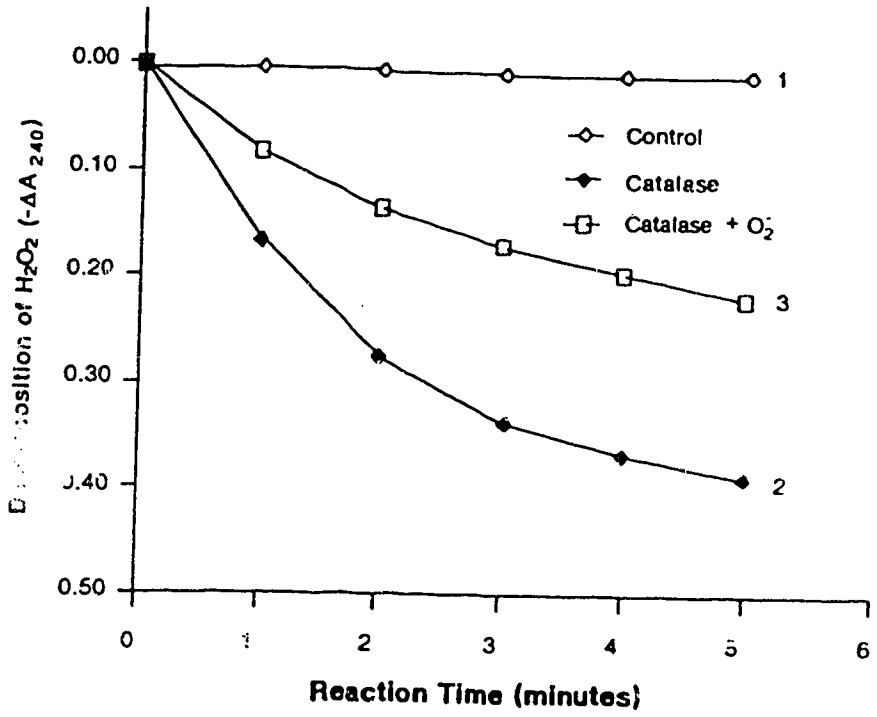


Figure 37: Inhibition of catalase by O_2^-. Reaction mixture contained 50 mM sodium carbonate, pH 10, 10 mM H_2O_2, 50 µM xanthine, and 0.1 mM EDTA. The reactions were initiated by the addition of catalase (2 units/ml). The catalase activity was calculated from the decomposition of H_2O_2 by observing the decrease of absorbance at 240 nm. Line 1 was the reaction of xanthine oxidase in the absence of SOD and catalase. Line 2 was the catalatic reaction course of free catalase in the absence of xanthine oxidase while line 3 was the catalatic reaction course recorded when xanthine oxidase was present from the outset.

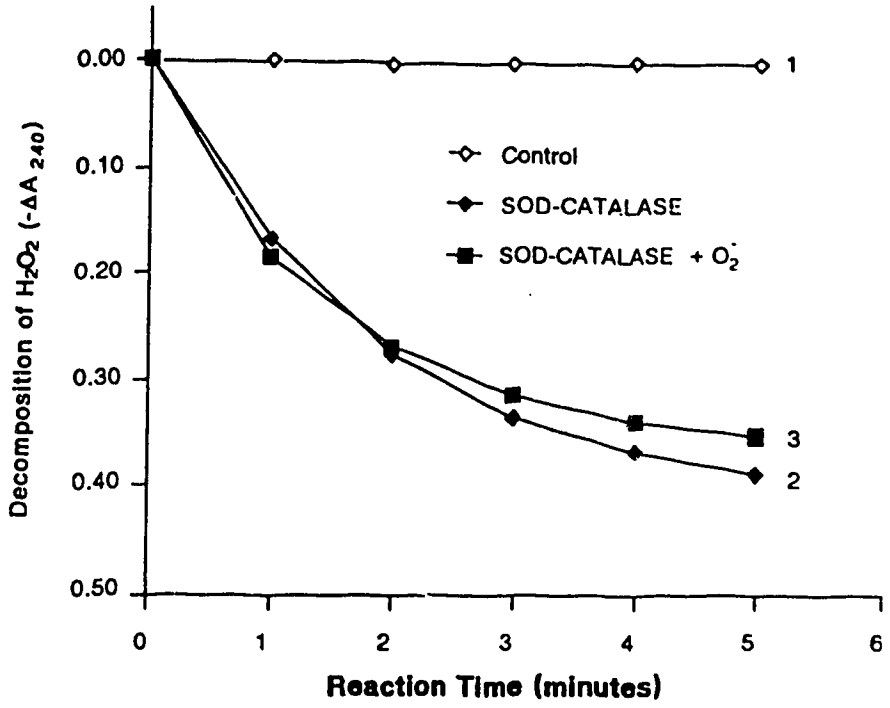


Figure 38: Resistance of the conjugate to O_2^- inhibition. Reaction mixture contained the same components as the experiment shown in Figure 37. The reactions were initiated by the addition of SOD-CATALASE conjugates. The catalase activity was calculated from the decomposition of H_2O_2 by observing the decrease of absorbance at 240 nm. Line 1 was obtained from the reaction of xanthine oxidase in the absence of SOD and catalase. Line 2 was the catalatic reaction course of conjugate in the absence of xanthine oxidase. Line 3 was the reaction of the same amount of conjugates while xanthine oxidase was present from the outset.

in H_2O_2 concentration after a 5-minute incubation suggests that the influence of the reaction of xanthine oxidase and spontaneous dismutation of superoxide on the H_2O_2 concentration was negligible. A possible explanation is that the production of hydrogen peroxide by xanthine oxidase and spontaneous dismutation of superoxide was very slow at pH 10 as suggested by Fridovich (455). Line 2 in Figure 37 was obtained following the reaction of catalase with H_2O_2 in the absence of xanthine oxidase. Line 3 was obtained when 5 nM xanthine oxidase was present from the onset. The decreased rate of decomposition of H_2O_2 (line 3) clearly indicates that the initiation of the xanthine oxidase reaction rapidly inhibited the activity of catalase. The experiment demonstrated in Figure 38 was performed under the same condition as in Figure 37. The comparison of line 2 with line 3 of Figure 38 indicates that catalase conjugated with SOD was not susceptible to inhibition by superoxide radicals, suggesting that SOD provided sufficient protection for vicinal catalase. This synergism between SOD and catalase in the conjugated form observed in vitro might be significant in vivo.

7.3.4. A Solution to Overcome SOD Toxicity (?)

We suggested that SOD might exert a toxic effect on tissue, possibly by enhancing toxic hydroxyl radical formation in the presence of a O_2^- generating system and metal ions via the Fenton reaction. Combined use of SOD and catalase has been shown to quench the Fenton reaction and suppress the formation of hydroxyl radicals in vitro (see section 6.3.5.). In this part of work, we present the determination of the inhibitory effect of the SOD-CATALASE conjugate on SOD and iron ion-mediated hydroxyl radical formation. ESR

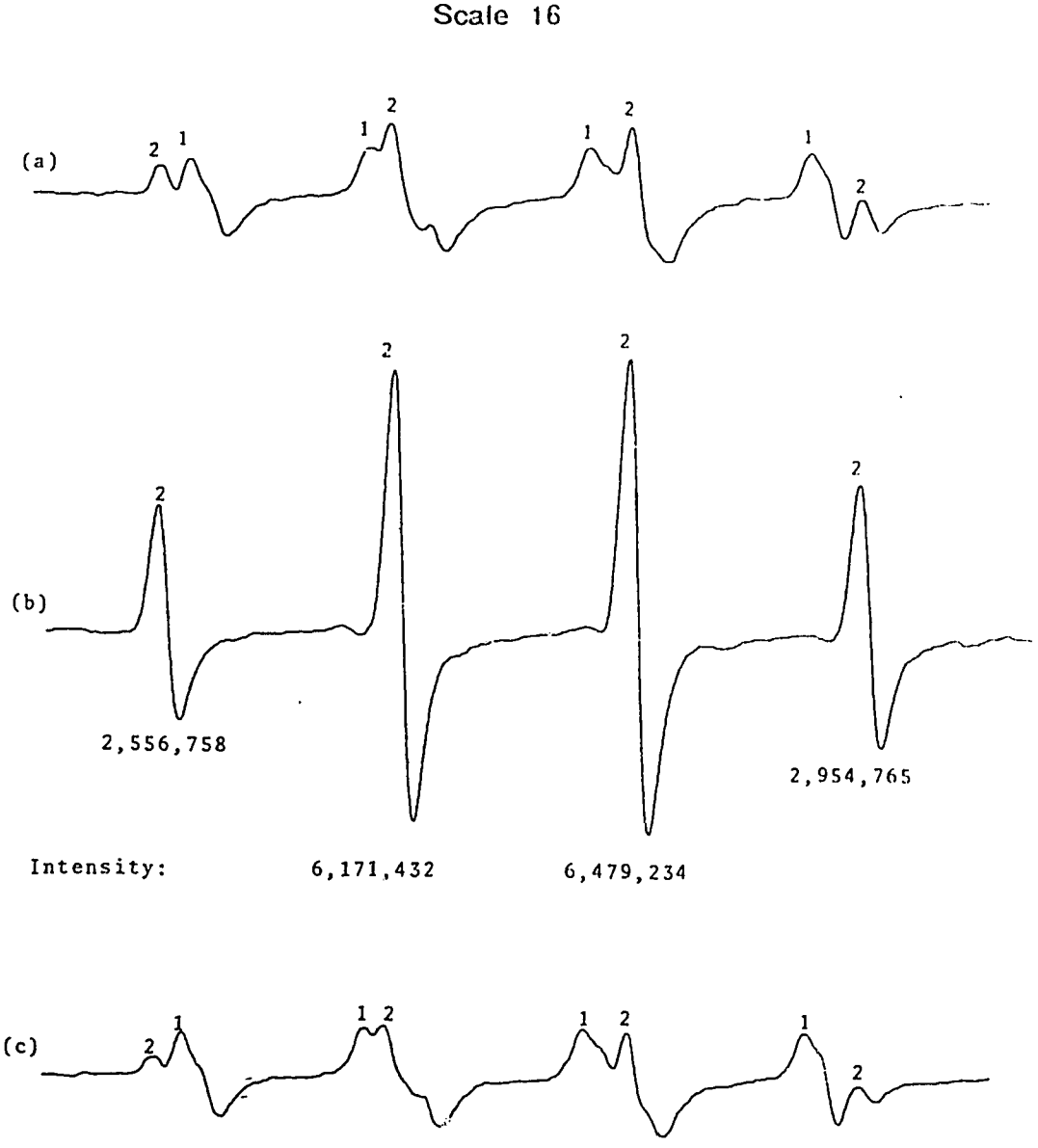


Figure 39: ESR spectra obtained following the reaction of xanthine with xanthine oxidase in the presence of (a), 20 μM Fe^{2+}; (b), 20 μM Fe^{2+} plus SOD (350 units/ml); (c), 20 μM Fe^{2+} plus catalase (70 units/ml).

