THESIS

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Assay of Superoxide Dismutase

in the Hyperoxic Retinal and

Choroidal Tissues of the Rainbow Trout

(Salmo gairdneri)

presented by

Graig Edward Eldred

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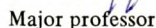
of the requirements for

Ph.D. degree in Physiology



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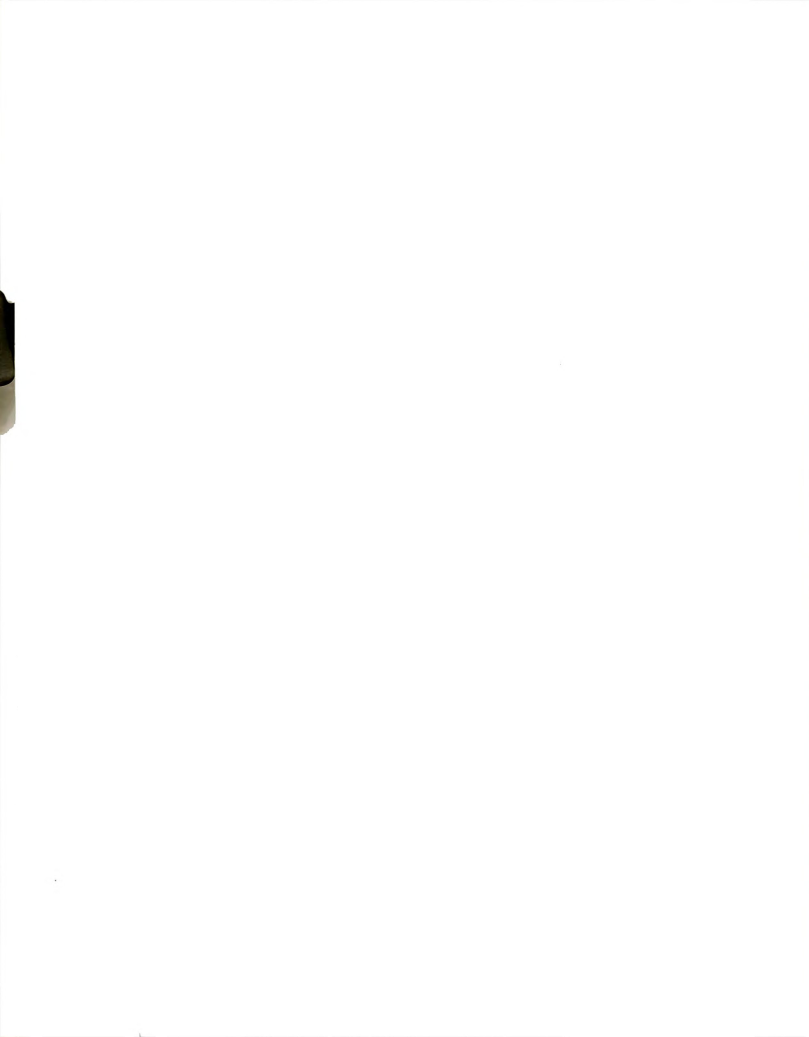

ASSAY OF SUPEROXIDE DISMUTASE IN THE HYPEROXIC RETINAL AND CHOROIDAL TISSUES OF THE RAINBOW TROUT (*SALMO GAIRDNERI*)

By

Graig Edward Eldred

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ABSTRACT

ASSAY OF SUPEROXIDE DISMUTASE
IN THE HYPEROXIC RETINAL AND
CHOROIDAL TISSUES OF THE RAINBOW TROUT
(SALMO GAIRDNERI)

By

Graig E. Eldred

Within the past decade, the superoxide dismutase (SOD) enzymes have been demonstrated to be of primary importance against the toxic effects of the superoxide anion and other free radicals generated therefrom. Free radicals are generated not only in normal metabolism, but also under exposure to such varied conditions as hyperbaric oxygen, ionizing and visible irradiation, paraquat poisoning, and senescence. Ocular tissues are known to be particularly susceptible to many of these agents.

In the current investigation, superoxide dismutase is assayed by its ability to inhibit the reduction of acetylated ferricytochrome c by superoxide anion generated by a xanthine-xanthine oxidase system. Analysis of variance applied to parallel-line assay techniques allows the assured detection of the presence of any endogenous

interfering substances within crude tissue extracts.

These procedures have been applied to the problem of determining physiological levels of SOD within the neural retina, photoreceptor outer segments, retinal pigment epithelium, and choroidal tissues of the rainbow trout, Salmo gairdneri. A countercurrent multiplier for oxygen within the choroidal layer of this species continuously subjects the ocular tissues to oxygen tensions twenty times those of arterial values.

It is shown that the parallel-line approach reveals the presence of substances which alter the reaction mechanism of the assay in the blood, choroidal tissues, retinal pigment epithelium and photoreceptor outer segments. The cytosolic form of SOD is seen to be relatively constant throughout the layers of the retina whereas the cyanide-insensitive form increases with distance from the vitreous. The role of superoxide dismutase as a cellular antioxidant is discussed in relation to other purported cellular antioxidants, and with special referrence to the physiological phenomena present in the retinal tissues.

The Antithesis:

"My dear colleagues, I had no purpose to swell this treatise into a large volume by quoting the names and writings of anatomists, or to make a parade of the strength of my memory, the extent of my readings, and the amount of my pains;..."

William Harvey, in the presentation of his treatise: Anatomical Studies on the Motion of the Heart and Blood to the Royal College of Physicians, 1628.

A conference glossary:

> "In presenting papers, when they say,
> '...careful statistical analysis...',
> they mean, 'After going through a
> dozen books, we finally found one
> obscure test that we could apply.'"
>
> Kritchevsky and Van der Wal (1960)

DEDICATION

—Ibid. plus two new.

—And to the post-war baby boom which continues to keep my professional and financial status and prospects tenuous indeed.

ACKNOWLEDGMENTS

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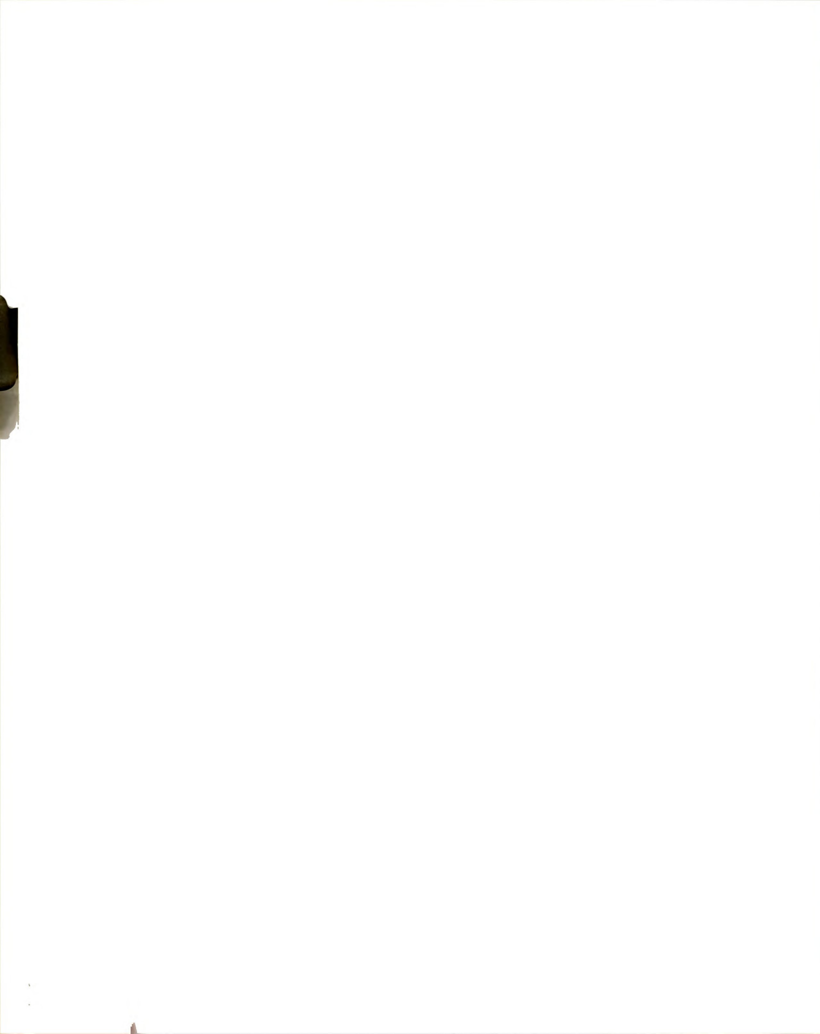

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INTRODUCTION

The pernicious effects of elevated oxygen tensions upon immature retinal vasculature and other retinal tissues has been a matter of concern for many years. In the early 1940's, high tolls of brain damage caused by cerebral anoxia was a common problem in premature infants. At the time a decision was made to include as standard treatment, incubation of the infants in an environment enriched in oxygen. But concomitant with this treatment, a unique form of blindness arose and it quickly became an epidemic (Silverman, 1977). The syndrome was termed retrolental fibroplasia. Its characteristic symptom was a heavily vascularized grayish membrane, appearing on the rear surface of each lens.

It was not until the early 1950's that the causative factor for this disease was decisively established to be exposure to elevated oxygen tensions (Ashton et al., 1953). The initial effect of the oxygen was shown to be a marked narrowing of the immature retinal vessels, followed by their obliteration. Upon return to a normal atmosphere, the blood vessels grew in a disorganized fashion, with a budding of new capillaries out of the retina into the vitreous body. In most cases this proliferative process subsided, leaving a normal retina,

but in some cases, extensive hemorrhages developed and fibrous scar tissue formed, causing the retina to become detached from its normal position and to billow out against the back of the lens (Ashton et al., 1954; Patz, 1975).

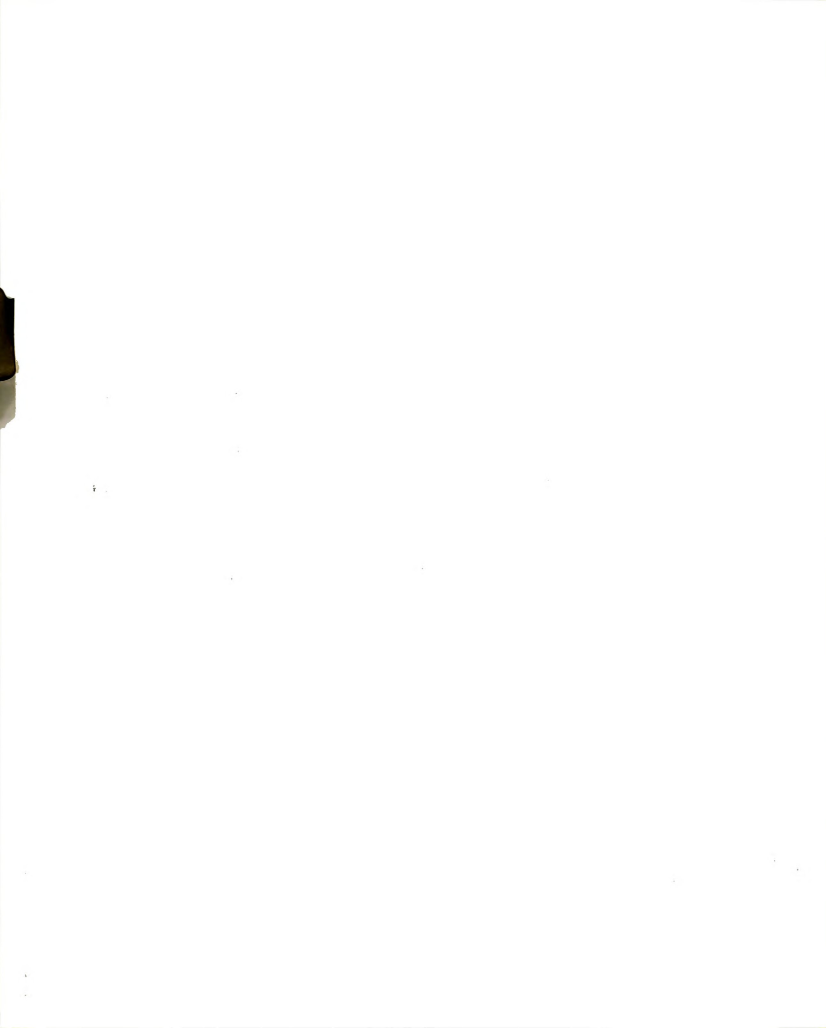
Upon elucidation of the problem, the oxygen therapy was curtailed but there was also an increased death rate among premature infants in the first 24 hours of life. Cross (1973) stated: "Severe cyanosis in the small premature baby became an increasingly common spectacle, and the anxieties induced by the Haldane dictum came more and more to mind---'anoxia not only stops the machine, but wrecks the machinery'." Based upon infant mortality statistics from the years 1935 through 1970, he concluded that each sighted baby gained may have cost some 16 deaths due to anoxia. As of 1977, a compromise had been struck whereby premature infants who need extra oxygen to survive without brain damage receive it, but in concentrations not seeming to give rise to blindness with as high a frequency (Patz, 1976; Silverman, 1977). Thus, the goal of elucidating the mechanisms of oxygen toxicity to the immature retinal vasculature remains of as great a clinical consequence as it was a quarter of a century ago.

Exposure to elevated oxygen tensions is deleterious not only to immature retinal vasculature, but also to other retinal tissues. Rabbits exposed to hyperbaric

oxygen display an extreme attenuation in the electroretinogram a- and b-waves (Noell, 1962; Bridges, 1966; Ubels et al., 1977). This decrease in ERG amplitude has been correlated with increased lipoperoxide levels (Yagi and Ohishi, 1977). Haugaard (1968) pointed out that lipid peroxidation is related to the toxic effects produced by high concentrations of oxygen in all tissues. Ultimately, the process ends in a histologically demonstrable degeneration of the visual cell layer of the retina (Noell, 1962; Yagi and Ohishi, 1977).

In summary, the gross manifestations of ocular oxygen toxicity are: 1) generalized vasoconstriction followed by obliteration of the immature vascular tissues, 2) a decrease in cellular function as manifested by the electroretinogram, 3) an increase in the lipid peroxide content of the tissues, and 4) histologically demonstrable cell death and degradation.

Nearly identical manifestations result from exposure to other cytotoxic agents. Noell (1955, 1958) reported that exposure of adult rabbits to a high concentration of oxygen at ambient pressure for a few days induced retinal degeneration similar to that induced by X-ray irradiation. Several workers have reported that X-ray irradiation induces peroxidation of lipids (Horgan et al., 1957; Wills and Wilkinson, 1967; Glavind and Faber, 1967). In these cases, the presence of molecular oxygen was



found necessary if X-ray irradiation were to have an effect.

Similarly, exposure to monochromatic light sources of various wavelengths in the visible range induces irreversible reduction of the ERG a-wave amplitude and degeneration of visual cells and pigment epithelium (Noell et al., 1966). A drastic disruption of the highly organized, lipid-rich, stacked membranous disk structure of the photoreceptor outer segments occurs upon only 24 hours exposure to relatively cold light at a brightness (750 ft-c.) well below the photocoagulating intensity and only slightly above that used for exhaustive bleaching of the photopigments (Kuwabara and Gorn, 1968). This light-induced disruption has also been correlated with an accumulation of lipid peroxides in the photoreceptor outer segments (Kagan et al., 1973). Again, the presence of molecular oxygen is requisite to the damaging effects of visible light (Noell et al., 1966).

Thus, the cellular and physiological manifestations of retinal oxygen toxicity, light-induced retinal damage, and damage due to ionizing radiations all appear to be essentially identical and all require the presence of, or are potentiated by, molecular oxygen. Gershman et al. (1954) postulated that both X-ray irradiation and oxygen poisoning produce lethal effects through a common mechanism, possibly through the formation of oxidizing free

radicals, highly reactive substances containing unpaired electrons. The destructive effects of visible light (i.e., photodynamic actions) are thought to be photosensitized oxidations initiated by the action of light upon a dye or natural pigment. The dye or pigment acts catalytically in a chain reaction which ultimately leads to the reaction of substrate with free oxygen to form a peroxide via activated and free radical intermediates (Slater, 1965; Foote, 1968). The free radicals formed in all of these cases are in turn responsible for producing irreversible damage to proteins and membrane lipids. In fact, these same free radicals may be causative agents of slower, ubiquitous aging processes (Harman, 1968; Sinex, 1977; Sanadi, 1977). In summary, then, it now appears that all of these toxicity problems result from a series of chemical reactions whereby oxygen is converted to a number of transient free radicals.

It was not until 1968 that an enzyme capable of protecting the cell against the primary free radical of oxygen was recognized. The enzyme was termed superoxide dismutase (SOD) (McCord and Fridovich, 1969). The isolation of this enzyme had been achieved prior to this date from a wide variety of tissues and it had been variously termed haemocuprein (Mann and Keilin, 1938), erythrocuprein (Markowitz et al., 1959), cerebrocuprein (Porter and Ainsworth, 1959), hepatocuprein (Porter

et al., 1964), and cytocuprein (Carrico and Deutsch, 1969). Yet its function remained unknown until J. McCord and I. Fridovich happened upon its true role subsequent to some frustrating and unsuccessful work on carbonic anhydrase (McCord and Fridovich, 1977).

In the brief, but lucrative, decade of SOD research that followed, it has been determined that all oxygen metabolizing cells encountered to date have evolved SOD in conjunction with other protective mechanisms that either minimize the production of free radicals, or alternatively, destroy them as rapidly as they are formed.

The goal of the present study was to develop an SOD assay which is applicable to very crude tissue extracts and not susceptible to the many possible interfering substances which are inevitably present in such preparations (Misra and Fridovich, 1977). Within the decade in which the importance of SOD has been known, many assays have been developed, but most have been employed in the determination of specific activities of purified extracts. Problems arise in assays for SOD due to the fact that whereas SOD is specific for the substrate, $O_2^{\cdot-}$, the substrate is not specific to the enzyme. Thus, apparent SOD activity can arise from the presence of various other enzymes and small molecules in crude tissue extracts.

An assay procedure has been developed which aids in detecting these problems when applied to crude tissue

extracts. It has been utilized in an investigation into the physiological levels of SOD within the ocular tissues of the rainbow trout, *Salmo gairdneri*. This species has long been used as a model in the study of ocular oxygen toxicity because of the unusually high oxygen tensions under which this visual system operates (Wittenberg and Wittenberg, 1962; Fairbanks et al., 1969). Levels of superoxide dismutases were determined in the choroidal layer, retinal pigment epithelium, photoreceptor outer segments, and neural retina of this species.

LITERATURE REVIEW

In reviewing the literature, only a very brief summary of SOD chemistry will be presented. The family of SOD enzymes will then be placed into cellular perspective, particularly in relation to other antioxidant mechanisms which have long been purported to be of significance in protecting the cell against the toxic effects of oxygen and other lethal agents. Attention will next be turned to the specific sources of concern within those ocular tissues which are of interest to the current study. Finally, the levels of SOD which have been measured in other tissues in the past decade will be tabulated.

Oxygen Reductions and the Superoxide Dismutases

All cells which can exist in an environment containing oxygen, or which utilize oxygen in metabolism, inevitably generate very reactive free radical intermediates of oxygen. This occurs in the normal course of events, without the encroachment of cytotoxic agents. During biological oxidations, most cellular oxygen is reduced by two-electron transfers via the cytochrome carriers to yield harmless water (H_2O). However, during electron transport to molecular oxygen via the mitochondrial respiratory chain, toxic univalent reduction products of oxygen may be formed indirectly, presumably as transient

intermediates on the active sites of such enzymes. The most important intermediate is the superoxide anion ($O_2^{\cdot}$). Superoxide anion formation has been detected in heart submitochondrial particles treated by succinate and NADH (Loschen et al., 1974; Tyler, 1975a). This species is also produced as a direct reduction product of various hydroxylation and oxygenation reactions within the general cytoplasm, for example, in the aerobic actions of the enzymes xanthine oxidase, aldehyde oxidase and many flavin dehydrogenases (Fridovich, 1975a). The NADPH oxidation system of liver microsomes (Fridovich and Handler, 1961) and ferredoxins (Misra and Fridovich, 1971a) also generate $O_2^{\cdot}$. The superoxide anion is produced in autoxidations of reduced flavins, hydroquinones and catecholamines as well (Michelson, 1977a). Although both non-haem iron and flavin groups have been proposed as sites of O_2 reduction to $O_2^{\cdot}$, non-haem iron sites appear to be far more effective reductants than are sites containing flavin (Misra and Fridovich, 1971b, 1972a).

Molecular oxygen is a relatively unreactive species. Unlike most other elements, oxygen has two unpaired electrons with parallel spins. The complete divalent reduction of one oxygen atom requires an electron pair having parallel but opposite spins to that in oxygen. Such a pair is a rarity,however, since most compounds offer pairs of electrons with antiparallel spins. For this

reason, ground state molecular oxygen is relatively unreactive. Therefore the normal reduction of oxygen must proceed through steps of univalent electron additions, thereby forming superoxide radical anions as intermediates in the ultimate divalent reduction process. This univalent reduction product is much more reactive in that the parallel spin restrictions do not exist for this species.

Although superoxide anion is a very reactive species itself, the real danger lies in the various side products which are formed as the superoxide anion spontaneously dismutates with itself and reacts with other species present within the cellular milieu. Superoxide anion may nonenzymatically lose an electron to give rise to singlet oxygen ($^1O_2^*$) and hydrogen peroxide (H_2O_2) (Stauff et al., 1963; Khan, 1970; Pederson and Aust, 1973; Fee and Valentine, 1977):

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + {}^1O_2^*$$

Singlet oxygen is an electronically excited oxygen molecule in which a valence electron is shifted from its normal bonding orbital to an orbital of higher energy in which the electron spins are paired (Wilson and Hastings, 1970; Maugh, 1973). Singlet oxygen may act as an initiator of lipid peroxidation (Howes and Steele, 1971; Dowty et al., 1973; Pederson and Aust, 1973). The lipoperoxides thus formed cause not only changes in lipid composition via self-perpetuating autoxidative chain reactions (May and

McCay, 1968a,b), but further serious damage to essential structural components such as lipoproteins and enzymes (Tappel, 1973; Desai and Tappel, 1963; Hochstein and Ernster, 1963). These are among the main reactions leading to widespread cell damage and death.

Superoxide anion and hydrogen peroxide have been postulated to further combine through what is known as the Haber-Weiss reaction to form the hydroxyl free radical ($OH^{\cdot}$) (Haber and Weiss, 1934):

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

The hydroxyl radical is the most potent oxidant known to mankind, capable of attacking virtually any of the organic substrates found in cells (Fong et al., 1973; Myers, 1973; Kellogg and Fridovich, 1975). The extent of damage attributable to this mechanism is currently a matter of active debate (Cohen, 1977; Fee and Valentine, 1977).

The role of the superoxide dismutase enzymes (SOD) is to prevent the accumulation of the superoxide radical ($O_2^{\cdot-}$). SOD catalyzes the following dismutation reaction:

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

The SOD enzymes are present in high concentrations and are extraordinarily active, suggesting that $O_2^{\cdot-}$ radicals are being continuously produced during the enzymatic reduction of oxygen by various enzymes and enzyme systems and are quickly removed (Fridovich, 1977a).

SOD is found in two forms, one in the extramitochondrial cytosol and another in mitochondria. The mitochondrial SOD of eukaryotes is similar to the SOD of many bacteria with respect to its characteristic content of Mn^{++} and many homologies in amino acid sequence. The cytosol form of SOD has quite a different structure and contains Cu^{++} and Zn^{++}. These differences lend support to the currently extant concept of a symbiotic bacterial ancestry to mitochondria (Cohen, 1973; Puget et al., 1977; Harris and Steinman, 1977; Lumsden et al., 1977).

The biological importance of superoxide dismutase in its capacity as a defense mechanism has been demonstrated by a number of studies conducted with prokaryotic bacteria. McCord et al. (1971) examined the distribution of SOD among three classes of micro-organisms: aerobes which utilize oxygen in their metabolism almost exclusively, aerotolerant organisms which have an anaerobic metabolism even when grown in air, and strict anaerobes which cannot survive in oxygen. In all cases the aerobic organisms contained the highest activity of SOD, followed by intermediate activity in the aerotolerant group. Strict anaer-contained no SOD, which may explain their inability to tolerate oxygen. Hence, this family of enzymes is of penultimate importance to all aerobic cells.

The molecular chemistry and reaction kinetics of SOD's have been reviewed many times in the past few years

(Malmstrom et al., 1975; Michelson et al., 1977; Fridovich, 1974a,b, 1975a,b, 1976, 1977a,b,c, 1978), so only the most general overview to the subject has been made here.

The Cellular Perspective

Although SOD has been emphasized in recent years as being the key cellular protective agent against oxygen toxicity and oxidative degradation, numerous other substances have been advanced for this role as well. Among these are catalase, peroxidase, ascorbate, reduced glutathione, vitamin E, selenium, glutathione peroxidase and succinate. The lipid components of the membrane have long been considered a major site of oxidative susceptibility as evidenced by the accumulation of autofluorescent "wear and tear" pigment (i.e., lipofuscin) in oxidatively damaged cells. In addition, many essential sulfhydryl-containing enzymes seem particularly susceptible to damage. The evidence accumulated in these areas must be unified into the overall scheme of cellular antioxidant protection as well. This discussion will lead to a much clearer understanding of the multifarious processes occurring within the reaction mixture of crude tissue extracts during the SOD assay procedure as well.

Catalase and Peroxidase

Granted the ubiquitous distribution of SOD and its importance in destroying superoxide anion radical, the reaction equation shows that in so doing, it produces hydrogen peroxide. Although hydrogen peroxide is a more stable and less reactive molecule, it is still highly

toxic to the cell since it can, by reaction with the superoxide anion or with Fe^{++} produce the extremely reactive hydroxyl free radical ($OH^{\cdot}$) (Weiss, 1953; Tappel, 1975; Cohen, 1977). Additionally, H_2O_2 absorbs ultraviolet light continuously from 410 nm to 200 nm and this absorption results in the photolytic decomposition into two $OH^{\cdot}$ radicals (Fee and Valentine, 1977).

Catalase and peroxidase have evolved as a second defense against a buildup of hydrogen peroxide (Feeney and Berman, 1976). They act to reduce H_2O_2 to harmless water. In emphasis of the secondary role played by these enzymes, neither catalase nor peroxidase share the ubiquity of distribution among aerobic and aero-tolerant organisms that is enjoyed by SOD. Catalase is found mainly in liver, kidney, and erythrocytes, and the peroxidases are a group of enzymes found in a variety of cell types, namely leukocytes, mammary, thyroid, and salivary glands, and most recently, in retinal pigment epithelium (Klebanoff, 1975; Paul, 1963; Pilz et al., 1976; Armstrong et al., 1975, 1978a). The difference between the two enzymes is that catalases can use H_2O_2 as both oxidant and reductant:

$$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2$$

whereas, peroxidases use some reductant other than H_2O_2 as hydrogen donor:

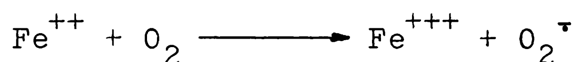
$$H_2O_2 + H_2R \longrightarrow 2H_2O + R$$

The physiological reductant may be glutathione, ascorbic acid, or cytochrome c. The physiological reductants are, in turn, renewable by the reduced forms of nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH).

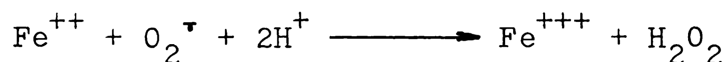
Alternative Scavengers of Free Radicals

Reduced glutathione, ascorbic acid, and cytochrome c readily accept electrons and may themselves serve a backup function by scavenging free radicals (McCord and Fridovich, 1969). In the case of cytochrome c, however, this effect is not related to its function in the respiratory chain in that the amount of added cytochrome c required for maximal protection of NADH oxidase activity during NADH treatment is about 500 times the amount of endogenous cytochrome c present in the submitochondrial particles (Tyler, 1975a).

Thiol compounds terminate free radical reactions by allowing the hydrogen on the —SH to be abstracted. The resultant thieyl radical, $-S^{\cdot}$, may then combine with another to form disulfide, —S—S— (Demopoulos, 1973a,b). In the cell, the major SH component is glutathione (Haugaard, 1968). Glutathione reacts very slowly with O_2, but in the presence of copper or iron ions glutathione reacts very rapidly with oxygen (Isherwood,1959). These metallic ions cause a catalytic conversion of O_2 to $O_2^{\cdot-}$, $OH^{\cdot}$ and other reactive intermediates (Michelson, 1977b):



$$Fe^{++} + O_2 \longrightarrow Fe^{+++} + O_2^{\cdot -}$$



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

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

$$Fe^{++} + OH^{\cdot} \longrightarrow Fe^{+++} + OH^{-}$$

Activated oxygen in the form of $O_2^{\cdot -}$ thus can cause hydrogen abstraction from the glutathione sulfhydryl groups (GSH = reduced glutathione, $GS^{\cdot}$ = glutathieyl radical, GSSG = glutathione dimer):

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

$$2GS^{\cdot} \longrightarrow GSSG$$

Thus, treatment with excess reduced glutathione will scavenge the superoxide anion thereby assisting in the protection against the effects of hyperbaric oxygen (Baeyens and Meier, 1978).

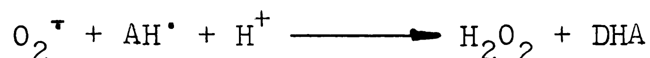
Glutathione is a stronger reducing agent than ascorbic acid and may also play a role in keeping ascorbate in its reduced form in the cell (Colowick et al., 1954; Mapson, 1959)(DHA = dehydroascorbic acid, AH_2 = ascorbic acid):

$$2GSH + DHA \longrightarrow GSSG + AH_2$$

Ascorbic acid is widely referred to as another free radical scavenger. The oxidation of ascorbic acid by $O_2^{\cdot -}$ occurs as follows (Epel and Neuman, 1973; Allen and Hall, 1973; Nishikimi, 1975):

$$O_2^{\cdot -} + AH_2 + H^{+} \longrightarrow H_2O_2 + AH^{\cdot}$$

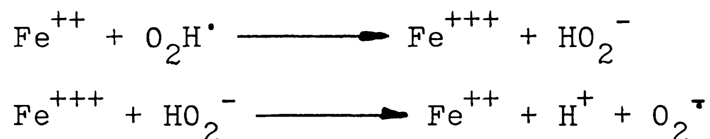
The $AH^{\cdot}$ radical is a semiquinone, but unlike the semiquinones of flavin and NADH (Vaish and Tollin, 1971; Land and Swallow, 1971; Misra and Fridovich, 1971b), $AH^{\cdot}$ is unable to reduce O_2 to $O_2^{\cdot-}$ (Nishikimi, 1975). Instead, it is further oxidized by yet another $O_2^{\cdot-}$:



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

Although the reaction rate of ascorbate oxidation is low as compared to that of the SOD reaction ($2.7 \times 10^5\ M^{-1}sec^{-1}$ vs. $1.9 \times 10^9\ M^{-1}sec^{-1}$), the rate is still 2.7 times the rate of spontaneous dismutation ($1 \times 10^5\ M^{-1}sec^{-1}$) (McCord et al., 1977b). It has been proposed that the velocity of the ascorbate reaction may become comparable to that of the SOD reaction in tissues of high ascorbic acid concentration (Nishikimi, 1975; Nishikimi and Yagi, 1977). This would have to be concentrated 7000 times that of the same tissue's SOD concentration. Treatment with large doses of ascorbic acid has been reported to protect against oxygen poisoning (Benbough, 1969; Jamieson and Van den Brenk, 1964).