spectra shown in Figure 39 was obtained following the reaction of xanthine with xanthine oxidase in the presence of 20 μM ferrous ion (spectrum a), 20 μM ferrous ion plus 3.5×10^5 IU/L SOD (spectrum b), or 20 μM ferrous ion plus 7×10^4 U/L catalase (spectrum c). SOD under this condition strongly promoted the hydroxyl radical signals while catalase had the potential to lower the formation of ·OH owing to the removal of hydrogen peroxide. Figure 40 demonstrates the comparison of the SOD-CATALASE conjugate with free SOD plus free catalase on attenuating SOD toxicity in terms of suppression of hydroxyl radicals. It is not surprising that the combination of SOD (3.5×10^5 IU/L) and catalase (7×10^4 U/L) caused the intensity of the third DMPO-OH peak to decrease from 6,479,234 (catalase free system) to 742,239 (Figure 40, spectrum a). Raising the concentrations of the enzymes resulted in a greater depletion of the DMPO-OH signal (Figure 40, spectrum c). It was exciting to find that SOD-CATALASE, at the same enzyme activity as the free SOD and catalase, inhibited hydroxyl radicals derived from the Fenton reaction more efficiently. Spectrum b in Figure 40 indicates that the intensity of the third DMPO peak was decreased to 532,091 by the conjugate containing 3.5×10^5 IU/L SOD activity and 7×10^4 U/L catalase activity compared with 742,239 from the free enzymes. Doubling the concentration of the conjugate caused the third DMPO-OH peak to further decrease to 241,944 (Figure 40, spectrum d) compared to 430,504 with the same activities of free enzymes (Figure 40, spectrum c). A more complete comparison of SOD-CATALASE conjugates with free enzymes is shown in Figure 41. In the presence of 20 μM Fe^{2+} (sufficient Fenton reaction catalyst) and the same enzyme activities, the conjugate is more effective than the free enzymes for the elimination of hydroxyl radicals.

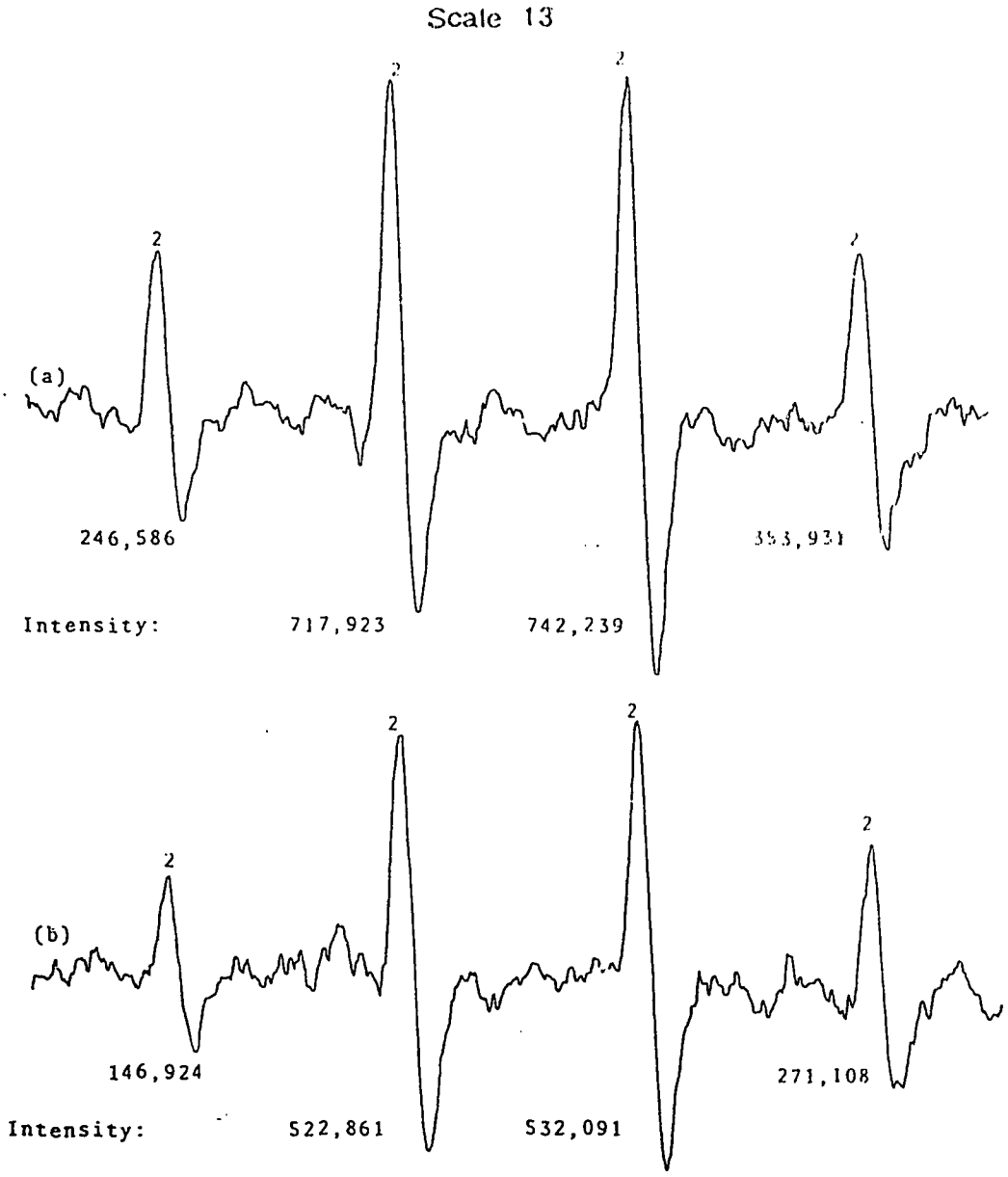


Figure 40: Comparison of the effect of SOD-CATALASE conjugate with free SOD and catalase on inhibiting hydroxyl radical formation. The ESR spectra were obtained following the reaction of xanthine with xanthine oxidase in the presence of 20 μM Fe^{2+} and (a) free SOD (350 U/ml) and catalase (70 U/ml); (b) SOD-CATALASE conjugate (equivalent enzyme activities as in spectrum a); (c) free SOD (700 U/ml) and catalase (140 U/ml); (D) SOD-CATALASE conjugate (equivalent enzyme activities as in spectrum c).

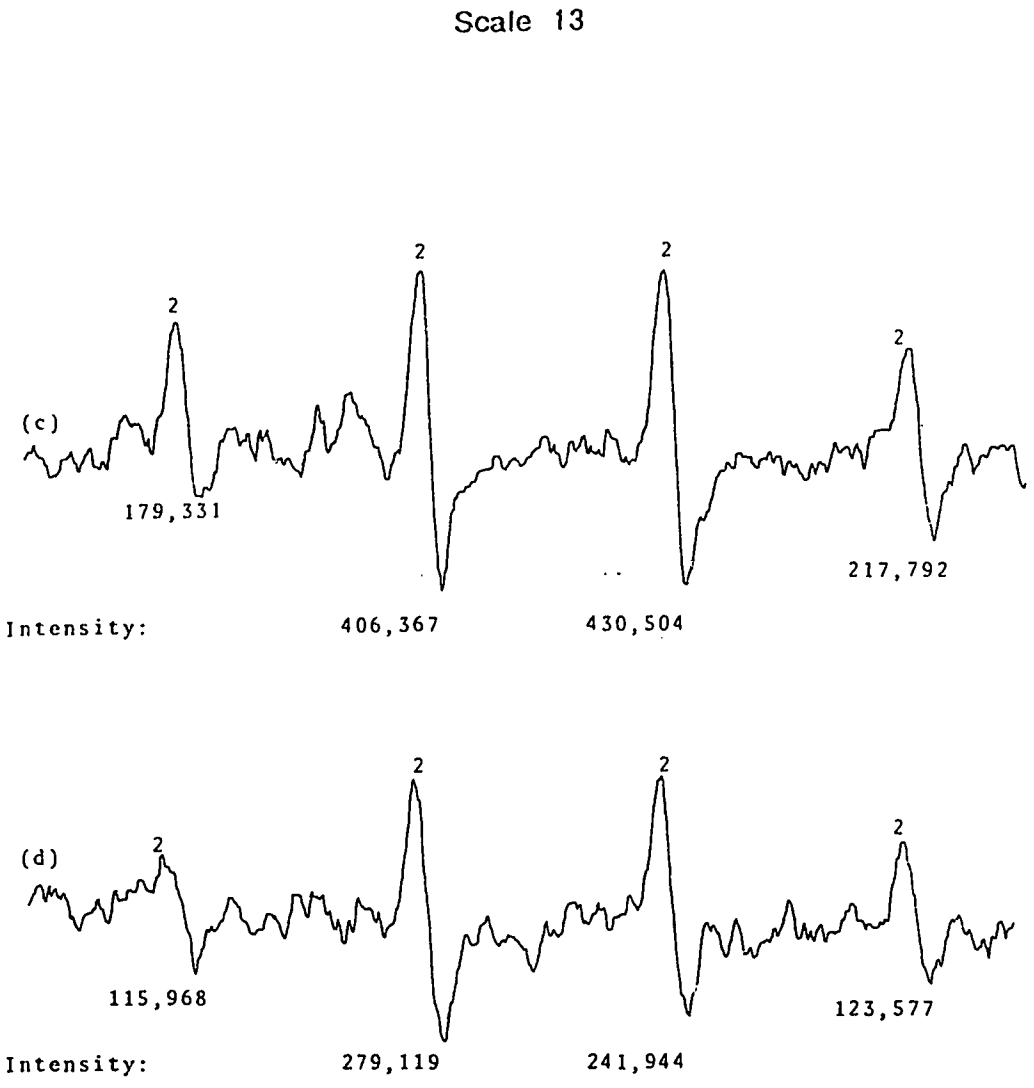


Figure 40: Continued.