On the other hand, it has also been shown that ascorbic acid readily oxidizes in the presence of molecular oxygen and this reaction is increased by the presence of metal ions such as Cu^{++} and Fe^{++}. A mixture of ascorbic acid and a metal complex functions as a source of $O_2H^{\cdot}$ radicals. This process is termed homolysis by redox coupling and can ultimately lead to $O_2^{\cdot-}$ generation (Slater, 1972):



$$Fe^{++} + O_2H^{\bullet} \longrightarrow Fe^{+++} + HO_2^{-}$$

$$Fe^{+++} + HO_2^{-} \longrightarrow Fe^{++} + H^{+} + O_2^{\bar{\cdot}}$$

Liver microsomes have been shown to peroxidize when exposed to ascorbic acid especially in the presence of added ADP-ferrous ions. This system is very sensitive to α-tocopherol and EDTA (Hochstein and Ernster, 1963). The effect of ascorbic acid on lipid peroxidation complicates studies on the biosynthesis of this vitamin for lipid peroxidation causes a decrease in the activity of L-gulonolactone oxidase which is part of the biosynthetic route to ascorbic acid (Chatterjee and McKee, 1965). Low concentrations of EDTA, however, prevent this peroxidation and allow accurate measurements of vitamin C synthesis to be made. The system is also responsive to hyperbaric oxygen (McCay et al., 1960). L-gulonolactone oxidase activity is increased upon exposure to hyperbaric oxygen and this, by increasing the synthesis of ascorbic acid, increases lipid peroxidation, causing a decrease in the polyunsaturated fatty acid (PUFA) content of rat liver microsomes. Currently, much work is being done on the effects of lipid peroxidation on photoreceptor and retinal function using a ferrous sulfate-ascorbate incubation medium to induce lipid peroxidation (Kozlov et al., 1972; Kagan et al., 1975; Novikov et al.,1975; Shvedova et al., 1979). The effect of ascorbic acid within the cell will depend, then, upon the concentrations present and upon the presence or absence of trace amounts of metal ions,

particularly non-haem iron.

Carotenoids quench triplet molecules efficiently (Chessin et al., 1966; Fujimori and Livingston, 1957). Carotenoid pigments have additionally been demonstrated to be quenchers of singlet oxygen in vitro (Foote and Denny, 1968) and may function similarly in vivo (Krinsky, 1971; Krinsky, 1974b). β-carotene in solution is an extremely efficient quencher for singlet oxygen (^{3}β-carotene = triplet state):

$$^1O_2^* + \beta\text{-carotene} \longrightarrow O_2 + {}^3\beta\text{-carotene}$$

In this reaction, one molecule of β-carotene quenches at least 100 molecules of singlet oxygen (Foote, 1968). A demonstration of this quenching mechanism was provided by experiments conducted by Krinsky (1974a). A mutant strain of bacteria deficient in carotenoid pigments was observed to be much more susceptible to killing by human polymorphonuclear (PMN) leukocytes than a comparable carotenoid-containing strain. These PMN leukocytes destroy ingested bacteria by generation of singlet oxygen which disrupts bacterial membranes (Allen et al., 1972; Maugh, 1973; Allen et al., 1974). Thus, the susceptibility of the mutant bacteria to destruction by PMN leukocytes may be due to an inability to quench singlet oxygen. There is some evidence to indicate that carotenoids may also function in eukaryotes to quench singlet oxygen. Matthews (1964) was able to protect against the lethal photodynamic effects

of intraperitoneally injected hematoporphyrin in mice when the animals were simultaneously injected with β-carotene. The protection afforded in mice to photodynamic lethality may be due to quenching of singlet oxygen, as many photo-oxidations proceed via singlet oxygen mechanisms (Foote, 1968).

Melanin

Melanin is a free radical itself, and as such, may have a role as a biological electron-transfer agent (Mason et al., 1960). Melanin granules are membrane-limited, compacted layers of melanopolymer. The melanin compound is an insoluble, high molecular weight polymer derived from the enzymatic oxidation of tyrosine and dihydroxyphenylalanine. On the basis of molecular orbital calculations, Pullman and Pullman (1961) postulated that melanin should be an extremely good electron acceptor. Melanin appears to be unique among free radicals in its relative stability, yet it can efficiently transfer electrons in a manner analogous to the processes in p- or n-type semiconductors (Longuet-Higgins, 1960). This has been demonstrated by its ability to transfer electrons from NADH to ferricyanide (Gan et al., 1976). Melanin can also bind paramagnetic transition metal ions such as manganese, copper, or iron (Sarna et al., 1976) all of which are essential cofactors for forms of copper-containing oxidases and superoxide dismutases (Malmstrom et al., 1975). Superoxide anion

and other free radical species containing one unpaired electron, are by definition themselves paramagnetic (Fee and Valentine, 1977), but whether melanin has an affinity for $O_2^{\cdot}$ for this reason alone is not known.

It is not yet known whether the free radical character of melanin is beneficial or detrimental to the cell. Mason et al. (1960) suggested that melanin may be able to protect the tissues against the reducing and oxidizing conditions which might otherwise liberate reactive free radicals capable of disrupting metabolism. This is consistent with the postulated photoprotective action of melanin (Edelstein, 1971).

On the other hand, incident radiation has been shown to stimulate the formation of free radicals in melanin. Mason et al. (1960) demonstrated that under conditions of ultraviolet radiation, black hair gives a strong increase in free radical content whereas unmelanized hair gives a poor response. Proctor et al. (1974) cite evidence suggesting that melanin can absorb this excited state (i.e., free radical) energy and channel it into processes yielding highly cytotoxic substances, including hydrogen peroxide. Additionally, Van Woert (1967, 1968) and Gan et al. (1974) have reported that melanin can oxidize NADH, while molecular oxygen is simultaneously reduced to H_2O_2. Through this mechanism, UV-generated hydrogen peroxide is responsible for the bleaching action of sunlight on hair. Similarly,

Cope et al. (1963) have demonstrated a reversible free radical generation in melanin granules from eyes by visible light.

<u>Lipid Peroxidation</u>

Many free radicals, regardless of their source, are capable of acting on biomembranes, and specifically on their polyunsaturated fatty acids (PUFA) (Tappel, 1975). The free radical chain reaction proceeds in three distinct steps (Pryor, 1973). First, is the initiation process in which the radicals are generated. Second, is a series of propagation reactions in which the number of free radicals is conserved. Finally, there is a series of termination reactions in which free radicals are destroyed (Bus, 1975) (LH = polyunsaturated lipid; $L^{\cdot}$ = lipid free radical; $LOO^{\cdot}$ = lipid peroxide radical; LOOH = lipid hydroperoxides):

Initiation:

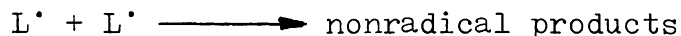
$$2LH + O_2^{\overline{\cdot}} \longrightarrow L^{\cdot} + H_2O$$

Propagation:

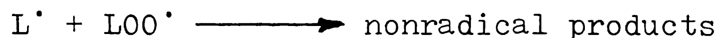
$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot}$$

$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot}$$

Termination:



$$L^{\cdot} + L^{\cdot} \longrightarrow \text{nonradical products}$$



$$L^{\cdot} + LOO^{\cdot} \longrightarrow \text{nonradical products}$$

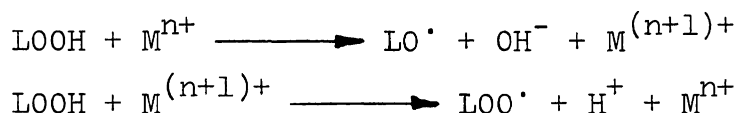
$$LOO^{\cdot} + LOO^{\cdot} \longrightarrow \text{nonradical products}$$

Unsaturated fatty acids are particularly susceptible to peroxidation because the presence of a double bond weakens the carbon-hydrogen bond on the carbon atom adjacent to the unsaturated carbon-carbon bond (Swern, 1961; Demopoulos, 1973a). As a result these allylic hydrogens are susceptible to abstraction by small amounts of oxidants or initiators (initiation reaction). Molecular oxygen can abstract an allylic hydrogen, but it first must be activated. The role of singlet oxygen as an initiator of lipid peroxidation has been confirmed by several investigators (Howes and Steele, 1971; Dowty et al., 1973; Pederson and Aust, 1973).

Once initiated, an avalanche of autocatalytic free radical chain reactions may occur. The lipid free radical ($L^{\cdot}$) is readily converted to lipid peroxide radical ($LOO^{\cdot}$) in the presence of molecular oxygen. The $LOO^{\cdot}$ is a highly reactive species which triggers a chain propagation reaction by interacting with an adjacent PUFA molecule in the membrane. This process, known as autoxidation, is self-perpetuating in the presence of oxygen, and plays the leading role in membrane damage.

The lipid hydroperoxides (LOOH) that are generated in the propagation step are unstable and decompose to form additional radical products. In a process called molecule-assisted homolysis, trace amounts of transition metal ions or hemeproteins capable of hydrogen bonding with

peroxide groups will weaken the oxygen-oxygen bond and cause decomposition of lipid hydroperoxides resulting in the generation of additional lipid free radicals (Holman, 1954; Heaton and Uri, 1961; Barber and Bernheim, 1967; Pryor, 1976) (M^{n+} = transition metal ion):



$$LOOH + M^{n+} \longrightarrow LO^{\cdot} + OH^{-} + M^{(n+1)+}$$
$$LOOH + M^{(n+1)+} \longrightarrow LOO^{\cdot} + H^{+} + M^{n+}$$

The catalytic decomposition of lipid hydroperoxides along with propagation reactions previously described are therefore auto-catalytic, i.e., more free radicals are renewed as reaction products.

In addition to initiating a peroxidative chain reaction, the lipid peroxide free radical ($LOO^{\cdot}$) can also decompose, forming highly reactive fragments such as malonaldehyde, a three carbon dialdehyde ($OHC\text{-}CH_2\text{-}CHO$). This substance readily forms cross-linkages with free amino groups of proteins, phospholipids, and nucleic acids, giving rise to high molecular weight fluorescent polymers, and at the same time immobilizing functionally important enzymes in cellular and subcellular membranes. These chemically damaged cell organelles appear to be autophagocytized by the cell's lysosomal system, but owing to their relative insolubility and indigestibility (or due to the lack of appropriate enzymes), they cannot be completely degraded. These indigestible biomolecules accumulate within the lysosomal compartment of the

cytoplasm and are recognized structurally as autofluorescent late-stage secondary lysosomes or residual bodies. Classically, they are known as lipofuscin or age pigments (Tappel, 1975; Feeney, 1978). They are morphologically heterogeneous due to variations in the molecular structure of the original damaged organelles.

The lipid peroxide radicals (LOO$^{\bullet}$) that are generated in the propagation step may themselves abstract hydrogen atoms from neighboring proteins, resulting in protein cross-linking to form polymers (Tappel, 1965) (P = protein):

$$LOO^{\bullet} + P \longrightarrow LOOH + P(-H)$$

$$P(-H) + P \longrightarrow P{-}P$$

Studies by Chio and Tappel (1969) have demonstrated that sulfhydryl enzymes are most susceptible to inactivation by lipid peroxidation, as a result either of alteration of membrane structure, or of protein-protein cross-linking.

Several membrane proteins like succinic dehydrogenase and β-hydroxy butyrate dehydrogenase may derive some of their structure from closely associated membrane lipids. In the presence of lipid peroxidation, and in particular, the termination reactions in which two adjacent fatty acids are joined in abnormal bonds, the enzyme structure may be sufficiently altered to affect activity (Demopoulos, 1973a). Both Na-K ATPase and rhodopsin are integral membrane proteins and their function is highly dependent upon the physico-chemical characteristics of their

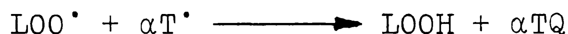
immediate lipid environment (Korenbrot, 1977).

The autocatalytic propagation reactions continue until substrate is depleted, sufficient termination reactions occur, or until intervention by another line of defense interposes itself to interrupt the chain reaction process (Demopoulos, 1973b). Certain substances, notably α-tocopherol (i.e., vitamin E), are able to intercept or terminate the autoxidative chain reaction and thereby protect the membrane against further damage. In general, antioxidants function by allowing a hydrogen to be abstracted from themselves rather than from the allylic hydrogen of an unsaturated lipid and thus act by interrupting the free radical chain reactions (Tappel, 1972) (αTH = α-tocopherol; αTQ = α-tocopherol quinone):

Antioxidant action:

$$LOO^{\cdot} + \alpha TH \longrightarrow LOOH + \alpha T^{\cdot}$$

Termination:



$$LOO^{\cdot} + \alpha T^{\cdot} \longrightarrow LOOH + \alpha TQ$$

The lipid hydroperoxides thus generated are still capable of decomposing into additional lipid and peroxide free radicals via the molecule-assisted homolytic reactions discussed above. To minimize this, another protective system exists in the cell which inactivates LOOH by converting it to a harmless hydroxy fatty acid (LOH). This reaction utilizes glutathione peroxidase, a selenium-containing enzyme.

Selenium and vitamin E are thought to act synergistically in protecting cell membranes from oxygen damage. The antioxidant function of selenium was proposed from early studies which demonstrated that vitamin E deficiency syndromes such as nutritional muscular dystrophy were reversed by addition of small amounts of selenium to the diet (Bieri et al., 1961; Scott, 1962; Scott, 1969). It has also been long known that the enzyme glutathione peroxidase could detoxify hydrogen peroxide and lipid hydroperoxides (LOOH) in vivo (Mitts and Randall, 1958; Cohen and Hochstein, 1963; Christopherson, 1969; O'Brien and Little, 1969). Recently, Rotruck et al. (1973) proposed that selenium was a necessary cofactor for glutathione peroxidase, as selenium deficient rats were unable to prevent hydrogen peroxide induced erythrocyte hemolysis. Purification of glutathione peroxidase from erythrocytes has demonstrated that the enzyme consists of four subunits, with one gram-atom of selenium per subunit (Flohe et al., 1973; Oh et al., 1974). Other recent investigations have shown that glutathione peroxidase activity is directly related to the levels of dietary selenium in rats (Chow and Tappel, 1974; Hafeman et al., 1974; Reddy and Tappel, 1974; Smith et al., 1974; Tappel, 1974) and in chicks (Noguchi, 1973; Tappel, 1974). Thus, the majority of the antioxidant activity of selenium appears to be mediated through glutathione peroxidase activity.

The importance of glutathione peroxidase activity in detoxifying lipid hydroperoxides has been demonstrated in studies in which animals were exposed to oxidant stress. It has been proposed that glutathione peroxidase may protect against lipid peroxidative damage since the activity of this enzyme was induced in rat lung after exposure to the oxidant gas, ozone (Chow and Tappel, 1972; Chow et al., 1974). It was also observed by these investigators that the enzyme activity of glutathione reductase and glucose-6-phosphate dehydrogenase was induced in response to ozone exposure, in addition to the induction of glutathione peroxidase. The three enzymes were referred to as the glutathione peroxidase system and are proposed to function as a unit in combating lipid peroxidation. The conversion of toxic lipid hydroperoxides to lipid alcohols by glutathione peroxidase is now thought to be linked to the activity of glutathione reductase and glucose-6-phosphate dehydrogenase which supply reducing equivalents in the form of reduced glutathione (GSH) and NADPH, respectively (Chow and Tappel, 1972).

Thus, the biochemical consequences of lipid peroxidation at the membrane level are exceedingly complex and involve not only the unsaturated lipids, but also the many different proteins that are an integral part of membranes. Similarly, free radicals are capable of directly damaging enzymes, amino acids (Mead, 1976) and mucopolysaccharides (Matsumura et al., 1966).

Role of SOD Protection against Lipid Peroxidation

From the above discussion, it is evident that much oxidative damage occurs within the hydrophobic, polyunsaturated fatty acid-rich, lipid environment of the membranes. Oxygen is 7-8 times more soluble in nonpolar media and thereby has an affinity for the hydrophobic midzone of membranes. Some of the most potent catalysts involved in lipid peroxidation (coordinated iron and hemeproteins) are found in association with membranes (Tappel, 1973). Yet, the superoxide dismutases are soluble enzymes and not available inside the hydrophobic inner membrane layers.

This apparent paradox is explicable by considering the rarely emphasized importance of the redox potentials involved in the reactions. The oxidation-reduction potential for the $O_2/O_2^{\cdot-}$ redox couple ($O_2 + e^- \longrightarrow O_2^{\cdot-}$) is -0.57V in aprotic solvents, but only -0.31V in protic solvents such as water (Fee and Valentine, 1977). Thus, although a paucity of biological reducing agents exist that are as strong as -0.57V, superoxide production can clearly be driven by a number of biological reducing agents in the aqueous cytoplasmic medium. Similarly, the charged, anionic $O_2^{\cdot-}$ would be unlikely to penetrate the lipophilic zone in which the PUFA exist, but if allowed to spontaneously dismutate to give rise to activated singlet oxygen, this species not only could, but would have an affinity for the lipophilic zone. The soluble mitochondrial and

cytosol forms of SOD are thus available precisely where needed. Their true role being to sequester $O_2^{\cdot}$ generated as enzymatic by-products either within the protic environment of the cytoplasm or on the hydrophilic zones of the membrane, and to catalytically dismutate these species before they spontaneously react to form: 1) the extremely reactive hydroxyl free radical, or 2) singlet oxygen, which would enter the hydrophobic zone to initiate lipid autoxidative chain reactions.

Mitochondrial Free Radical Formation

The organelle primarily concerned with cellular oxygen utilization is the mitochondrion. Within this structure are the enzymes responsible for the tricarboxylic acid cycle which acts to provide reducing equivalents in the form of NADH to the electron transport chain. Electron transport chain redox enzymes are a series of highly organized, integral membrane proteins occurring within the inner mitochondrial membrane. The electrons are passed sequentially from NADH dehydrogenase to ubiquinone to the b cytochromes, cytochrome c, cytochrome a, and finally to oxygen in a divalent reduction step. During this transfer, all cellular ATP is generated.

During oxidation of substrate by mitochondria (Boveris et al., 1972) and during NADH oxidation by submitochondrial particles (Hinkle et al., 1967) small amounts of H_2O_2 are formed. This H_2O_2 is very likely to originate from the

dismutation of $O_2^{\cdot}$ generated in the respiratory chain (Flohe et al., 1977). Cytochrome b-type pigments (Minakami et al., 1964), cytochrome oxidase (Fridovich and Handler, 1961; Chance and Leigh, 1977), and NADH dehydrogenase (Tyler, 1975a) have been implicated in this $O_2^{\cdot}$ production.

The b-type cytochromes are part of an isopotential pool with an apparent midpoint potential of zero volts (Wikstrom, 1973). Two flavoproteins, two iron-sulfur proteins and ubiquinone are also components of this pool. Of these, however, only cytochrome b_{566} in the reduced state readily reacts with oxygen and since this cytochrome is a monovalent redox catalyst, the primary product of its reaction with oxygen will be $O_2^{\cdot}$ (Flohe et al., 1977). This reaction is not, however, likely to be of importance under normal conditions. Submitochondrial particles (SMP's) are obtained by sonic disruption of intact mitochondria. The end result is a membrane vesicle turned inside out. SMP's only produce H_2O_2 when in the presence of antimycin A and a suitable substrate (succinate, malate, α-ketoglutarate) (Loschen et al., 1974; Azzi et al., 1975). Antimycin A induces a selective reduction of cytochrome b_{566} by blocking electron flow on the oxygen site of the b-type cytochromes (Loschen et al., 1973a,b; Slater, 1973). Only when cytochrome b_{566} is reduced do uncoupled intact mitochondria or SMP's produce H_2O_2 via $O_2^{\cdot}$ (Flohe et al., 1977). Also, in the presence of ADP, cytochrome b_{566} is

completely oxidized, but in the presence of excessive amounts of ATP, it is reduced and can give rise to $O_2^{\overline{\cdot}}$ (Flohe et al., 1977). Thus, although cytochrome b_{566} is capable of generating $O_2^{\overline{\cdot}}$ under artificially manipulated conditions, this is unlikely to be a major site for $O_2^{\overline{\cdot}}$ production under normal conditions.

An iron-sulfur component of respiratory chain-linked NADH dehydrogenase is the most likely site of oxygen reduction (Gutman et al., 1972). That the respiratory chain NADH dehydrogenase is capable of reducing O_2 to $O_2^{\overline{\cdot}}$ is consistent with the E_o' value of the $O_2/O_2^{\overline{\cdot}}$ couple of -0.31 V (Boyer et al., 1977). The superoxide anion formed here is in turn capable of reacting with and inhibiting NADH dehydrogenase activity and thereby, the entire NADH oxidation system (Tyler, 1975a). No evidence is seen that inhibition is due to lipid peroxidation in this case. The mechanism of inactivation has been attributed to the oxidation or some other modification of the Type III thiol group (i.e., susceptible sulfhydryl site) of the NADH dehydrogenase complex (Tyler, 1975a). The effect of $O_2^{\overline{\cdot}}$ appears to be largely confined to the NADH branch of the respiratory chain (Tyler, 1975a). Hyperbaric oxygen has the ability to inhibit enzyme systems related to ATP production (Thomas et al., 1963), to depress ATP concentrations in brain tissue (Sanders et al., 1966), and inhibit reduction of NADH (Chance et al., 1965). Excess

NADH within preparations of submitochondrial particles causes a progressive and substantial inhibition of the NADH oxidation system and oxygen is required for this inactivation to occur (Tyler, 1975a).

In mitochondria, the TCA cycle which is responsible for NADH production is regulated in part by succinate dehydrogenation. Although this reaction is not usually the rate-setting step in the cycle, it competes with NAD-linked reactions in donating electrons to the electron transport chain and thus may affect the integration of the dehydrogenation reactions of the cycle (Lehninger, 1975). The entry point of the succinate pathway circumvents the NADH dehydrogenation step which generates the $O_2^{\cdot-}$ anion. Succinate administration has been demonstrated to protect against both acute and chronic oxygen toxicity (Sanders et al., 1965; Sanders et al., 1972; Sanders and Currie, 1971; Block, 1977), and its mode of action has been correlated with its ability to stimulate ATP production (Sanders et al., 1972). Succinate oxidation remains unaffected by NADH treatment (Tyler, 1975a). The failure of succinate oxidation to cause respiratory chain inactivation (Tyler, 1975a) suggests that the formation of $O_2^{\cdot-}$ during cytochrome b_{566} and cytochrome oxidase activity is unlikely to produce harmful effects on the respiratory chain (Markossian and Nalbandyan, 1975). The succinate pathway allows continued production of ATP while bypassing

the $O_2^{\overline{\cdot}}$-generating NADH dehydrogenation step.

This same mechanism probably operates during the protective action seen with reduced glutathione treatment (Baeyens and Meier, 1978). Both reduced and oxidized glutathione can be converted to succinate via a glutathione succinate shunt (Roberts et al., 1958), thereby stimulating oxidative metabolism again avoiding reliance upon the labile NADH dehydrogenation reaction.

Thus, the ability of exogenous succinate treatment to prevent oxygen toxicity appears to be related to its ability to supervene in the control of energy transport through the respiratory chain. The potential advantage of succinate treatment over SOD administration has been demonstrated by Block (1977). Whereas intraperitoneal injection of SOD is apparently unable to achieve access to the crucial mitochondrial particles, succinate does.

The mitochondrial matrix contains the mangano-SOD and glutathione peroxidase (Flohe et al., 1977) but lacks catalase (Tyler, 1975b). It may be concluded that under normal conditions, by protecting the relatively small quantities of NADH dehydrogenase present in cells (Cremona and Kearney, 1964; Tyler, 1975a) against inhibition by $O_2^{\overline{\cdot}}$, mitochondrial superoxide dismutase ensures that the key role of NADH dehydrogenase in electron transport activity and energy conservation reactions is maintained in the presence of oxygen. Glutathione peroxidase then

further metabolizes the product of $O_2^{\overline{\cdot}}$ dismutation, i.e., H_2O_2.

Mitochondrial SOD is crucial in the protection of tissues against oxygen toxicity. Induction of SOD by elevated oxygen tensions occurs both in prokaryotic bacteria (Gregory and Fridovich, 1973a,b) and yeast (Gregory et al., 1974), and in eukaryotes. Rosenbaum et al. (1969) demonstrated that exposure of rats to 85% O_2 for 7 days prolonged the survival time compared to nonpretreated rats when these rats were transferred to 100% O_2. SOD activity in the lungs of rats exposed to 85% O_2 for 7 days was increased 50% compared to controls. Furthermore, the rate of tolerance development to 100% O_2 for rats pretreated with 85% O_2 closely paralleled increases in pulmonary SOD activity (Crapo and Tierney, 1974). Thus, a correlation between enhanced SOD activity and resistance to elevated O_2 has been demonstrated in both prokaryotic and eukaryotic organisms. In pulmonary macrophages, 70% of the total SOD activity is normally in the form of cyanide-insensitive mitochondrial mangano enzyme (Rister and Baehner, 1976). When SOD is induced in both in vivo and in vitro neonatal rat lung tissues by hyperoxic treatment the increase is due entirely to increased synthesis of the mangano-SOD (Stevens and Autor, 1977).

The Ocular Standpoint

To recap for a moment, oxygen toxicity, X-ray irradiation, and UV and visible light damage---seemingly unrelated phenomena---may in fact have a common mechanism through which they cause cellular destruction, namely, the production of free radicals. The protective actions of SOD, catalase and peroxidase are supported by b-carotene, ascorbic acid, reduced glutathione, vitamin E, and the glutathione peroxidase system. The true role of melanin in free radical reactions is uncertain, but the current concensus seems to be that melanin is probably neither a source nor a sink for free radicals, but rather an efficient transfer agent for a variety of ionic species.

Even though ocular tissues have a normal complement of protective mechanisms against free radicals, if any step in the defense system fails, or if the defense system is inundated due to the incursion of agents which act to acutely stimulate the production of free radicals, the tissues would be unable to cope with the continuous production of cytotoxic species resulting in widespread membrane and protein damage.

Free Radical Reactions and the Role of Antioxidants in the Retinal Pigment Epithelium

In proposing a justification for abnormally high or low levels of SOD activity within any specific cell type,

it is instructive to review known functional and biochemical peculiarities of that tissue. The main functions of the retinal pigment epithelium (RPE) are:

1) to serve as a light screen for the photoreceptor outer segments during photopic vision (Murray and Dubin, 1975),

2) to regulate the flow of ions between choroidal blood and the neural retina (Miller and Steinberg, 1976, 1977),

3) to store and convert retinol (Vitamin A) to the biologically active esterifies 11-cis form (Dowling, 1960; Krinsky, 1958),

4) to synthesize the mucopolysaccharides and glycoproteins found in the extracellular matrix surrounding the photoreceptor cells (Young and Bok, 1970; Berman, 1964), and

5) to phagocytose and dispose of shed photoreceptor outer segments (POS) (Young and Bok, 1969; Hogan, 1972).

The RPE is rich in mitochondria, and ATP is required for many of the functional roles described above. In particular, the RPE has been shown to have a Na/K ATPase pumping mechanism (Riley et al., 1978); phagocytosis is an energy dependent process (Silverstein et al., 1977); and ATP is also involved in the migration of pigments along the microtubules in the light and dark adaptation processes (Murray and Dubin, 1975). Thus, with a large

metabolic demand, one would expect a large amount of mitochondrial Mn-SOD.

In addition to the mitochondrial electron transport chain as a site for $O_2^{\cdot}$ production, the phogocytic activity may also involve free radical processes for which the cellular antioxidant mechanisms have been modified. Photoreceptor outer segment disks are periodically detached and phagocytized by the RPE and incorporated into their lysosomal system (Young and Bok, 1969). Various cell organelles are similarly autophagocytized and undergo lysosomal digestion (Frank and Christensen, 1968).

Based upon morphological evidence alone, lysosomal enzymes have been purported to be solely responsible for the degradation of phagocytosed outer segment disks. Seven forms of pigmented inclusions within the RPE are associated with these phagocytic and degradative processes (Feeney, 1978): phagosomes, phagolysosomes, secondary lysosomes, lipofuscin granules, melanin granules, melanolipofuscin, and melanolysosomes. Phagosomes are the initial phagocytic vacuole containing ROS disks. Primary lysosomes fuse with the phagosome membrane and as the initial degradation begins, autofluorescent lipid breakdown products become detectable. At this stage, the inclusions are referred to as phagolysosomes. Older phagolysosomes in which the degradation is at a more advanced stage are termed secondary lysosomes. In the eyes of young humans,

these secondary lysosomes continue to diminish in size and disappear (Feeney, 1978). In old eyes, however, they represent intermediates in lipofuscin formation. They are autofluorescent and contain extractable lipid and proteinaceous material, the latter of which presumably represent lysosomal enzymes and undigested opsin.

Lipofuscin granules are the fluorescent age pigments (Sinex, 1977; Sanadi, 1977). The lipids of the lipofuscin granules, originate mainly from phospholipids of photoreceptor outer segment (POS) disks which are rich in PUFA (Berman et al., 1974; Anderson and Maude, 1970; Anderson et al., 1976). With increasing age the quantity of fluorescent granules increases (Streeten, 1961). By the fifth decade of life RPE cells are strikingly fluorescent, and lipofuscin granules predominate in the cytoplasm (Streeten, 1961; Feeney, 1978). Fusions between primary lysosomes and lipofuscin granules continue and are common in older eyes, suggesting that the overall degradative process involves repeated injection of lysosomal enzymes. Lipofuscin granules also coalesce with large phagolysosomes (Feeney, 1978). Thus, the initial fusion of lysosomes with phagosomes is only one of several attempts to hydrolyze the membranous material. Despite the residual body designation, the lipofuscin compartment is a dynamic intracellular compartment capable of many different interactions with other cellular inclusions of the RPE cytoplasm.

The RPE melanin granules are synthesized prenatally, achieve maturity before the first decade of life (Toda and Fitzpatrick, 1972; Moyer, 1969) and thereafter are generally thought to be unchanging structures in the cytoplasm of these essentially non-dividing cells. This is in contrast to the melanosomes of uveal and dermal origin which show ultrastructural and enzymatic properties suggestive of continual synthesis during life (Feeney et al., 1965; McGovern and Russel, 1973). Feeney (1978), however, postulated that the melanin of the RPE is undergoing autophagic remodelling if not actual degradation. This process is evidenced in the presence of two types of melanin-containing complex granules. Melanolysosomes are melanin with a cortex of non-lipid, enzyme-reactive material. Melanolipofuscin is melanin with a cortex of lipofuscin. These indicate that melanin commonly becomes incorporated into the lysosomal system and undergoes modification or degradation there. Melanin polymer, although highly insoluble, is linked to protein which is amenable to enzymatic digestion (Zimmerman, 1977). Disassembly of melanin via dissolution of this protein matrix is suggested since various degrees of compaction of the layers of melanopolymer are seen in the melanolipofuscin granules (Feeney, 1978).

As stated previously, from this morphological evidence, lysosomal enzymes appear to be solely responsible for the degradation of phagocytosed outer segment disks leaving no

need for free radical mechanisms. Contrary to what one would expect for such a task, however, the RPE lysosomal enzymes differ neither in composition nor in quantity from the rest of the normal neural retina (Siakotos et al., 1978). Lysosomes of the RPE do contain acid lipases (Rothman et al., 1976; Hayasaka et al., 1977) which hydrolyze fats by attacking the ester linkages between glyceral and fatty acids. This does not, however, account for the further degradation of PUFA into the autofluorescent products seen in the RPE lipofuscin granules. In short, the lysosomal system appears not to be equal to the task required.

As emphasized throughout this discussion, PUFA are particularly amenable to degradation via free radical mechanisms. In fact, free radicals have been shown to be damaging to lipids in the rod outer segments (Kagan et al., 1973). In addition, the autofluorescent material which accumulates is considered to be the end product of free radical-mediated autolytic degradations of polyunsaturated fatty acids (Tappel et al., 1973). Hydrogen peroxide, hydroxyl free radicals, lipid radicals are all capable of destroying rhodopsin as well. In fact, retinal is easily autoxidized and, as such is inhibitory to lysosomal enzymes such as b-glucuronidase (Christner et al., 1970). Thus, a free radical mechanism would be beneficial in this case for the degradation and disposal of the phagocytized photoreceptor outer segments.

There is a well-established precedent for the involvement of oxygen free radical mechanisms in the phagocytic activities of mammalian blood neutrophils and monocytes. Baboir et al. (1973) showed that phagocytizing neutrophils elaborate $O_2^{\cdot}$. This may interact with H_2O_2 to form hydroxyl radical ($OH^{\cdot}$), a possibly important part of the bactericidal mechanism (Johnston et al., 1973). A role for singlet oxygen ($^1O_2^*$) in bacterial killing has also been proposed (Allen et al., 1972; Webb et al., 1974; Johnston et al., 1975; Rosen and Klebanoff, 1976).