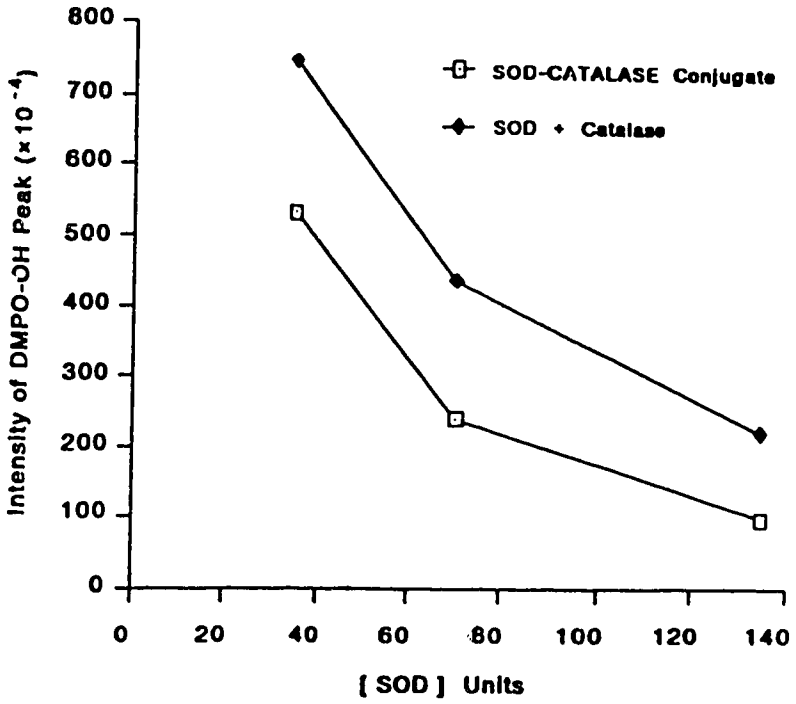


Figure 41: Inhibition of hydroxyl radical formation by the combinationof SOD and catalase or SOD-CATALASE conjugate. The intensities of DMPO-OH peaks were derived from the ESR spectra following the reaction of xanthine/xanthine oxidase. The 100 µl of reaction system contained 0.4 mM xanthine, 100 mM DMPO, 20 µM Fe^{2+}, and 6 nM xanthine oxidase. The same amount of free SOD and catalase or the conjugates were added to the system for comparing their effect on the inhibition of hydroxyl radical formation.

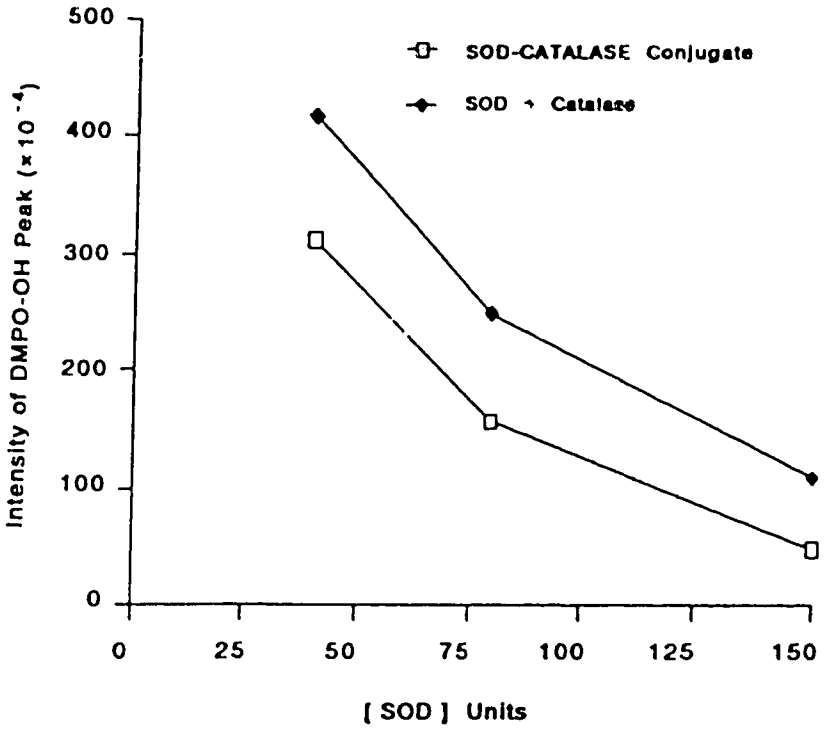


Figure 42: Inhibition of hydroxyl radical formation by the combination of SOD and catalase or SOD-CATALASE conjugate. The intensities of DMPO-OH peaks were derived from the ESR spectra following the reaction of xanthine/xanthine oxidase. The 100 µl of reaction system contained 0.4 mM xanthine, 100 mM DMPO, 0.5 mM Fe^{3+}, and 6 nM xanthine oxidase. The same amount of free SOD and catalase or the conjugates were added to the system for comparing their effect on the inhibition of hydroxyl radical formation.

Replacing Fe^{2+} with Fe^{3+}, the SOD-CATALASE conjugate also shows the same benefit in terms of inhibiting hydroxyl radical formation. In Figure 42, enzyme activity has been plotted against the hydroxyl radical signals in the presence of 0.5 mM Fe^{3+}. The graph clearly indicates that the conjugate exhibited a stronger potential in preventing the formation of hydroxyl radicals than the combination of free SOD and catalase under the same conditions.

7.4. Discussion

Catalase might be an ideal carrier to allow the SOD to perform in vivo without the risk of eliciting a potential toxicity. The SOD-catalyzed superoxide dismutation reaction is an important source of hydrogen peroxide. Cross-linking catalase with SOD enables it to abolish the hydrogen peroxide right after its production, decreasing the chance of hydrogen peroxide feed-back to inhibit SOD activity or diffuse away to form the more toxic hydroxyl radicals. Our spin trapping and ESR experimental observations in vitro have provided evidence to support this contention. SOD-CATALASE conjugates have shown an excellent ability to prevent the formation of hydroxyl radicals induced by an excess SOD and iron. This ability is greater for the conjugates than that for the same amount of free enzymes and thus the conjugates appear to be a more effective antioxidant.

While there is a no fixed recipe for the preparation of SOD-CATALASE conjugates, there are a few basic principles which we follow. We attempt to start with an activity ratio of SOD to catalase of 5:1 in order to decrease the possiblity of catalase-catalase polymer and free catalase in the end product. The temperature of the reaction medium is maintained at 4° C in order to slow down

the rate of turnover of the enzyme during the cross-linking process. Glutaraldehyde is usually our cross-linking agent of choice since it appears to be the most gentle, allowing for the greatest preservation of enzyme activity. The resultant conjugate is separated from free enzyme by molecular sieve chromatography and further ultrafiltration through XM 300 filters (300,000 m.w. cutoff). The SOD-CATALASE conjugate can be stored at 4° C for months without losing its enzymatic activity.

We have prepared conjugates with different activity ratios. In a test tube with xanthine/xanthine oxidase as a source of superoxide radicals, we found that the conjugates with activity ratios of SOD to catalase of 7:1; 5:1; and 3:1 all had higher efficacy for inhibiting hydroxyl radical production compared with equivalent amounts of free enzymes. When the activity ratio of SOD to catalase is raised to 10:1, there was a drop in the efficacy of the conjugate to inhibit the formation of hydroxyl radicals. This loss of efficacy may be contributed to a result of the amount of catalase in the conjugated form, which was not sufficient to cope with the elevated concentration of hydrogen peroxide. SOD-CATALASE conjugate with an activity ratio of SOD to catalase of 1:1 (or 1:5) showed the similar efficacy as the free enzymes in controlling hydroxyl radicals derived from the Fenton type reaction. A possible explanation for this observation is that the high activity ratio of free SOD to free catalase (1:1 or 1:5) would allow more catalase molecules to surround the SOD molecule in the reaction system, so that the hydrogen peroxide resulting from SOD-catalyzed dismutation might be scavenged by free catalase in situ (like the performance of catalase in the conjugate). However, this situation is unlikely to occur in vivo because the local concentration of catalase in the organism (e.g. surrounding the SOD) will not be maintained as high as in the test tube, mainly owing to rapid inactivation and

tissue distribution of the enzyme. While in the case of the conjugates, catalase has been "immobilized" to SOD, which keeps the majority of catalase at an effective site to attenuate the potential toxicity of SOD.
Therefore, it is possible that the conjugates with a higher catalase to SOD activity ratio may still have the advantage over the equivalent amount of free SOD and catalase on preventing oxygen free radical damage in vivo.

CuZnSOD is inhibited by hydrogen peroxide while catalase is inhibited by superoxide radicals. These inhibitions provide the basis for synergistic interactions between catalase and SOD. In any reaction system generating both superoxide and hydroxyl radicals, the effectiveness of catalase would be enhanced by SOD, which prevents the conversion of the active catalase into the relatively inactive form. In a reverse synergism, catalase prevents the inactivation of SOD caused by hydrogen peroxide. SOD and catalase clearly constitute a mutually protective set of enzymes for coping with the oxygen free radical. From this point of view, conjugating SOD with catalase is no doubt a good choice, and SOD-CATALASE conjugates might have great potential in strengthening antioxidant defense in vivo.

8. SOD and SOD-CATALASE Conjugate in Isolated Working Hearts

8.1. Introduction

The isolated perfused heart is a useful experimental model for studies involving ischemia/reperfusion injury, in that many of the variables which can affect heart function are controlled. In addition, unlike tissue preparations such as the isolated papillary muscle, oxygen and substrates are delivered to the perfused heart via an intact capillary circulation. The most commonly used isolated perfused heart preparation is the Langendorff rat heart (456). In these hearts, perfusate is supplied to the heart via an aortic cannula by a retrograde flow down the aorta. The main advantages of the method are that the heart can be kept beating for hours, that it is easily accessible for a variety of measurements and that chemically defined media can be used for perfusion. The major disadvantage of the Langendorff preparation is the inability to vary levels of external work during perfusion. This difficulty was overcome by development of the isolated working heart preparation (457). In this model, the perfusate is delivered to the heart via a cannula in the left atrium, and is subsequently ejected from the left ventricle into the cannulated aorta. A major advantage of the modified working heart preparation is the ability to alter both left atrial filling pressure and aortic resistance. This feature is an important property when studying the response of the heart to drugs and hormones.

We have carried out the comparative study of free SOD and SOD-CATALASE conjugates on scavenging oxygen free radical species in vitro. Data from the last chapter demonstrated that SOD-CATALASE conjugates can effectively

decrease the hydroxyl radical formation caused by the Fenton type reaction while an excess of free SOD has the potential to induce ·OH. In this part of the work, we studied the effects of different doses of free SOD and SOD-CATALASE conjugates on reperfusion recovery of the isolated working hearts subjected to transient global ischemia followed by aerobic reperfusion.

8.2. Materials and Methods

8.2.1. Materials

SOD-CATALASE conjugates were prepared as described in section 7.2.2.4.. Male Sprague Dawley rats (250-300 g) were provided by the Health Sciences Laboratory, Animal Services (University of Alberta, Canada), bovine serum albumin (fraction V) and other chemicals (reagent grade) were purchased from Sigma Chemical Company (St Louis, MO U.S.A.).

A diagrammatic representation of the perfusion apparatus is shown in Figure 43. The entire apparatus is jacketed and maintained at 37° C. Perfusate is pumped from the perfusate reservoir to an oxygenator. Oxygenated perfusate enters the left atrium via a left atrial line. Perfusate that is pumped into the oxygenator but does not enter the heart overflows back into the reservoir. The height of the overflow above the heart is the preload to which the left atrium is exposed. Perfusate that does enter the heart flows into the left ventricle and is subsequently ejected from the aorta. The ejected perfusate enters a compliance chamber (simulating aortic compliance) and is subsequently pumped by the heart to the aortic overflow height that is chosen. Once the perfusate reaches this height it overflows back into the perfusion reservoir. A pressure transducer

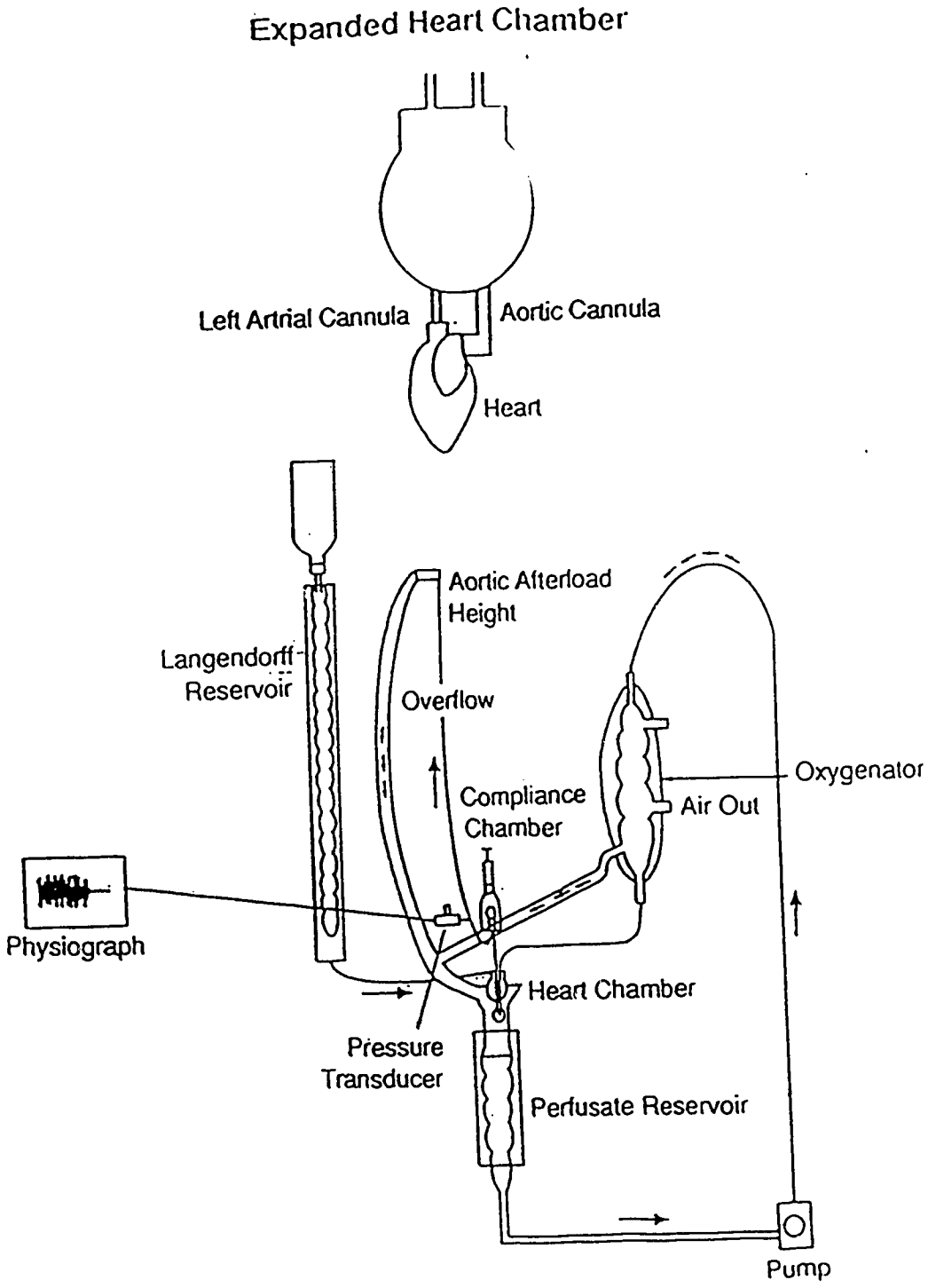


Figure 43: The perfusion apparatus for working heart model. For a detailed explanation of diagram see text.

monitors the pressure of the aortic outflow line. The signal is recorded on a physiograph for sebsequent analysis.

The composition of Krebs-Henseleit buffer for the perfusion is given below:

Krebs-Henseleit Composition (pH7.4):

$NaHCO_3$	25 mM	NaCl	118 mM
KCl	4.7 mM	KH_2PO_4	1.2 mM
$MgSO_4$	1.2 mM	$CaCl_2$	2.5 mM
EDTA	0.5 mM	Glucose	11 mM

For the working heart, Krebs-Henseleit buffer contains 3% bovine serum albumin and 1.2 mM palmitate.

8.2.2. Methods

Cannulation Procedure

Male Sprague Dawley rats were injected i.p. with 0.6 ml of euthanol. This results in the rats losing consciousness within 1 to 2 minutes. When the rat totally lacks sensation the thoracic cavity was opened and the entire heart quickly removed and immersed in cold Krebs-Henseleit buffer. Once the heart was in the buffer, the aorta was located using fine forceps. The entire heart was then suspended from the aortic cannula by placing the opening of the aorta around this cannula. A retrograde perfusate was initiated by opening the perfusate line which originated from the Langendorff reservoir. The entire procedure from opening the thoracic cavity to initiating a Langendorff perfusion did not exceed 2 minutes.

Perfusion Protocol

The heart was initially perfused retrogradely via the aorta for 10 minutes with Krebs-Henseleit buffer, pH 7.4, gassed with 95% O_2-5% CO_2. The heart is normally beating spontaneously at approximately 180 to 220 beats/min. During the Langendorff perfusion, the heart was trimmed of extra tissue and the entrance to the left atrium identified and cannulated to the left atrial cannula. Following this procedure the heart was switched to the working mode by opening the left atrial and aortic afterload lines while simultaneously closing off the Langendorff perfusion line. The heart was perfused at 80 mm Hg aortic afterload and a 11.5 mm Hg left atrial preload with Krebs-Henseleit buffer containing 3% bovine serum albumin, 1.2 mM palmitate (control group) and SOD or SOD-CATALASE conjugates as required (SOD-treated group). Aortic pressure development was measured with a pressure transducer attached to the aortic outflow line. Function was assessed by changes in heart rate and cardiac function throughout the perfusion period.

Ischemic Heart Perfusion

After 15 minutes of work, global ischemia was induced by clamping off the left atrial and aortic flow. After 30 minutes of no-flow ischemia, left atrial and aortic flow was restored with working buffer containing 3% bovine serum albumin, 1.2 mM palmitate and SOD or conjugates. Recovery of mechanical function was monitored for a minimum of a further 30 minutes.

Statistical Analysis

Statistical analysis was performed using analysis of variance, followed by comparison of group means using group t tests. Statistical significance was set at $p < 0.05$.

8.3. Results

8.3.1. Recovery of Heart Rate after Reperfusion

The diagram in Figure 44 demonstrates that preischemic base line values of heart rate did not differ significantly between the control group (227 ± 12 beats/min) and SOD-treated group (232 ± 8 beats/min). Following 30 minutes of reperfusion of isolated working hearts subjected to a 30-minute period of ischemia, the control group recovered 39 ± 11% of base heart rate. The presence of SOD in the perfusion medium at a concentration of 77 IU/ml did not significantly improve the recovery of heart rate in the perfused hearts. The heart rate of the SOD-treated group recovered 44 ± 12% of base value (Figure 44). Increasing the concentration of SOD to 770 IU/ml in the perfusion medium as shown in Figure 45, at the end of a 30-minute reperfusion, the heart rate recovered to 46 ± 16% of base value which was not significantly different from the control groups (39 ± 11%).

The heart rate measured before the onset of ischemia was 218 ± 15 beats/min in the SOD-CATALASE conjugate-treated group (77 IU/ml of SOD and 15 units/ml of catalase respectively). Throughout the 30-minute reperfusion, the heart rate was significantly higher in the SOD-CATALASE conjugate group (Figure 46) than in the control or free SOD-treated group (Figure 44). At the end of the reperfusion period, the heart rate in the group
treated with conjugates preserved 98 ± 10% (215 ± 23 beats/min) of base value compared with 39 ± 11% of base value in controlgroups and 44 ± 12% of base value in free SOD treated groups. Addition of the conjugates containing 770 IU/ml SOD and 150 units/ml of catalase to the perfusion medium also produced

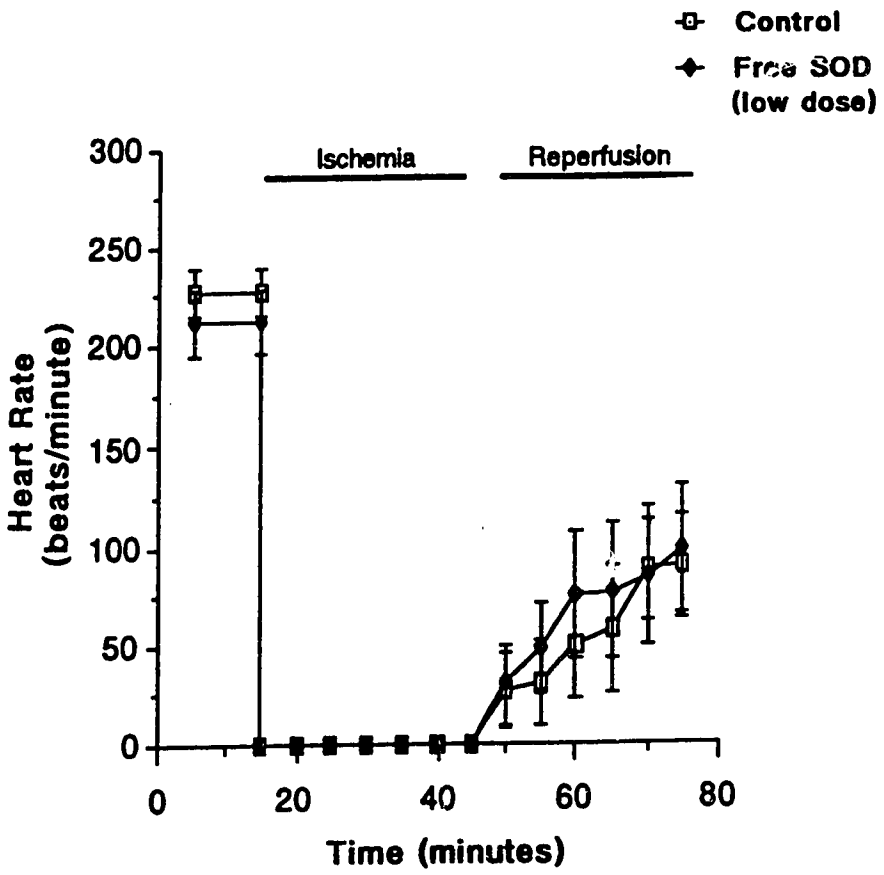


Figure 44: Effect of free SOD (77 IU/ml) on heart rate in isolated working hearts. A 10-minute Langendorff perfusion was initiated with Krebs-Henseleit buffer (pH 7.5) containing 11 mM glucose. Hearts were then switched to the working mode for 10 minutes with buffer containing 3% bovine serum albumin, 11 mM glucose and 1.2 mM palmitate. Hearts were perfused at a left filling pressure of 11.5 mm Hg and a hydrostatic afterload of 80 mm Hg. When used, the SOD (77 IU/ml) was added directly to the perfusate. Aerobic work continued under these conditions for an additional 10 minutes. The preload and afterload lines were then clamped inducing global ischemia. Following 30-minute ischemia the hearts were reperfused for 30 minutes. Values are the mean ± SEM of at least six hearts in each group.