In polymorphonuclear (PMN) leukocytes, triggering of the $O_2^{\cdot}$-generating system occurs at the cell surface (Roos et al., 1977). An NADPH oxidase may be the primary enzyme in this reaction (Curnutte et al., 1975; DeChatelet et al., 1975). It has been suggested that NADPH oxidase is of lysosomal origin (Patriarca et al., 1973; Hohn and Lehrer, 1975). But, as Roos et al. (1977) point out, a lysosomal localization presents some teleological problems. In theory, during attack on an ingested microorganism, $O_2^{\cdot}$ is generated inside the phagosomes via a lysosomal NADPH oxidase. Yet, there is no way for the cytosol-produced NADPH substrate to enter the phagosome. Additionally, a lysosomal localization for an $O_2^{\cdot}$-generating system does not account for the high amounts of $O_2^{\cdot}$ and H_2O_2 found in the extracellular milieu upon triggering the oxidative metabolism of these cells (Fridovich, 1972). This would mean that these products

would have to pass through the cytosol which contains SOD to reach the extracellular environment.

An alternative localization for NADPH oxidase is on the plasma membrane (Johnston et al., 1975; Baehner et al., 1975; Roos et al., 1976, 1977). In this case, NADPH oxidase is proposed to be a membrane spanning enzyme where the NADPH would come from the cell interior and the O_2 from the cell exterior, releasing $NADP^+$ inside, but $O_2^{\cdot}$ and H^+ on the outside of the cells. In the case of phagosome formation, NADPH does not need to pass through the phagosomal membrane, since the phagosome is surrounded by a piece of inverted plasma membrane. By this mechanism, there is a directed release of $O_2^{\cdot}$ to the inside of the phagosomes and to the outside of the cells.

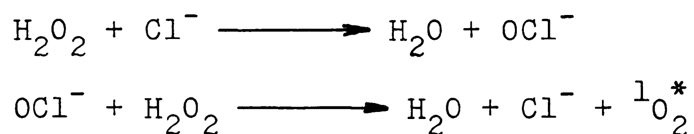
If similar mechanism were operating within the retinal pigment epithelium one might expect this to be reflected in the cellular antioxidant levels. Catalase and glutathione peroxidase are absent from RPE cells, whereas peroxidase is present (Armstrong et al., 1978a). There are two forms of peroxidase in the RPE, a soluble form with a pH optimum of 5.0 and a membrane-bound or insoluble enzyme having a pH optimum of 7.4. This is in contrast to the peroxidases of the neural retina whose pH optima for both soluble and bound forms is 7.4 (Armstrong et al., 1978a,b). Peroxidase levels have been found in dogs to be 30 to 150 times higher in the RPE than in the retina (Armstrong et al., 1978b).

In a syndrome termed canine ceroid lipofuscinosis (CCL), there is a specific and significant reduction in peroxidase actitity. The initial pathogenic event seems to be an inability of affected cells to cope with peroxidative damage at an early stage. With age and progression of the disease the reduced peroxidase activity manifests itself in an increase in lipofuscin accumulation (Armstrong et al., 1978b), followed by an exaggerated, yet furtive, attempt by the cells to digest accumulating lipopigments by increased titers of acid lipase and acid phosphatase (Siakotos et al., 1978). Hence, in this disease, it is a failure of the cellular antioxidant mechanism (peroxidase in this case) rather than in the lysosomal system which gives rise to the accumulation of lipofuscin in the RPE.

It is tempting to speculate from the above discussion that a free radical-initiated autoxidation of the PUFA of ROS membranes takes place during or immediately after phagocytosis of shed ROS via the RPE. Melanin may also be involved in free radical transfer as implicated by the presence of the melanolipofuscin and melanolysosome granules (Feeney, 1978). In senescence (Streeten, 1961; Feeney et al., 1965; Sinex, 1977; Sanadi, 1977), in vitamin E deficiency states (Hayes, 1974), and in CCL (Armstrong et al., 1978b), the process whereby phagocytized POS disks are degraded and converted to lipofuscin granules is likely to

be the consequence of a failure of some of the free radical protective mechanisms.

Lysosomal involvement would remain important in that the vitamin E and SOD of the POS require degradation and disposal prior to proceeding with free radical-mediated degradation of PUFA. Additionally, the lysosomal acid hydrolases (esp., β-galactosidase and N-acetyl-β-glucosaminidase) would be important in the catabolism of rhodopsin and of the mucopolysaccharides (glycosaminoglycans) and glycoproteins found in the extracellular matrix surrounding the photoreceptor cells (Berman and Bach, 1968; Berman, 1971). In fact, if the situation were analogous to that in the PMN leukocytes, lysosomes could introduce myeloperoxidase into the vacuole which would serve to enhance the overall effectiveness of oxygen metabolites (Johnston and Lehmeyer, 1977) (MPO = myeloperoxidase):



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

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

Assuming for the moment that a complex system of free radical production and disposal is operating continuously in these cells, this does not necessarily imply that there will be elevated cytosolic Cu-Zn SOD. In fact, PMN leukocytes have been found to contain quantitatively less SOD than most other cell types (Salin and McCord, 1977). The superoxide production associated with this activity is not produced within or directed toward the cell interior,

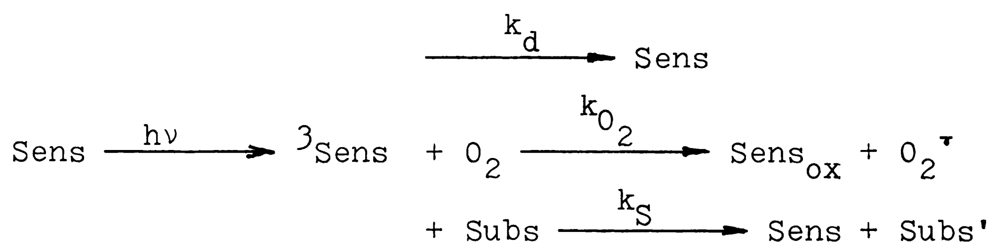
but rather to the outside of the cell and to within the phagolysosomes. As discussed previously, $O_2^{\cdot}$ is not likely to diffuse into or across the hydrophobic zone of membranes, but if allowed to spontaneously dismutate to $^1O_2^*$, this will enter the membrane and cause the initiation of lipid peroxidative autolysis. Also, if there is a paucity of PUFA and/or a large amount of chain terminating reagents within the membrane, this autolytic damage would not be as extensive. Such may be the case in the PMN leukocyte and RPE plasma membranes.

Significance of Antioxidants in the Photoreceptor Outer Segments

If the RPE is generating $O_2^{\cdot}$ by a mechanism analogous to that of PMN leukocytes, $O_2^{\cdot}$ will be released into the matrix surrounding the POS. This may not be a matter for concern for the POS just as it is not for the PMN leukocytes themselves. The cell membrane appears to be an effective barrier to the diffusion of $O_2^{\cdot}$ back into the cell. If the composition of that membrane is low in PUFA it would not be susceptible to damage. Shvedova et al. (1979) have exposed retinas in vitro to exogenous sources of $O_2^{\cdot}$ and demonstrated that the ERG a-wave was relatively resistant and the photoreceptor cell membrane potential remained unaffected. They were also unable to visualize any ultrastructural changes in the POS membranes. Thus, elevated SOD within the POS (Hall and Hall, 1975) is

probably a defense against an endogenous source of $O_2^{\cdot}$.

Although there are no sources for the metabolic generation of oxygen free radicals within the POS, this structure may be a major site for photodynamically induced free radical generation. This mechanism involves the excitation of a sensitizer molecule by photons into an excited singlet or triplet state. Most photosensitized oxidations proceed by way of the triplet sensitizer since the triplet state has a much greater lifetime than the excited singlet state (Foote, 1968). The most effective sensitizers are those which give rise to a long-lived triplet state in high quantum yield. The sensitizer triplet may then react with oxygen, with a specific substrate, or it may decay spontaneously to the ground state (Sens = ground state sensitizer; ^{3}Sens = sensitizer triplet; Subs = specific substrate; Subs' = substrate-sensitizer reaction product) (Foote, 1968):



The predominance of one pathway over another depends upon the relative oxygen and substrate concentrations and the reaction rates k_S, k_{O_2}, and k_d. In oxygen- or air-saturated solutions, the oxygen concentration is in the range of 10^{-2}M to 10^{-3}M (Livingston and Owens, 1956). No matter

what the sensitizer, k_{O_2} seems to be very close to 10^9 $M^{-1}sec^{-1}$ (Gollnick, 1968). This is a very high rate constant so that the reaction with oxygen will predominate unless $k_s(S)$ is close to 10^6 to 10^7 sec^{-1} (Foote, 1968). The reaction with oxygen may involve a process of electron transfer to oxygen to form superoxide anion (Weiss, 1935) or it may involve the formation of singlet oxygen (Foote, 1968).

Although never reported directly in the literature as such, all of the components for this process are present within the photoreceptor outer segments. Grady and Borg (1968) have demonstrated free radical formation within the visual pigment upon illumination. The incidence of a photon upon the 11-cis retinal excites it into a triplet state in which there is no energy barrier to rotation to the all-trans form (Knowles and Dartnall, 1977). This is of prime importance in photoisomerization. This isomerization process is equivalent to a sensitizer triplet decay reaction. The triplet excited state of retinal has also been found to accept an electron to be converted to a retinyl radical anion (Grady and Borg, 1968) which leads to further breakdown products of retinal (Sack et al., 1972). One destination for the electron is a transfer from the chromophoric group to the tryptophan moeity of opsin, to form a linear free radical. The quantum yield for the formation of this species is only about 0.1 and so it

does not seem to be on the main pathway of the bleaching sequence (Knowles and Dartnall, 1977). This is equivalent to the sensitizer-substrate interaction.

No one has reported the production of $O_2^{\cdot}$ or $^1O_2^*$ via reaction of triplet rhodopsin with oxygen, however, several observations are suggestive of this process. The outer segments are a major site of primary light-induced damage in the retina (Noell et al., 1966; Kuwabara and Gorn, 1968). Continuous cool illumination with even ordinary fluorescent lights leads to profound changes in photoreceptor function and morphology. Noell and associates (1966) postulated that photosensitized oxidations leading to the formation of lipid peroxides could play an important role in light damage, and direct evidence supporting this hypothesis was recently provided using the frog (Kagan et al., 1973). It was shown that lipid hydroperoxides were formed in POS after only 30 minutes exposure to light in vivo. The structural damage is due to peroxidation of POS membrane fatty acids, the principal one being docosahexaenoate, a 22-carbon fatty acid containing six unsaturated double bonds (22:6) (Nielsen et al., 1970; Anderson and Risk, 1974). This species is highly susceptible to autoxidation by the mechanisms described above. The maximum concentration of lipid hydroperoxides was found to occur at 508 nm gradually decreasing to the level of non-illuminated controls at shorter (455 nm) as well as longer (668 nm)

wavelengths. Light exposure at 508 nm leads to 95% bleaching of the rhodopsin in POS. As free radical lipid peroxidation is initiated, increasing damage to the membranes would be expected in time possibly leading ultimately to the physical release of fragments from the POS. Indeed photoreceptor shedding has been found to be controlled or initiated by cyclic lighting (LaVail, 1976; Basinger et al., 1976).

In addition to the observation of typical oxygen-mediated lipid peroxidation processes, the POS are known to possess defenses against these reactions. Vitamin E is an integral component of POS membranes, and its depletion adversely affects these polyunsaturated fatty acid-rich membranes, especially when challenged with oxygen (Farnsworth and Dratz, 1976). This has been substantiated by Hayes (1974) who showed extensive degeneration of rod outer segment membranes in vitamin E deficient monkeys with a concomitant buildup of lipofuscin in the pigment epithelium. Peroxidase is absent in the canine POS (Armstrong et al., 1978b), but superoxide dismutase is approximately 400 times as active in bovine and frog POS (Hall and Hall, 1975) as anywhere else in the retina. Thus, $O_2^{\cdot}$ may very well be reduced via a photoactivation mechanism, thereby explaining the presence of such large amounts of SOD in the outer segments. The question whether rhodopsin is a direct sensitizer for

the photooxidation of associated phospholipids (Blasie, 1972; Kagan et al., 1973) is yet to be resolved.

One other source of peroxidizing free radicals exists in the POS. Micro-amounts of non-haem iron have been found in vivo (Fujishita, 1962) as well as comparatively high concentrations (0.5 mM) of ascorbate (Daemen, 1973). These are precisely the components of a system for the induction of lipoperoxidation in proportions optimal for the catalysis of the free radical oxidation of lipids (Kagan et al., 1975).

Whether or not rhodopsin is the source of the lipid peroxidation, the lipoperoxide free radicals observed upon illumination must be controlled, otherwise free radicals are known to be capable of destroying rhodopsin (Chio and Tappel, 1969). Also, like other integral membrane proteins, the function of rhodopsin depends very largely upon the integrity of the membrane surrounding the molecule (Korenbrot, 1977).

Significance of Antioxidants in the Neural Retina

The retina is transparent and Varma et al. (1977) postulated that it is thus uniquely susceptible to photodynamic generation of $O_2^{\cdot}$ in addition to that generated by normal processes of tissue oxidative metabolism. The photochemical generation of $O_2^{\cdot}$ is known to take place by the interaction of ambient oxygen and photoexcitable

substances like riboflavin and flavin adenine dinucleotide (FAD). These are universal cell constituents. The primary site of light-induced retinal damage appears, however, in the photoreceptor cells and retinal pigment epithelium. The ganglion, horizontal, amacrine, and Mueller cells escape the damaging effects of visible light at intensities and durations which destroy the other cell types (Noell et al., 1966). The neural retina atrophies only long after the complete obliteration of these two layers (Noell et al., 1966; Kuwabara and Gorn, 1968). This evidence suggests that photosensitization of intracellular flavin compounds is possible, but not a major source of $O_2^{\cdot}$ within the cell.

The retina is peculiar in its exceedingly high rate of metabolic activity (Noell, 1958) being exceeded only by cancer tumor tissue and embryonic tissue, This elevated rate of metabolism would in turn be expected to result in correspondingly elevated superoxide anion production. A portion of this metabolic activity is accountable in terms of anaerobic glycolysis and hexose monophosphate (HMP) shunt activity (Hoffert et al., 1974; Masterson et al., 1978). The HMP shunt serves as an important alternate glucose metabolic pathway as it provides for 1) the production and disposal of pentoses used, for example, in nucleic acid synthesis, and 2) the generation of NADPH which is used in many reductase systems, notably in lipid synthesis which is essential for membrane formation. Within the

retina, there must be a large amount of lipid production which continues well past the developmental stages since the photoreceptor outer segments are continually renewed. Roos et al. (1977) demonstrated that SOD induces a marked activation of the HMP shunt, presumably through activation of the glutathione peroxidase pathway by increased amounts of H_2O_2. Phagocytizing neurtrophils undergo a cyanide-insensitive "respiratory burst" consisting of oxygen consumption, generation of H_2O_2, and activation of the HMP shunt (Klebanoff, 1975). Thus, even though there is a very high rate of metabolism within the retina, this may not be reflected in a very large supply of mitochondrial SOD.

Embryologically, the neural retina is directly derived from central nervous tissue. Thus, the antioxidant mechanisms of the two tissues might be expected to be similar. Fried and Mandel (1975) found that the SOD in crude extracts of whole beef retina had approximately the same activity as that from the brain. Brain mitochondria appear to have an oxygen metabolism different from that of other tissues (Sorgato et al., 1974). Neither H_2O_2 nor $O_2^{\overline{\cdot}}$ are produced in rat brain mitochondria, and catalase is low in concentration.