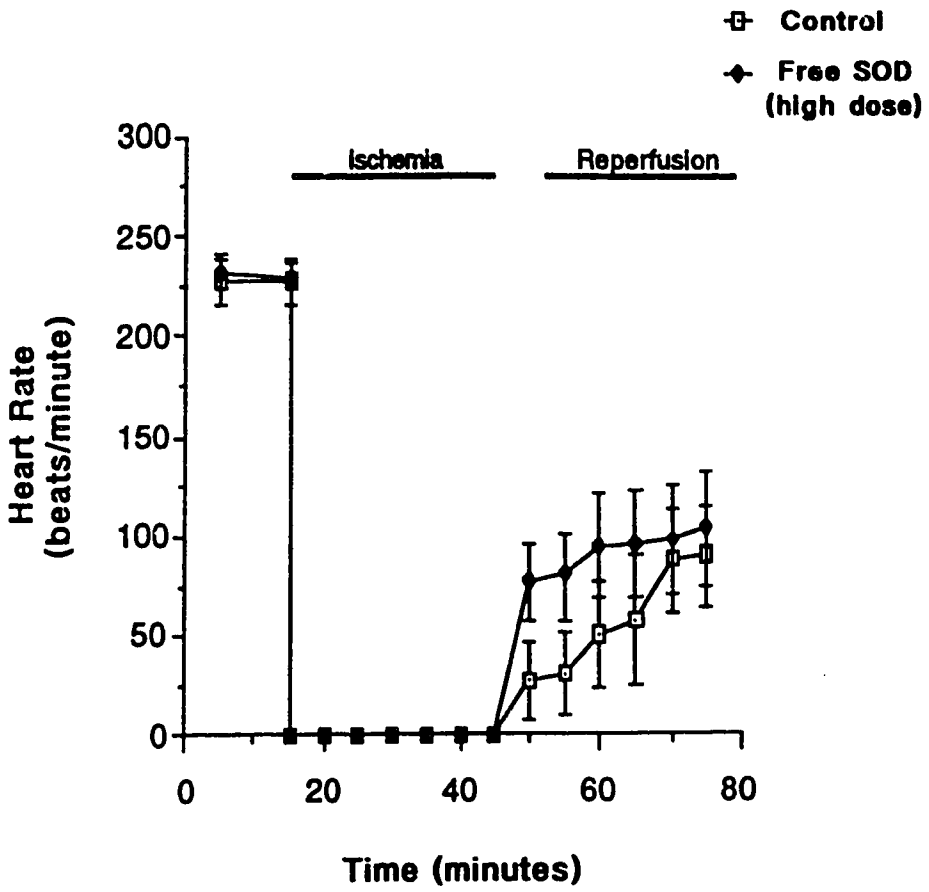


Figure 45: Effect of free SOD (770 IU/ml) on heart rate in isolated working hearts. A 10-minute Langendorff perfusion was initiated with Krebs-Henseleit buffer (pH 7.5) containing 11 mM glucose. Hearts were then switched to the working mode for 10 minutes with buffer containing 3% bovine serum albumin, 11 mM glucose and 1.2 mM palmitate. Hearts were perfused at a left filling pressure of 11.5 mm Hg and a hydrostatic afterload of 80 mm Hg. When used, the SOD (770 IU/ml) was added directly to the perfusate. Aerobic work continued under these conditions for an additional 10 minutes. The preload and afterload lines were then clamped inducing global ischemia. Following 30-minute ischemia the hearts were reperfused for 30 minutes. Values are the mean ± SEM of at least six hearts in each group.

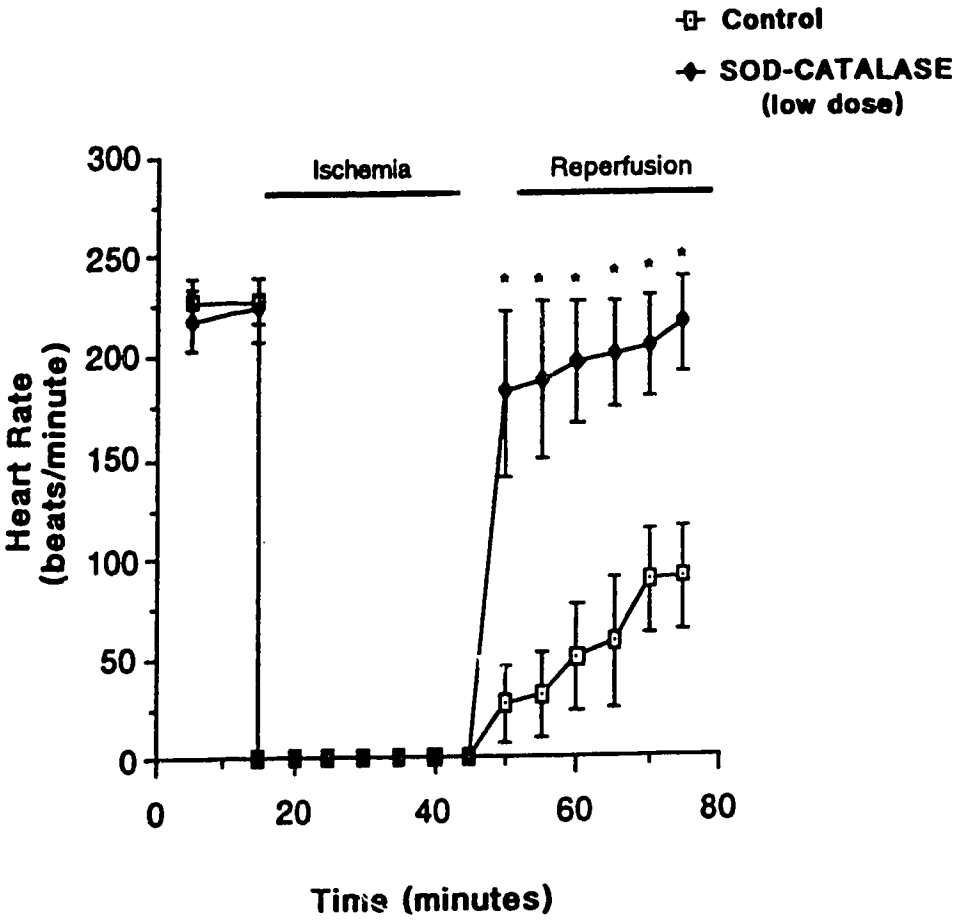


Figure 46: Effect of SOD-CATALASE (low dose) on heart rate in isolated working hearts. A 10-minute Langendorff perfusion was initiated with Krebs-Henseleit buffer (pH 7.4) containing 11 mM glucose. Hearts were then switched to the working mode for 10 minutes with buffer containing 3% bovine serum albumin, 11 mM glucose and 1.2 mM palmitate. Hearts were perfused at a left atrial filling pressure of 11.5 mm Hg and a hydrostatic afterload of 80 mm Hg. When used, the SOD-CATALASE conjugate (77 IU/ml SOD, 15 units/ml catalase) was added directly to the perfusate. Aerobic work continued under these conditions for an additional 10 minutes. The preload and afterload lines were then clamped inducing global ischemia. Following 30-minute ischemia, the hearts were reperfused for 30 minutes. Values are the mean ± SEM of at least five hearts in each group. *Significantly different from control hearts.

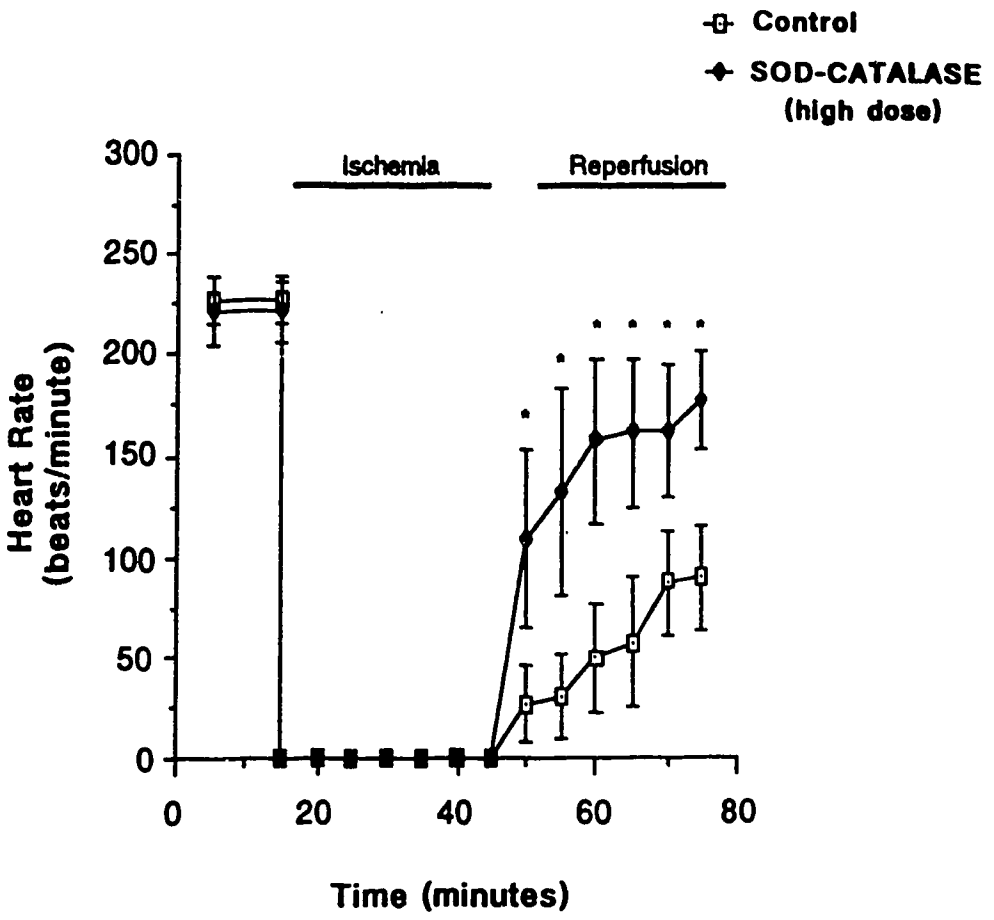


Figure 47: Effect of SOD-CATALASE (high dose) on heart rate in isolated working hearts. A 10-minute Langendorff perfusion was initiated with Krebs-Henseleit buffer (pH 7.4) containing 11 mM glucose. Hearts were then switched to the working mode for 10 minutes with buffer containing 3% bovine serum albumin, 11 mM glucose and 1.2 mM palmitate. Hearts were perfused at a left atrial filling pressure of 11.5 mm Hg and a hydrostatic afterload of 80 mm Hg. When used, the SOD-CATALASE conjugate (770 IU/ml SOD, 150 units/ml catalase) was added directly to the perfusate. Aerobic work continued under these conditions for an additional 10 minutes. The preload and afterload lines were then clamped inducing global ischemia. Following 30-minute ischemia, the hearts were reperfused for 30 minutes. Values are the mean ± SEM of at least five hearts in each group. *Significantly different from control hearts.

a significant increase in recovery of heart rate in the reperfused hearts. The heart rate of perfused hearts treated with this dose of conjugate recovered to 178 ± 25 beats/min (81 ± 11% of base value) following reperfusion (Figure 47).

8.3.2. Recovery of Cardiac Function after Reperfusion

Cardiac function, as measured by the heart rate (HR) peak systolic pressure (PSP) product, was similar in the control and free SOD (77 IU/ml or 770 IU/ml) treated groups before the onset of ischemia. After 30 minutes of global no-flow ischemia, the presence of free SOD in the perfusion medium did not result in a significantly increased postischemia recovery of cardiac function. Following reperfusion, the recovery of cardiac function was 40 ± 10% in control hearts, 48 ± 13% and 25 ± 15% in hearts treated with 77 IU/ml and 770 IU/ml free SOD respectively (Figures 48 and 49). The effect of SOD-CATALASE conjugates on the recovery of cardiac function in reperfused hearts is shown in Figures 50 and 51. In the group of hearts treated with a conjugate containing 77 IU/ml SOD activity and 15 units/ml catalase activity, cardiac function rapidly recovered to 74 ± 18% of the preischemic value within 5 minutes, 80 ± 15% after 15 minutes, and 85 ± 9% at the end of a 30-minute reperfusion (Figure 50). The group of hearts which received the conjugate containing 770 IU/ml SOD activity and 150 units/ml catalase activity, recovered 58 ± 23% of preischemic cardiac function 10 minutes after reperfusion, and 80 ± 12% after 30-minute reperfusion (Figure 51). Both groups of conjugate-treated hearts have demonstrated a significantly greater recovery of cardiac function than the control group hearts.

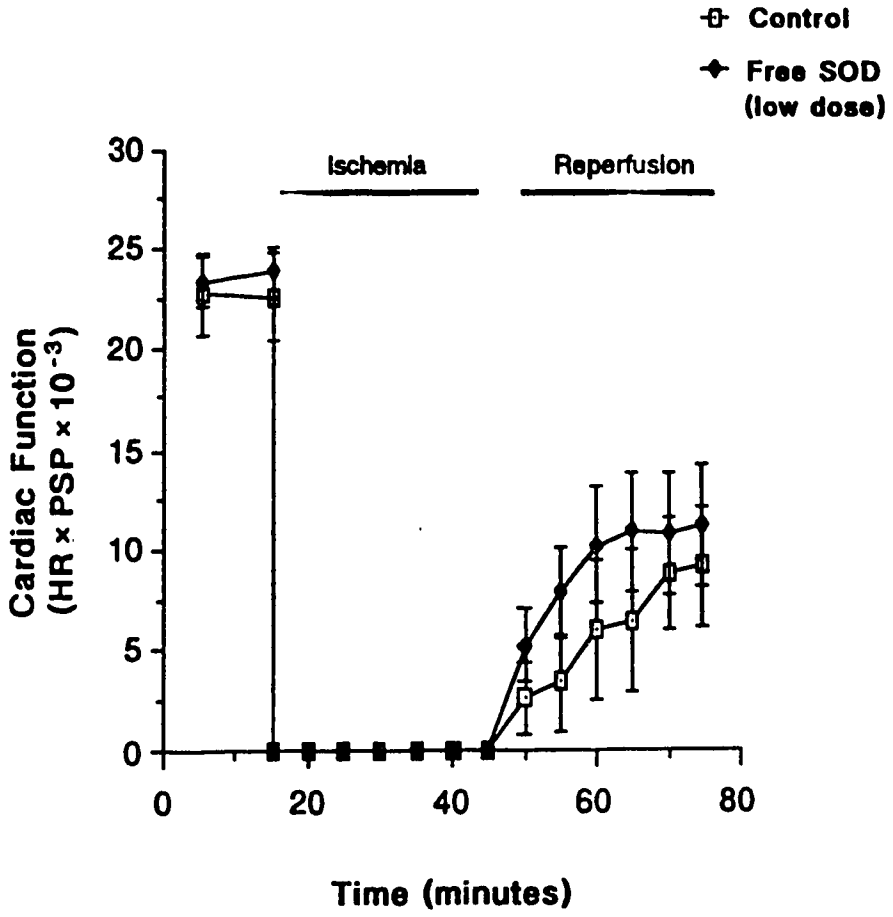


Figure 48: Effect of free SOD (77 IU/ml) on cardiac function in isolated working hearts. A 10-minute Langendorff perfusion was initiated with Krebs-Henseleit buffer (pH 7.4) containing 11 mM glucose. Hearts were then switched to the working mode for 10 minutes with buffer containing 3% bovine serum albumin, 11 mM glucose and 1.2 mM palmitate. Hearts were perfused at a left atiral filling pressure of 11.5 mm Hg and a hydrostatic afterload of 80 mm Hg. When used, the SOD (77 IU/ml) was added directly to the perfusate. Aerobic work continued under these conditions for an additional 10 minutes. The preload and afterload lines were then clamped inducing global ischemia. Following 30-minute ischemia, the hearts were reperfused for 30 minutes. Values are the mean ± SEM of at least six hearts in each group.

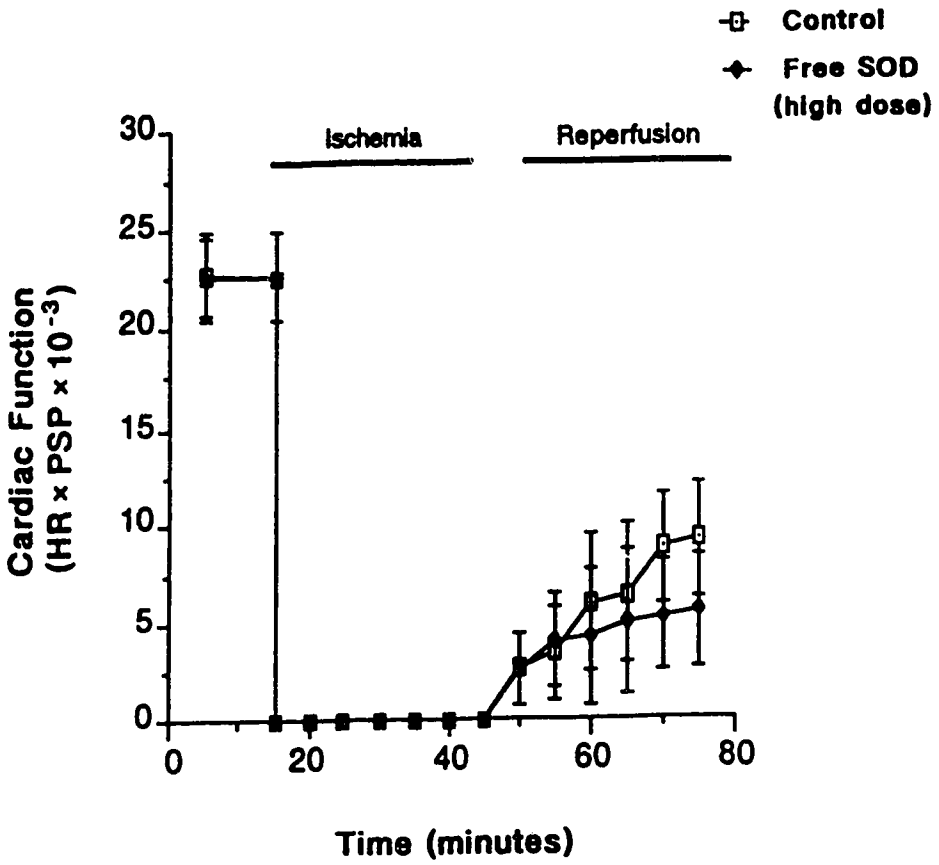


Figure 49: Effect of free SOD (770 IU/ml) on cardiac function in isolated working hearts. A 10-minute Langendorff perfusion was initiated with Krebs-Henseleit buffer (pH 7.4) containing 11 mM glucose. Hearts were then switched to the working mode for 10 minutes with buffer containing 3% bovine serum albumin, 11 mM glucose and 1.2 mM palmitate. Hearts were perfused at a left atrial filling pressure of 11.5 mm Hg and a hydrostatic afterload of 80 mm Hg. When used, the SOD (770 IU/ml) was added directly to the perfusate. Aerobic work continued under these conditions for an additional 10 minutes. The preload and afterload lines were then clamped inducing global ischemia. Following 30-minute ischemia, the hearts were reperfused for 30 minutes. Values are the mean ± SEM of at least six hearts in each group.