Catalase and peroxidase are also present in both the brain and neural retina. The importance of peroxidases is again demonstrated in the syndrome of canine ceroid

lipofuscinosis (CCL) (Armstrong et al., 1978b; Siakotos et al., 1978). This is a hereditary metabolic disease with secondary retinal degeneration occurring in a particular strain of English setter. Massive deposits of autofluorescent lipo-pigments (i.e., lipofuscin) accumulate in neurons of the central nervous system and the neural retina (Koppang, 1973/74). Toward the end of the CCL disease, the brain is grossly atrophic weighing about 70% of that of normal controls. The brain loss is caused by neuronal degeneration and neuronal death. The retinal cells, however, remain mostly intact but do contain excessive intracellular lipopigment. Lysosomal acid hydrolase activities have been implicated in normal lipofuscin generation (Feeney, 1978), but Armstrong et al. (1978b) demonstrated a specific and significant decrease in peroxidase with age and the progression of the disease. Hence, in this disease, a failure of the cellular antioxidant mechanisms gives rise to the accumulation of lipofuscin in the neural retina.

In addition to catalase and peroxidase, high concentrations of ascorbic acid are found in the retinas of many species (Heath et al., 1961). In rats, ascorbic acid is transported into the retina by an energy-dependent, sodium sensitive process (Heath and Fiddick, 1966) to a concentration on the order of 20 to 30 times that of the plasma. The function of ascorbic acid in the eye is not

yet known (Heath, 1962). It may have a subsidiary role as a cellular antioxidant (Nishikimi, 1975).

Significance of SOD in the Choroid

The choroidal layer is composed of a rich vascular network embedded within a connective tissue matrix in which are found numerous melanocytes. Like dermal melanocytes, uveal melanosomes show ultrastructural and enzymatic properties suggestive of continual synthesis during life (Feeney et al., 1965; McGovern and Russel, 1973). No lipofuscin granules are seen in choroidal melanophores (Streeten, 1961). The function of these melanophores is not yet known. They probably play a part in the absorption and reflection of whatever light passes the retinal pigment epithelium or in through the sclera from the cranial regions (Hogan et al., 1971). The connective tissue and associated fibroblasts may for the present discussion be considered rather inert to the discussion of oxygen toxicity and antioxidant mechanisms.

The vascular tissue is of prime interest to the current discussion. In retrolental fibroplasia, it was seen that the immature retinal vasculature was the most sensitive to elevated arterial oxygen tensions. Only the proliferative retinal endothelia are susceptible, this susceptibility being lost upon maturation (Patz, 1976). The choroidal circulation remains unaffected in RLF because it is largely developed at birth and out of the proliferative

phase. By the eleventh prenatal week arteries have branched extensively throughout the human choroid (Hogan et al., 1971). In contrast, the retinal vasculature is still proliferating at birth particularly in the vicinity of the ora serrata, and the most drastic effects are seen precisely in these proliferating areas (Ashton et al., 1954).

The endothelial damage is thought to be either a direct cytotoxic effect of oxygen, or a secondary response to the reduced retinal blood flow associated with severe vasoconstriction (Patz, 1975). In vitro studies of cultured retinal vascular endothelia indicate that lysosomal lability is an early sign of acute hyperbaric oxygen exposure (Tripathi et al., 1974). Although a role for vitamin E was considered by early investigators, results were inconclusive (Patz, 1976). The other components of the armamentarium that protect tissues from oxygen toxicity need also to be studied in seeking to understand the special vulnerability of immature retinal vascular endothelium.

Survey of SOD Levels

In compiling some of the published levels of SOD activity, one must first understand some of the pitfalls of the assay procedures involved. Therefore, some of the most widely used spectrophotometric methods of SOD assay and associated problems will be reviewed prior to tabulating

the reported data.

Methods for the determination of SOD activity exploit the ability of this enzyme to scavenge $O_2^{\cdot}$ radicals which would otherwise reduce a suitable electron accepting indicator. Most assays have been developed for characterizing purified enzyme. Relatively few attempts have been made to develop modified assay approaches for quantifying levels in crude extracts.

A vast array of substances occur within crude extracts which are capable of interfering with the assay reactions. Problems arise from the fact that whereas SOD appears to be specific to the substrate superoxide anion, the substrate is not specific to the enzyme. Any substance other than SOD or the indicator which will react with $O_2^{\cdot}$ to any significant degree, or any substance other than the designated $O_2^{\cdot}$-generating system which can give rise to $O_2^{\cdot}$ will either yield erroneously high apparent SOD activity or mask actual activity. Any substance in a crude extract which acts directly upon the indicator substance will also yield spurious readings.

Apparent SOD activity can arise from the presence of significant amounts of various small molecules such as FAD, FMN, riboflavin, NADH, and Fe^{+++} (Michelson, 1977a). Catalase interferes with the assay reaction to some degree though the affinity of catalase for superoxide anion is at least tenfold less than that of SOD (Kovacs and Matkovics,

1975). Peroxidase can be reduced by $O_2^{\cdot}$ to form oxyperoxidase (Sawada and Yamazaki, 1973) which may in turn break down again to give rise to free $O_2^{\cdot}$ radical (Rotilio et al., 1975). Thus, both catalase and peroxidase can give rise to apparent SOD activity. As discussed previously both ascorbate and reduced glutathione may react with $O_2^{\cdot}$ and thereby may also interfere with the assay.

The original SOD assay procedure of McCord and Fridovich (1969) utilizes a system of xanthine and xanthine oxidase for the generation of $O_2^{\cdot}$ radicals, and ferricytochrome c as an indicating electron acceptor. The presence of SOD is characterized by an inhibition of the rate of cytochrome c reduction.

Modified versions of the cytochrome c method have been discussed by McCord et al. (1973), Wesser et al. (1972) and Wesser and Voelcker (1972). McCord et al. (1977a) point out that cytochrome c reductases and oxidases are present in membranes of mitochondria, microsomes and nuclei and must be accounted for. Cyanide is often included in the reaction mixture in high enough concentrations (0.5 mM) to inhibit endogenous cytochrome peroxidases, yet too low to inhibit Cu-Zn SOD (Gregory and Fridovich, 1973a). Azzi et al. (1975) used acetylated cytochrome c in the reaction mixture. This is unrecognizable to the endogenous oxidases and reductases for which cytochrome c is a substrate, but it remains reducable by $O_2^{\cdot}$.

In the nitroblue tetrazolium (NBT) photoreduction assay, NBT is used as the indicator and the aerobic photoreduction of certain dyes or flavins in the presence of suitable electron donors such as EDTA can be used to generate superoxide radicals (McCord et al., 1973; Beauchamp and Fridovich, 1971). In this case SOD prevents the formation of colored insoluble blue formazan. The NBT photoreduction method can be applied to detect SOD activity in polyacrylamide gels. For crude extracts, however, NBT reductases within mitochondria, microsomes and nuclei prevent its application as a useful assay (Hatefi, 1963; Mii and Green, 1954).

Co-oxidation of epinephrine to adrenochrome was used as an early assay (McCord and Fridovich, 1969). However, it was later found that not only co-oxidation, but also autoxidation can lead to the formation of adrenochrome (Misra and Fridovich, 1972a).

Misra and Fridovich (1977) developed a color augmentation assay using dianisidine, rather than the typical color inhibition assays. This procedure was purported to be free of any interference problems in crude homogenates, however, others have not found this to be the case (Eldred, unpublished observations; S. Aust, personal communications).

A chemiluminescent reaction produced in an oxygen-hypoxanthine-xanthine oxidase-luminol system is inhibitable by SOD and this has also been used as an assay technique

(Puget and Michelson, 1974). A minimum concentration of about 0.2 ng erythrocyte SOD can be estimated by this technique.

Each of these assay procedures defines one unit of SOD activity to be that quantity required to inhibit the reaction rates by 50%, but this means that the unit of activity is peculiar to the specific assay. In reviewing the reported levels, it also becomes evident that no standardization has been established for the expression of the measured activities; some expressing it in terms of units of activity per milligram of protein, and others per mg whole tissue, or per mg DNA, or per gram of hemoglobin, or per 10^6 cells, etc. This makes comparison of findings difficult if not impossible.

Finally, one error commonly made is a failure to account for erythrocytic activity. In some tissues, such as the liver (Castagna, 1965), this source of activity could be very large and lead to erroneous results. Hemoglobin in large amounts is reported to interfere with many assays and should be removed prior to the determination. Bannister et al. (1977) have found the Tsuchihashi procedure, based upon the precipitation of hemoglobin with a mixture of ethanol and chloroform, to be the best method for this purpose.

Some of the SOD levels are tabulated in Tables 1,2 and 3. Although it is difficult to compare these values because

of the varying units involved, several enlightening observations have been made in these studies. Allen and Hall (1973) state that in spinach chloroplasts, there are 5 to 10 molecules of SOD per electron transport chain. Just as in the mitochondrial electron transport chain, there are several steps which are energetically capable of giving rise to $O_2^{\cdot}$. In several oxygen toxicity studies it has been demonstrated that specific and significant induction of manganic SOD synthesis occurs which correlates with increased resistance to oxygen toxicity, while no such change in cupro-zinc SOD activities take place (Fridovich, 1978). There are species-specific differences in specific tissue levels. For instance, McCord et al. (1977b) state that the human liver contains 20 times the amount of Mn-SOD, but less than half the amount of Cu-Zn SOD contained in an equivalent weight of rat liver (Tyler, 1975b; Peeters-Joris et al., 1975; Panachenko et al., 1975). On the average there is twice as much Mn-SOD as Cu-Zn SOD in primate livers, but the ratio varies considerably from individual to individual (McCord et al., 1977b). They conclude by stating that "fluctuations in Mn SOD content are particularly intriguing in view of the quite constant concentrations of Cu-Zn SOD found from tissue to tissue, and organism to organism."

TABLE 1: Survey of some reported SOD levels. Activities are total of all forms unless otherwise specified.

Tissue and Treatment	Assay*	Level	Ref.**
Aerobic bacteria:			
Holobacterium salinarium	1	2.1 U/mg prot.	1
Escherichia coli	1	1.8 U/mg prot.	1
Escherichia coli B			
Grown in N_2	1a	3.8 U/mg prot.	2
Grown in air	1a	13.5 U/mg prot.	2
Grown in 1 atm. O_2	1a	21.2 U/mg prot.	2
Grown in 5 atm. O_2 45 min exposure[2]	1a	42.5 U/mg prot.	2
Grown in 5 atm. O_2 19 hr. exposure[2]	1a	92.8 U/mg prot.	2
Escherichia coli K-12 his^-			
Grown in N_2	1	6.0 U/mg prot.	4
Grown in 20% O_2	1	6.1 U/mg prot.	4
Grown in 100% O_2	1	5.5 U/mg prot.	4
Azotobacter chroococcum (an aerobic soil bacterium capable of N_2 fixation)			
Grown in 5% O_2	1c	3700 μg SOD/mg prot.	3
Grown in 20% O_2	1c	1100 μg SOD/mg prot.	3
Grown in 30% O_2	1c	700 μg SOD/mg prot.	3
Anaerobic aerotolerant bacteria:			
Streptococcus mutans	1	0.5 U/mg prot.	1
Streptococcus fecalis	1	0.8 U/mg prot.	1
Grown in 0 atm O_2	1a	0.8 U/mg prot.	2
Grown in 1 atm O_2	1a	3.5 U/mg prot.	2
Grown in 5 atm O_2	1a	7.0 U/mg prot.	2
Grown in 10 atm O_2	1a	9.5 U/mg prot.	2
Grown in 15 atm O_2	1a	12.0 U/mg prot.	2

* See Table 2

** See Table 3

TABLE 1: Continued

Tissue and Treatment	Assay*	Level	Ref.**
<u>Obligate anaerobic bacteria</u>:			
Clostridium sp.	1	0.0 U/mg prot.	1
Veillonella alcalescens	1	0.0 U/mg prot.	1
<u>Eukaryotic yeast</u>:			
Saccharomyces cerevisiae			
Grown in N_2	1	1.3 U/mg prot.	4
Grown in 20% O_2	1	2.5 U/mg prot.	4
Grown in 100% O_2	1	8.6 U/mg prot.	4
<u>Eukaryotic fungi</u>:			
Neurospora crassa	1a	44 U/mg prot.	5
<u>Chloroplasts</u>:			
Spinach	6	500 U/mg chlorophyll	6
<u>Erythrocytes</u> (Hemoglobin-free supernatants):			
Porcine	3	216 U/mg prot.	8
Normal Human	2a	2720 U/gm hemoglobin	7
Downs syndrome			
Trisomy G21	2a	4930 U/gm hemoglobin	7
Unbalanced translocation G21/22	2a	4410 U/gm hemoglobin	7
<u>Blood platelets</u>:			
Normal Human			
Mn SOD	4	6.44 U/mg prot.	9
Cu-Zn SOD	4	40.30 U/mg prot.	9
Downs syndrome Trisomy 21			
Mn SOD	4	4.31 U/mg prot.	9
Cu-Zn SOD	4	62.79 U/mg prot.	9

* See Table 2

** See Table 3

TABLE 1: Continued

Tissue and Treatment	Assay*	Level	Ref.**
Phagocytic Cells:			
Polymorphonuclear leukocytes			
Without Cyanide	1b	17.58 U/mg prot.	10
With Cyanide	1b	17.25 U/mg prot.	10
Monocytes			
Without Cyanide	1b	30.93 U/mg prot.	10
With Cyanide	1b	28.29 U/mg prot.	10
Pulmonary alveolar macrophages			
Without Cyanide	1b	39.99 U/mg prot.	10
With Cyanide	1b	34.80 U/mg prot.	10
Cu-Zn SOD	1a	0.9 U/10^6 cells	11
Mn SOD	1a	2.1 U/10^6 cells	11
Whole Lung:			
Turtle	3	2.20 U/mg prot.	13
Rabbit	3	6.81 U/mg prot.	13
Mouse	3	6.86 U/mg prot.	13
Rat			
Cu-Zn SOD	5	109 µg/whole lung	12
Cu-Zn SOD after 7 days exposure to 85% O_2	5	155 µg/whole lung	12
Control	3	100 U/mg DNA	14
Perfused lung	3	6.9375 U/mg prot.	15
Pretreated 1 day with 85% O_2	3	113 U/mg DNA	14
Pretreated 3 days with 85% O_2	3	118 U/mg DNA	14
Pretreated 7 days with 85% O_2	3	133 U/mg DNA	14

* See Table 2

** See Table 3

TABLE 1: Continued

Tissue and Treatment	Assay*	Level	Ref.**
Heart:			
Turtle	3	3.01 U/mg prot.	13
Rabbit	3	3.76 U/mg prot.	13
Mouse	3	3.18 U/mg prot.	13
Liver:			
Turtle	3	13.16 U/mg prot.	13
Rabbit	3	14.21 U/mg prot.	13
Mouse	3	14.50 U/mg prot.	13
Hog	3a	315 U/mg prot.	8
Human	1a	44 U/mg prot.	16
Skeletal Muscle:			
Turtle	3	1.08 U/mg prot.	13
Rabbit	3	1.92 U/mg prot.	13
Mouse	3	2.41 U/mg prot.	13
Brain:			
Turtle	3	3.98 U/mg prot.	13
Rabbit	3	6.33 U/mg prot.	13
Mouse	3	6.20 U/mg prot.	13
Fetal rat (14 days)			
forebrain & telencephalon	1a,4	46 pmol/mg prot.	17
cerebellum, pons & medulla	1a,4	35 pmol/mg prot.	17
Fetal rat (18 days)			
forebrain & telencephalon	1a,4	41 pmol/mg prot.	17
cerebellum, pons & medulla	1a,4	27 pmol/mg prot.	17
Adult rats (male)			
telencephalon	1a,4	58 pmol/mg prot.	17
forebrain	1a,4	56 pmol/mg prot.	17
cerebellum & pons	1a,4	54 pmol/mg prot.	17
medulla	1a,4	56 pmol/mg prot.	17

* See Table 2

** See Table 3

TABLE 1: Continued

Tissue and Treatment	Assay*	Level	Ref.**
Adult rat (female)			
telencephalon	1a,4	43 pmol/mg prot.	17
forebrain	1a,4	56 pmol/mg prot.	17
cerebellum & pons	1a,4	59 pmol/mg prot.	17
medulla	1a,4	73 pmol/mg prot.	17
Eye:			
Bovine			
iris	2	30.30 μg prot./U SOD	18
lens cortex	2	0.23 μg prot./U SOD	18
lens nucleus	2	0.15 μg prot./U SOD	18
sclera	2	27.8 μg prot./U SOD	18
vitreous	2	15.8 μg prot./U SOD	18
choroid	2	20.6 μg prot./U SOD	18
retina	2	27.8 μg prot./U SOD	18
cornea	2	0 μg prot./U SOD	18

* See Table 2

** See Table 3

TABLE 2: Assays reported in Table 1.