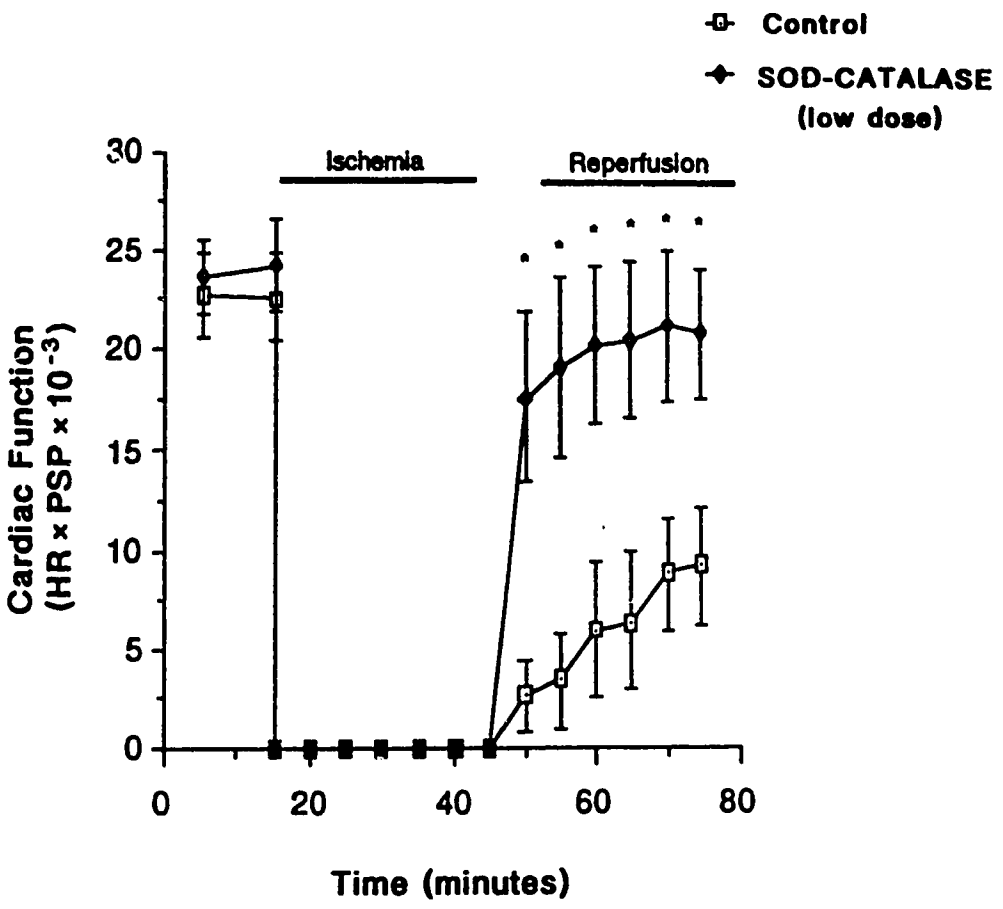


Figure 50: Effect of SOD-CATALASE conjugate (low dose) on cardiac function in isolated working hearts. A 10-minute Langendorff perfusion was initiated with Krebs-Henseleit buffer (pH 7.4) containing 11 mM glucose. Hearts were then switched to the working mode for 10 minutes with buffer containing 3% bovine serum albumin, 11 mM glucose and 1.2 mM palmitate. Hearts were perfused at a left atrial filling pressure of 11.5 mm Hg and a hydrostatic afterload of 80 mm Hg. When used, the SOD-CATALASE conjugate (77 IU/ml SOD, 15 units/ml catalase) was added directly to the perfusate. Aerobic work continued under these conditions for an additional 10 minutes. The preload and afterload lines were then clamped inducing global ischemia. Following 30-minute ischemia, the hearts were reperfused for 30 minutes. Values are the mean ± SEM of at least five hearts in each group. *Significantly different from control hearts.

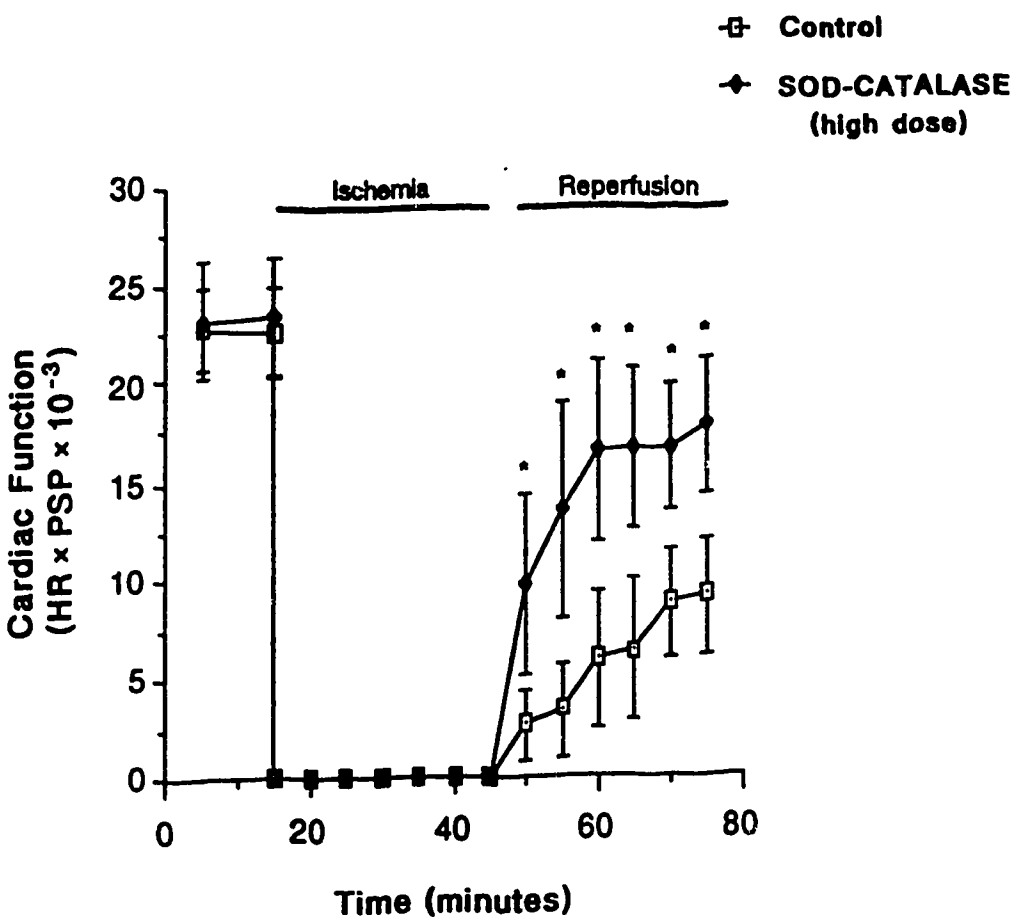


Figure 51: Effect of SOD-CATALASE conjugate (high dose) on cardiac function in isolated working hearts. A 10-minute Langendorff perfusion was initiated with Krebs-Henseleit buffer (pH 7.4) containing 11 mM glucose. Hearts were then switched to the working mode for 10 minutes with buffer containing 3% bovine serum albumin, 11 mM glucose and 1.2 mM palmitate. Hearts were perfused at a left atrial filling pressure of 11.5 mm Hg and a hydrostatic afterload of 80 mm Hg. When used, the SOD-CATALASE conjugate (770 IU/ml SOD, 150 units/ml catalase) was added directly to the perfusate. Aerobic work continued under these conditions for an additional 10 minutes. The preload and afterload lines were then clamped inducing global ischemia. Following 30-minute ischemia, the hearts were reperfused for 30 minutes. Values are the mean ± SEM of at least seven hearts in each group. *Significantly different from control hearts.

Table 10 provides a summary of the data collected from the hearts undergoing ischemia and reperfusion. Comparing a control group (ischemia/reperfusion) with the low dose SOD-treated group, it appears that this particular dose of SOD does not significantly improve the recovery of cardiac function of reperfused hearts. In the high dose SOD treated group, half of the reperfused hearts completely failed to regain cardiac function and the other half of the reperfused hearts partially recovered to the base value. Although a more convincing conclusion may need more experimentation, the present data suggest that a high dose of SOD has an apparent toxicity on the isolated reperfused hearts. The SOD-CATALASE conjugates (either low or high dose) significantly prevented the depression of cardiac function of reperfused hearts. Following a 30-minute reperfusion, the cardiac function of both sets of conjugate-treated groups returned to 80% of base value.

Table 10: Cardiac Function *

Experimental Group	% Return to Base Value Following Reperfusion**
Control	35 ± 8
Low Dose SOD	44 ± 10
High Dose SOD	20 ± 18 (0-50) ***
Low Dose SOD-CATALASE	85 ± 9
High Dose SOD-CATALASE	80 ± 12

* Cardiac function is defined as the heart rate times the peak systolic pressure.

** Minimum of 8 hearts in each group except for the high dose SOD-CATALASE group having only 5 hearts (values represent mean ± SEM.

*** The range for the high dose SOD hearts was much larger than for the others and there were 4 complete failures.

Low dose SOD = 77 units/ml

High dose SOD = 770 units/ml

The low and high dose SOD-CATALASE conjugates had 77 units/ml and 770 units/ml of SOD respectively. Keeping the constant activity ratio of SOD/catalase of 5:1.

8.4. Discussion

The effect of treatment with SOD on protection of the isolated reperfused hearts has been the center of controversy for a number of years. Ambrosio and colleagues (458) have shown that after receiving 60,000 IU of SOD as a bolus and a continuous infusion of 100 IU/ml for 15 minutes at the time of reflow, Langendorff perfused rabbit hearts improved recovery of myocardial metabolism and function. They reported that after 45 minutes of reperfusion, myocardial phosphocreatine recovered to 89 $\pm$ 8% of control in SOD-treated hearts and to 83 $\pm$ 6% in free SOD/free catalase treated hearts compared with 65 $\pm$ 5% in untreated hearts. Recovery of isovolumic left ventricular developed pressure was 68 $\pm$ 5% of control in SOD-treated hearts and 66 $\pm$ 6% in SOD/catalase treated hearts, compared with 48 $\pm$ 6% in untreated hearts. McCord et al. (415) have shown that SOD at 60 IU/ml concentration suppressed creatine kinase release in Langendorff rat hearts by 72% and SOD at 15 IU/ml suppressed lactate dehydrogenase release in Langendorff rabbit hearts by 54% of the controls. However, Myers et al. found a divergent result (459) showing that SOD (30 mg/ml) alone did not significantly reduce creatine kinase loss in isolated buffer-perfused rabbit hearts. Nejima et al. (460) reported that treatment of conscious dogs with SOD or SOD and catalase neither increased the recovery of wall thickening in the ischemic zone nor reduced infarct size when compared with a control group. The overall data dealing with SOD and the rationale for its use during ischemia/reperfusion has been reviewed by R. Engler and E. Gilpin (462).

Although we have not carried out a systematic SOD dose-response study, we did assess the effect of two SOD doses on isolated reperfused hearts. One was

a low dose of 77 IU/ml, a concentration similar to most experiments with beneficial results, and the other was a high dose of 770 IU/ml. The results in Table 10 demonstrate the recovery of cardiac function in isolated reperfused hearts under different conditions. It shows that the difference in preservation of cardiac function between low dose SOD-treated hearts and the control (without SOD) hearts was not significant, so that the low dose of SOD did not have the protective effect on the reperfused hearts. We observed that a high dose of SOD appears to exacerbate the free radical injury which is in agreement with McCord's laboratory reports (415,420). In Table 10, in comparison with the control and low dose SOD-treated hearts which all exhibited a depressed mechanical function (but not complete failure), only half of high dose SOD-treated hearts preserved partial cardiac function following 30-minute reperfusion. The other half of the high dose SOD-treated hearts completely failed. We suggest that this wide range of recovery in cardiac function (from complete failure to 50% return) may be due to an apparent toxicity of a high dose SOD for the reperfused hearts, although we need more values on the SOD dose-response study to reach a convincing conclusion.

The administration of SOD during the period of ischemia/reperfusion, either simply failed to improve the recovery of myocardial function in isolated reperfused hearts or in fact may have caused more damage at the higher free SOD concentrations. These results imply that H_2O_2 or ·OH may act as the major damaging species in the genesis of reperfusion injury in our model. Considering that a high dose of SOD speeds up the dismutation of superoxide radicals mainly from the xanthine/xanthine oxidase reaction, hydrogen peroxide may accumulate in the rat heart tissue during the period of reperfusion. The presence of a large concentration of hydrogen peroxide may overwhelm cellular

defense and cause lethal cell injury by two primary mechanisms. The first mechanism is related to the peroxidation of membrane phospholipids by H_2O_2. It has been shown that upon exposing hepatocytes to hydrogen peroxide, the cell membrane phospholipids were readily peroxidized by H_2O_2. As a result, there was a good correlation between the amount of MDA (product of lipid peroxidation) accumulated in the cultures and the number of dead cells (461). The second mechanism, and perhaps the more important one, was that hydrogen peroxide damages cells through the generation of the more toxic hydroxyl radical via the Fenton reaction. This notion is supported by our earlier data using ESR and spin trapping methods (see section 6). It has been demonstrated that the acceleration of dismutation of superoxide radicals by SOD resulted in a dramatic enhancement of hydroxyl radicals in the presence of a low concentration of metal ions. Considering these results and the potential harm caused by the increased concentration of H_2O_2, it appeared that including catalase in our intervention (conjugate) wouid have an impressive beneficial effect on avoiding the direct toxicity of hydrogen peroxide and the possible amplified toxicity due to hydroxyl radicals through the Fenton reaction. Our results indicating that SOD-CATALASE conjugates are more efficient in preventing depressed mechanical functions including both heart rate and cardiac function in reperfused hearts (Figures 46,47,50,51) support this hypothesis. At the present time, we can not eliminate the possibility of the direct toxicity of superoxide radicals in the isolated reperfused hearts, but we think that it is more critical to avoid the hydroxyl radical than its less reactive precursor, the superoxide radical. SOD-CATALASE conjugate may provide an ideal means to achieve this purpose.

9. General Conclusions

The ubiquity and abundance of SOD in aerobes and its paucity in anaerobes suggest that SOD plays a vital defensive role in the prevention of oxygen free radical damage. However, the therapeutic effect of exogenous SOD is restricted in vivo presumably due to very rapid blood clearance of the enzyme and potential immunogenicity and toxicity of the enzyme. We have described an approach to prepare a soluble polymer of SOD and human serum albumin using glutaraldehyde as a cross-linking agent. The SOD-Albumin polymers have the following important advantages over native SOD: (a) the polymers markedly prolong plasma half-life of enzymatic activity (4 hours) compared with native SOD (10 minutes), (b) the polymers increase the resistance to H_2O_2 inhibition, (c) the polymers are more stable under a variety of storage conditions, (d) the polymers have lower immunological reactivity compared with native SOD. The SOD-Albumin polymers may provide new possibilities for the application of SOD in medicine.

It has been suggested that oxygen free radicals mediate tissue damage associated with a variety of pathologic conditions. Of the available methods for the detection of free radicals, spin trapping offers the opportunity to simultaneously measure and distinguish a variety of biologically generated free radicals. In this technique, a nitrone or a nitroso compound reacts with a short-lived free radical to form a relatively long-lived spin-trapped adduct which is detectable by conventional electron spin resonance spectroscopy. We have used the spin trapping agent DMPO and ESR to study the generation of oxygen free radicals from enzymatic and cellular sources. The ESR spectra obtained from the reaction of xanthine with xanthine oxidase exhibit both a DMPO-

superoxide adduct (DMPO-OOH) signal and a DMPO-hydroxyl adduct (DMPO-OH) signal . Addition of the hydroxyl radical scavenger DMSO or the hydrogen peroxide scavenger catalase decreases the intensity of DMPO-OH. Addition of superoxide dismutase only inhibits the DMPO-OOH while the combination of SOD and catalase completely inhibits all free radical spin-trapped adduct signals. These observations suggest that the reaction of xanthine oxidase produces hydroxyl radicals as well as superoxide radicals and hydrogen peroxide. The ESR spectra obtained from the PMA-stimulated human neutrophils have shown that the generation of superoxide radicals by stimulated neutrophils is a progressive process. However, the ESR data do not support the hypothesis that neutrophils directly produce hydroxyl radicals. The main experimental evidence is (a) all spin-trapped adduct signals can be scavenged by superoxide dismutase; (b) catalase has no effect on the spin-trapped adduct signals; (c) in the presence of the hydroxyl radical scavenger DMSO, an accumulation of DMPO-CH_3 has not been observed following a continuous stimulation of neutrophils. We suggest that the DMPO-OH peak and the small DMPO-CH_3 peak in the ESR spectra following the stimulation of neutrophils are due to the decomposition of DMPO-OOH.

The study of the permeability of oxygen free radicals to membranes may lead to a greater understanding of the detrimental effect of free radicals and the appropriate design of effective antioxidant therapy. We have used lipid vesicles as a lipid bilayer model and erythrocyte ghosts as a biological membrane model. Superoxide dismutase was entrapped in lipid vesicles and erythrocyte ghosts. By incubating SOD lipid vesicles with an external source of superoxide radicals, we found that SOD inside the lipid vesicles is unable to scavenge extravesicular superoxide radicals unless the lipid bilayer is lysed by

deoxycholate. This result indicates that the lipid bilayer is impermeable to superoxide radicals. In contrast, superoxide radicals generated outside the erythrocyte ghosts can interact with SOD enclosed in the ghosts. Intact SOD ghosts have been shown to inhibit external superoxide radicals as effectively as the hypotonically relysed SOD ghosts. Thus our data indicate that superoxide radicals can cross the erythrocyte membrane and are likely to permeate biological membranes. The future trials of antioxidants may pay special consideration to block the pathway of superoxide radicals through the membrane and quench free radicals in situ.

SOD may exacerbate the oxygen free radical damage at high doses. The interpretations for this phenomenon have not been proven. One hypothesis from our laboratory is that an excess of SOD may cause an over-production of hydrogen peroxide, which in turn results in an "explosion" of hydroxyl radicals via the Fenton reaction. Iron may play a vital role in this mechanism. In order to verify this hypothesis, we have used the xanthine/xanthine oxidase system as a source of oxygen free radicals. The effect of iron on the production of hydroxyl radicals has been studied either in the presence or absence of SOD. The results have shown that in the absence of SOD, ferrous ion only slightly increases the generation of hydroxyl radicals compared with the control. However, the combination of SOD and ferrous ion induces huge DMPO-OH adduct signals. This effect of Fe^{2+} on the enhancement of hydroxyl radicals can be observed at a concentration of Fe^{2+} as low as 2 μM. Ferric ion has exhibited a similar reaction pattern of enhancing the production of hydroxyl radicals in the presence of SOD. Since Fe^{3+} must be reduced to Fe^{2+} for catalyzing the formation of ·OH from H_2O_2, relatively higher concentrations of Fe^{3+} (millimolar range) are needed. These results imply that SOD, in the presence of iron, has a

high toxic potential since the product of SOD-catalyzed dismutation of superoxide radicals, hydrogen peroxide, is reduced by iron to form more toxic hydroxyl radicals. From a physiological perspective, iron is carefully handled in higher organisms. It is transported in the ferric state and bound to transferrin. It is mainly stored in the ferric state by ferritin, a protein found in virtually all tissues and in plasma. However, the superoxide radicals and organic free radicals are capable of reducing ferritin-bound iron to the ferrous state, whereupon it is released. Therefore, the pathological production of superoxide radicals may liberate sufficient free iron which participates in the Fenton reaction and exacerbate tissue damage.

We have studied the dose-response of SOD on the enhancement of hydroxyl radical formation in the xanthine/xanthine oxidase system using ESR and spin trapping techniques. In the absence of iron, SOD does not increase the DMPO-OH adduct signal at the given doses. In the presence of fixed concentrations of Fe^{2+}, the DMPO-OH peaks are proportional to the SOD doses until a plateau is reached. This implies that high doses of SOD increase the availability of hydrogen peroxide for the Fenton reaction to form more hydroxyl radicals. In the presence of Fe^{3+}, catalytically active Fe^{2+} results from the reduction of Fe^{3+} by superoxide radicals. When the concentration of Fe^{3+} is 0.1 mM, Fe^{3+} can not compete with SOD for available superoxide. As a result, an inhibition of DMPO-OH adduct signals is observed due to the retardation of the superoxide-mediated reduction of Fe^{3+} to Fe^{2+}. At higher concentrations of Fe^{3+} (0.3 and 0.5 mM), Fe^{3+} competes with SOD for superoxide and is reduced to Fe^{2+}. Therefore the increase of SOD actually enhances the formation of hydroxyl radicals by providing more hydrogen peroxide.

Other evidence which supports our hypothesis is derived from the combined application of SOD and catalase. The hydrogen peroxide production promoted by SOD can be effectively eliminated by catalase. Therefore, the addition of catalase effectively decreases the huge DMPO-OH peaks induced by SOD and iron. This result suggests that simultaneous administration of SOD and catalase may attenuate the potential toxicity of SOD.

In order to improve pharmacological properties of native SOD and catalase and efficiently use them, we have cross-linked SOD with catalase. The SOD-CATALASE conjugate is stable and enzymatically active, has greatly increased clearance time and has increased resistance to inhibitors. In terms of inhibiting the formation of hydroxyl radicals in the presence of iron, ESR spectra have shown that the conjugates appear to be more effective than the combination of the equivalent free enzymes. We have assessed the protective effect of SOD-CATALASE conjugates in an isolated heart model of ischemia/reperfusion. Following a 30-minute period of global ischemia, the ischemic hearts are reperfused with free SOD or SOD-CATALASE conjugates. The results demonstrate that a low dose of SOD does not improve functional recovery of the reperfused hearts. Heart rate and cardiac function remain depressed in low dose SOD-perfused hearts compared with that of buffer-perfused hearts. The high dose of SOD appears toxic to the reperfused hearts. Our data have demonstrated that half of the ischemic hearts reperfused with a high dose of SOD failed completely, and the other half only partially recovered their cardiac function. In contrast, the ischemic hearts perfused with the conjugates (either low or high dose) have significantly increased functional recovery in terms of heart beat and cardiac function. At the end of reperfusion, the heart rate in the conjugate-treated group preserves an average of 90% of pre-ischemia values

compared with an average of 40% in the control or free SOD-treated groups. Cardiac function in the conjugate-treated group (either low or high dose) recovers up to 80% compared with 35% in the control group, 44% in the low dose SOD-treated group and 20% in the high dose SOD-treated group. It appears therefore that the catalase "immobilized" on SOD can scavenge hydrogen peroxide immediately after its generation and thereby prevent it from reacting with iron to form hydroxyl radicals. This characteristic may account for the beneficial effect of the SOD-CATALASE conjugate for tissue protection. Our data in vitro and in vivo suggest that the SOD-CATALASE conjugate seems more amenable for use as a therapeutic agent.

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