Assay

1 Cytochrome c assay:

$O_2^{\cdot-}$-generating system:	xanthine-xanthine oxidase
Monitoring system:	$O_2^{\cdot-}$ reduces ferricytochrome c to ferrocytochrome c which absorbs at 550 nm
Unit:	The amount of extract required to inhibit the rate of reduction of cytochrome c by 50%
Original Source:	McCord and Fridovich (1969)
Objections:	Cytochrome c oxidases and peroxidases are prevalent in crude homogenates.

1a Modification:

2 to 5 x 10^{-5} M cyanide is included in the reaction mixture to inhibit cytochrome c peroxidases Gregory and Fridovich, 1973a)

1b Modification:

1 mM sodium azide is included in the reaction mixture to inhibit cytochrome c oxidases. Cyanide insensitive SOD was measured at the final cyanide concentration of 1 µM (Rister and Baehner, 1976).

1c Modification:

Interference by peroxidases and cytochrome oxidases is eliminated by the use of acetylated cytochrome c. The unit of SOD activity is also redefined as the amount of SOD that gives 25% inhibition of the rate of acetylated cytochrome c reduction at pH 9 at 29°C (Buchanan and Lees, 1976).

2 Nitroblue tetrazolium assay:

$O_2^{\cdot-}$-generating system:	xanthine-xanthine oxidase or univalent reduction of O_2 by photoinduced riboflavin
Monitoring system:	Soluble nitroblue tetrazolium forms an intensely blue insoluble precipitate (blue formazan) upon reduction

TABLE 2: Continued

Assay

2 NBT Assay: cont'd

Unit:	That quantity of SOD required to inhibit the rate of NBT reduction by 50%.
Original Source:	Beauchamp and Fridovich (1971)
Objections:	NBT is susceptible to direct reduction by certain reductants and dehydrogenases likely to be found in crude homogenates via mechanisms not inhibitable by SOD (McCord et al., 1977a). The formazan is insoluble in aqueous solution and quickly precipitates (Fried, 1975).

2a Modification:

The unit is standardized against BESOD (Winterbourn et al., 1975).

3 Epinephrine-adrenochrome assay:

$O_2^{\overline{\cdot}}$-generating system:	At pH 10.2 epinephrine autooxidizes via a free radical chain reaction involving $O_2^{\overline{\cdot}}$ as a chain-mediating species.
Monitoring system:	Adrenochrome appears as an end product and is detectable spectrophotometrically.
Unit:	The amount of SOD required to cause a 50% inhibition of adrenochrome formation.
Original Source:	Misra and Fridovich (1972a)
Objections:	Not only autoxidation, but also cooxidation of epinephrine occurs in the reaction mixture. In working with crude homogenates, significant assay variability occurs due to unidentified reactants, chain

TABLE 2: Continued

Assay

3 Epinephrine assay: cont'd

breakers, etc. (McCord et al., 1977a).

3a Modification:

Activity is reported after one purification step: ammonium sulfate precipitation

4 Chemiluminescent Luminol assay:

$O_2^{\cdot}$-generating system:	xanthine-xanthine oxidase
Monitoring system:	Chemiluminescence of luminol is induced by $O_2^{\cdot}$
Unit:	That quantity of SOD required to inhibit the maximal light intensity by 50%.
Original Source:	Puget and Michelson (1974)
Comments:	Very sensative: 0.2 ng/ml (Puget et al., 1977)

5 Antibody titrations:

Specific for Cu-Zn SOD

6 Oxygen uptake studies:

Assay method unclear (Allen and Hall, 1973).

TABLE 3: References for Table 1.

Ref. No.	Reference
1	McCord et al. (1971)
2	Gregory and Fridovich (1973a)
3	Buchanan (1977)
4	Gregory et al. (1974)
5	Misra and Fridovich (1972b)
6	Allen and Hall (1973)
7	Kedziora et al. (1977)
8	Bartkowiak et al. (1979)
9	Sinet et al. (1975)
10	Rister and Baehner (1976)
11	Stevens and Autor (1977)
12	Crapo (1975)
13	Simon et al. (1977)
14	Crapo and Tierney (1973)
15	Crapo and Tierney (1974)
16	McCord et al. (1977b)
17	Bohnenkamp and Wesser (1975)
18	Crouch et al. (1978)

MATERIALS AND METHODS

Animals

Ocular tissues were collected from 150 to 300 gm commercially cultured rainbow trout, Salmo gairdneri (Midwest Fish Farming Enterprises, Inc., Harrison, Michigan). Fish were held in fiberglass tanks at $12 \pm 1.0^{\circ}C$, with a continuous flow of aerated water. The animals were exposed to light-dark periods of 16 and 8 hours, respectively.

Dissection Procedures

The trout were dark-adapted and all enucleation and dissection was carried out under dim red illumination. All dissections were done in a plastic dish. The retinal pigment epithelial cells should not come into contact with glass during the dissection procedure. The trout were single pithed by cervical section prior to enucleation. The optic nerve was trimmed to the level of the sclera and the lamina cribosa was trimmed away. The anterior segment of the eye was separated at a level just posterior to the limbus. The vitreous body in S. gairdneri is very viscous and adheres tenaciously to the surface of the retina so no attempt was made to remove this portion. The neural retina was separated from the RPE by gently teasing out the retina with a pair of forceps. Upon

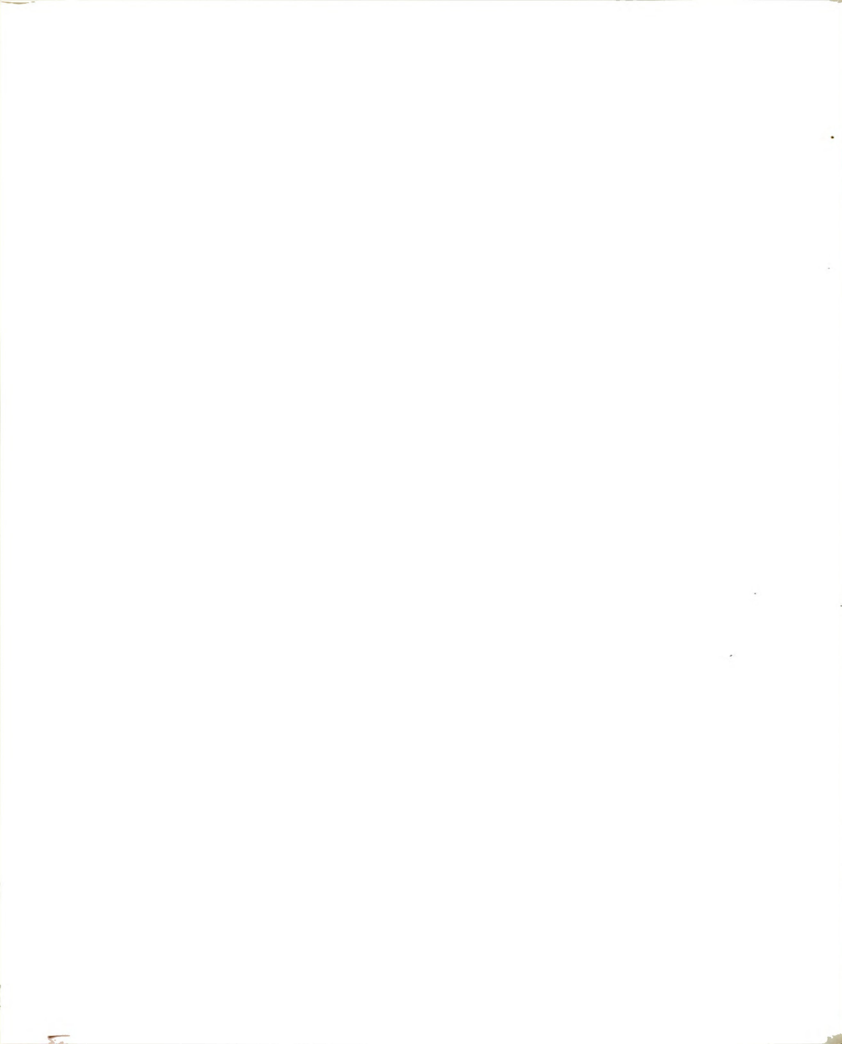
removal from the eyecup, a small camel-hair brush was used to gently remove any clinging RPE from the surface of the retina. This was saved to be pooled with the rest of the RPE fraction. The detached neural retina was transferred to a polycarbonate centrifuge tube containing phosphate buffered saline (PBS; 0.6% NaCl in 0.05M phosphate buffer, pH 7.8 at $0^{o}C$ with 0.4M sucrose) and stored on ice.

Next, several milliliters of 0.4M sucrose were added to the eyecup and a camel hair brush was used to brush the eyecup surface in order to detach the retinal pigment epithelium. The detached cells in sucrose were collected by aspiration and fresh 0.4M sucrose was added to the eyecup. The brushing and recovery of detached cells was repeated once more. Aspiration was done with a 500 ml polyethylene wash bottle modified by cutting the outlet tube to within 1 inch of the inside surface of the cap. The bottle can be employed as a suction device but cannot discharge its contents.

Finally, the remining choroidal layer was removed and pooled in PBS over ice in a polycarbonate centrifuge tube.

Tissue Fractionation

The desired final fractions from these dissected tissues were 1) neural retina, 2) photoreceptor outer segments (POS), 3) retinal pigment epithelium (RPE), and 4) choroidal layer. Further fractionation procedures were adapted from



the techniques described by Hall and Hall (1975) and Siakotos et al. (1978).

Photoreceptor Outer Segments

The POS were separated from the neural retina by mild agitation (Hall and Hall, 1975). The tube containing neural retinas was filled with PBS such that the volume of solution was twice the volume of the retinas. The tube was then capped, inverted twenty times to liberate the POS and centrifuged in the cold ($4^{o}C$) for 5 min. at 50g. The supernatant containing the POS was removed. Two volumes of PBS were added to the pellet and the procedure was repeated. The pooled supernatants were then centrifuged at 50g for 10 min. to remove retinal debris. The POS were pelletized by centrifugation at 2000g for 10 min.

Retinal Pigment Epithelial Fraction

The RPE suspension as dissected is contaminated with outer segments and with red blood cells derived from the choriocapillaris. The RPE are known to be phagocytic (Hollyfield and Ward, 1974) whereas neither the RBC's nor POS posses this ability. Like other phagocytic cells (Rabinowitz, 1964) the RPE attach to glass surfaces and can be reversibly detached by the addition of bovine serum albumin. This then, serves as the basis for a method of separating the RPE from non-RPE contaminants (Siakotos et al., 1978).

The RPE suspension was poured onto columns packed with

glass beads averaging 210 μm diameter (Class IV A Microbeads, Cat. No. 710, Cataphote-Ferro, Inc., Jackson, MS). The beads were pretreated with concentrated hydrochloric acid, washed with distilled water until free of acid, followed by a wash with 1M Tris base, again with distilled water and finally acetone, and dried at room temperature. The columns used were a series of four 600 ml, 90 mm diameter Pyrex brand Buchner funnels with coarse (40-60 μm pore size) fritted discs. These funnels were packed with beads by pouring dry beads into the column containing 0.4M sucrose to a bed height of 5-6 cm. Siakotos et al. (1978) emphasize that the quantity of cells per cm of column diameter is critical. Four 90 mm diameter columns packed as described above were found necessary to separate the RPE yield for 70 trout eyes.

The beads at $4^{o}C$ were washed with several aliquots of cold 0.4M sucrose solution and then the suspension of brushed RPE cells were applied directly to the columns. Once the sucrose level had fallen to within 1 cm of the top of the column bed, a second volume of sucrose was added and the upper few cm of the column bed was gently stirred. This was followed by a third wash volume of sucrose. By this method erythrocytes, outer segments and fragmented RPE debris are removed from the intact RPE cells.

Next, the column was inverted to empty the adsorbed RPE cell-glass bead mixture into a plastic beaker. To

detach the washed RPE cells from the glass beads, one volume (i.e., volume of glass beads) of 1% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, A-4503) plus 2% Dextran (Sigma Chemical Co., St. Louis, MO, D-4751) in 0.2M sucrose was added and the bead-cell mixture was stirred gently with a glass stirring rod. The glass beads were allowed to settle and the supernatant sucrose containing the RPE cells was poured off into polycarbonate centrifuge tubes. The RPE cell-glass beads were washed again. The pooled 1% BSA-Dextran-sucrose-RPE cell suspension was centrifuged at 1000g for 10 min. to pelletize the RPE cells. These RPE pellets were then resuspended in small volumes of PBS, pooled together, and recentrifuged both to remove the contaminating albumin protein and to end with one final RPE pellet.

Choroidal Fractions

Finally, the choroidal tissues were treated identically to the neural retina, being capped, inverted, centrifuged at 50g for 10 min., and rewashed, in order to remove as many red cells as possible. The amount remaining trapped within the choroidal vasculature after this procedure was significant.

Perfused choroidal tissues were also prepared as follows. Trout were anaesthetized to stage 5 (Jolly et al., 1972) using tricaine methane sulfonate (MS-222; Finquel, Ayerst Laboratories, Inc., N.Y., N.Y.) and the heart was

then exposed by a mid-ventral incision. The apex of the ventricle was cut off and the auricle clipped to prevent the buildup of back pressure. The blunt bevel tipped cannula (PE 240) was then introduced into the conus arteriosus and gently advanced into the ventral aorta until meeting the first bit of resistance and then withdrawn slightly. The fish were then perfused for 6 minutes at a stroke volume of 0.1 ml/stroke and pulse rate of 45 strokes/min. The perfusate was a Cortland's saline solution (Appendix I; Wolf, 1963) containing 2 USP units per ml heparin plus 0.3% procaine hydrochloride, pH 7.2. Procaine is used to prolong the anticoagulant action of heparin (Loomis et al., 1952). The eyes were then enucleated and choroids collected, washed and centrifuged as described above.

Cell Disruption and Extraction Procedures

The pelletted neural retina, POS, RPE, and choroidal tissue were resuspended in approximately 5 ml PBS and sonicated over ice in three consecutive 10 second periods at a power setting of 60 watts (Sonifier Cell Disruptor, Model W-185-C, fitted with a Micro Tip, Branson Instruments, Inc., Danbury, Conn., 06810). Additionally, 3 ml whole blood was obtained from the caudal vein of trout and diluted to 5 ml with PBS and similarly sonicated.

After centrifugation at approximately 20,000g for

1 hour the supernatants of all blood and tissue fractions were dialyzed (Appendix II) against 2 liters of saline-free phosphate buffer (0.5M, pH 7.8 at 0°C) for at least 12 hours. These extracts (i.e., dialyzed supernatants) were then frozen over dry ice, lyophilized and stored at -20°C until used.

Assay Procedures

Proteins were assayed via the Lowry method (Oyama and Eagle, 1956) (Appendix III).

Standard bovine erythrocyte superoxide dismutase (BESOD) was obtained from Sigma Chemical Co. (#S-8254; Lot 16C-8030; 2900 U/mg protein; 2880 U/mg solid; assayed as per McCord and Fridovich (1969)).

Tissue superoxide dismutase was assayed on the basis of its ability to inhibit the reduction of ferricytochrome c (Sigma Chemical Co., St. Louis, MO, C-2506) by superoxide anions generated by the xanthine-xanthine oxidase system (McCord and Fridovich, 1969). The rate of reduction was followed at 550 nm using a recording spectrophotometer (Beckman Model DB-G Grating Spectrophotometer, Beckman Instruments, Inc., Fullerton, Calif., 92634). Cytosolic SOD was distinguished from mitochondrial SOD by the former's sensitivity to 2.0mM KCN (Fridovich, 1977c). For uninhibited SOD determinations, cyanide at a concentration of 0.5 mM was included in the reaction mixture to aid in

the elimination of interference by cytochrome oxidase (Stevens and Autor, 1977; McCord et al., 1973) (Appendix IV). The cytochrome c was also heavily acetylated so that it would no longer be recognizable as a substrate for cytochrome oxidases and reductases, yet still be reducable by superoxide anion (Azzi et al., 1975; Minakami et al., 1958) (Appendix V). This latter modification also increases the sensitivity of the assay approximately twofold (McCord et al., 1977a).

The extent of acetylation of cytochrome c was measured by the ninhydrin reaction (Hirs, 1967; Moore, 1968) (Appendix VI). The final concentration of the acetylated cytochrome c was determined in the reduced form at 550 nm (Azzi et al., 1975; Massey, 1959; van Gelder and Slater, 1962)(Appendix VII).

The sample extract and standard were diluted for SOD determination such that the data could be analyzed via the parallel line assay and statistical design procedures described by Finney (1952) and Bliss (1952). These references also describe the appropriate potency and ED_{50} calculations.

Erythrocytes contain significant amounts of SOD and in those extracts with erythrocytic contamination, the contribution of SOD activity from this source must be subtracted from the total activity. This is accomplished by spectrophotometrically measuring the oxyhemoglobin

content of the sample (Castagna, 1965; Hohorst et al., 1959) (Appendix VIII). The erythrocytic SOD contamination would then be quantitated from predetermined levels of SOD per unit oxyhemoglobin.

Another source of extraneous SOD activity enters as a contaminant in the cytochrome c. The cytochrome c available through Sigma Chemical Co. has been reported to contain trace amounts (less than 0.01%) of cuprozinc SOD (McCord et al., 1977a). This is detectable as an enhanced rate of cytochrome c reduction in the presence of 2 mM KCN in the reaction mixture. This too must be taken into account (Appendix IX).

Finally, as a check for nonspecific binding or compensating interferences which would not be detected as deviations from parallelism, the actual assayed level of combined reference and standard which yields 50% inhibition is compared to the level predicted from the individual standard and extract determinations (Appendix X).

RESULTS

SOD Assay Behavior and Analysis

The assay used in the current study is based upon the competition between superoxide dismutase and cytochrome c for the superoxide anion generated in the xanthine-xanthine oxidase reaction system. Cytochrome c is reduced upon reaction with an electron from the superoxide anion and the product absorbs light at a wavelength of 550 nm with a millimolar extinction coefficient of 27.7. Figure 1 demonstrates the change in absorbance at 550nm of native non-acetylated cytochrome c with time in the absence of SOD and in the presence of several different concentrations of purified bovine erythrocyte superoxide dismutase (BESOD). As originally established, one unit of SOD activity was defined by this assay to be that quantity required to inhibit the rate of reduction of ferricytochrome c by 50%.

In the present study, the initial reduction rate (i.e., over two to six minutes) was found to decline exponentially with increasing SOD concentrations (Figure 2). Thus, the plot of reaction rate versus the log of the concentration of SOD (hereafter referred to as log dose SOD) generates a straight line (Figure 3). The assay response is considered to be the percent inhibition of the rate of reduction of cytochrome c, thus this data may be replotted as percent

inhibition vs. log-dose SOD to yeild a typical linear log dose-response curve (Figure 4). To avoid the statistical bias introduced into the data by percentage values, the percentage response is converted to probit response using readily available statistical tables (Figure 5). Finally, for convenience of plotting and for ease of future statistical calculations, the dosages may be arbitrarily coded into a linear scale (encoding and decoding procedures are described in Appendix X). The use of acetylated cytochrome c (ACc) was seen to enhance the sensitivity of the assay and to again yield a linear log dose-probit response curve over a range of from 5% to 95% inhibition (Figure 6).

To apply this assay, it is assumed that the SOD of the extract differs from the standard only in that it is dissolved in a different diluent which is completely inert in the reaction mixture. In the present case, the assumed inertness of the crude tissue extract SOD diluent is particularly suspect. A vast array of compounds are inevitably present in crude extracts and may be expected to react with the $O_2^{\overline{\cdot}}$. To repeat, whereas the SOD is specific for $O_2^{\overline{\cdot}}$, the reverse is far from the case.

Were the extract different from the standard only by a dilution factor, their log dose-response curves should coincide if the concentration of the extract could be adjusted to exact equality with the standard. Thus, if the extract preparation differs from the standard only in

FIGURE 1.--Absorbance at 550 nm vs. time in the presence of various concentrations of bovine erythrocyte SOD. Data was collected using native cytochrome c. Units of activity refer to those defined by the Sigma standard.

FIGURE 1

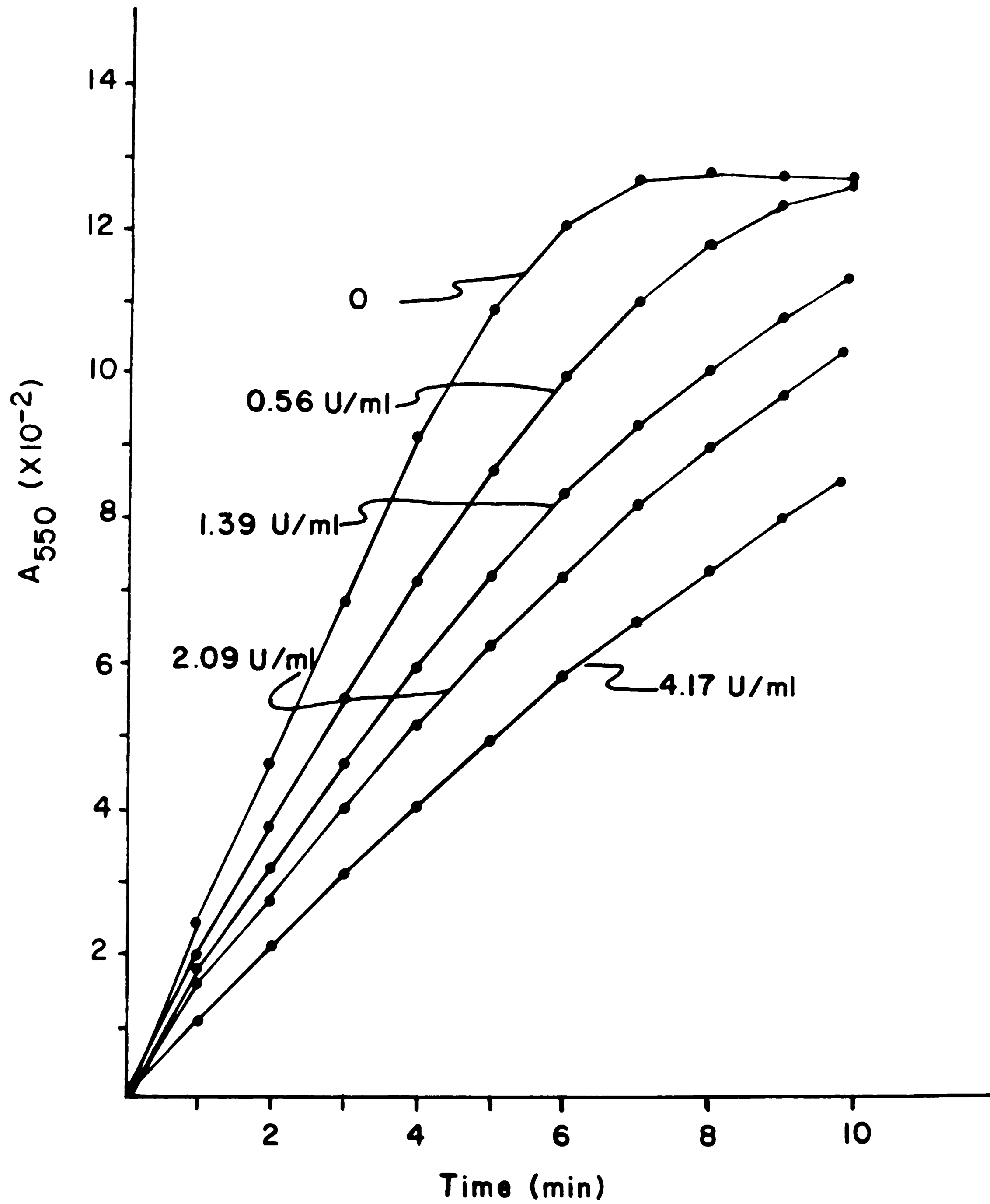


FIGURE 2.--Rate of native cytochrome c reduction vs. bovine erythrocyte SOD concentration. Data are plotted as means with the range for four replications at each concentration. The rate of reduction over the first two to six minutes as measured by the change in absorbance at 550 nm per minute is designated as V_o. The "Basal Rate" represents the rate of reduction in the absence of SOD.

FIGURE 2

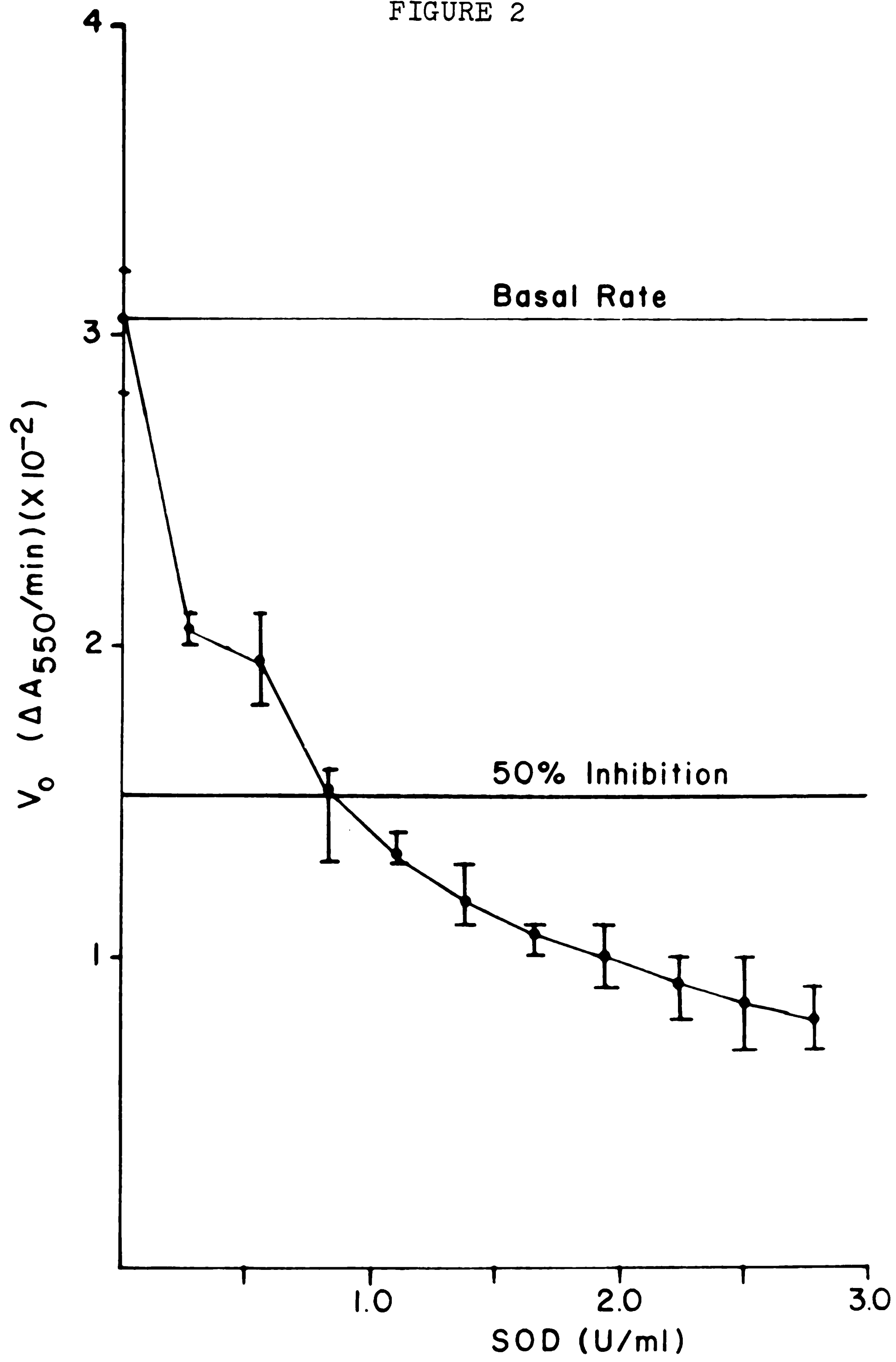


FIGURE 3.--Rate of native cytochrome c reduction vs. log bovine erythrocyte SOD concentration. Data are replotted from Figure 2.

FIGURE 3

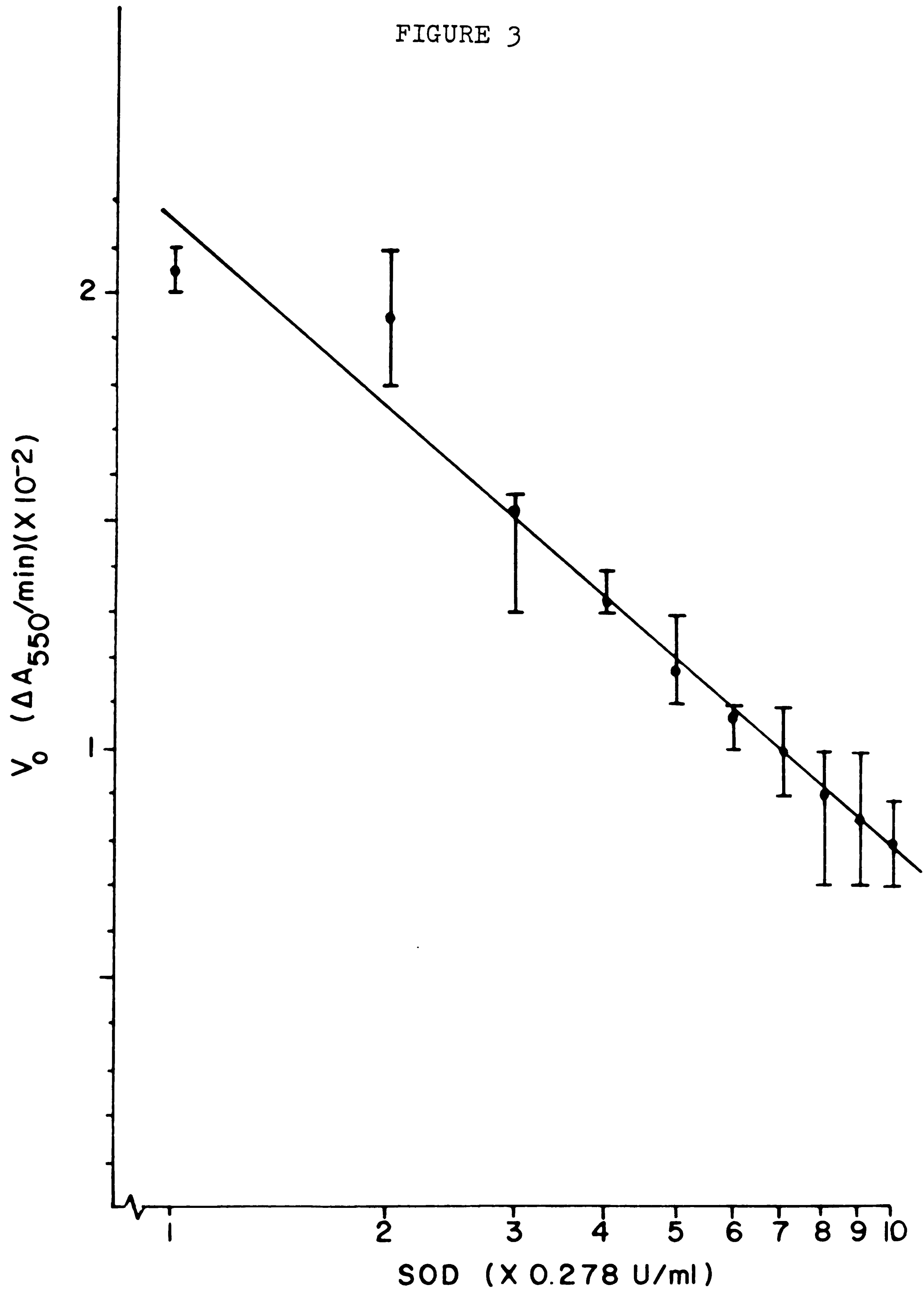


FIGURE 4.--Percent inhibition of the rate of cytochrome c reduction vs. log standard BESOD concentration. Mean data only are replotted from Figure 2.

FIGURE 4

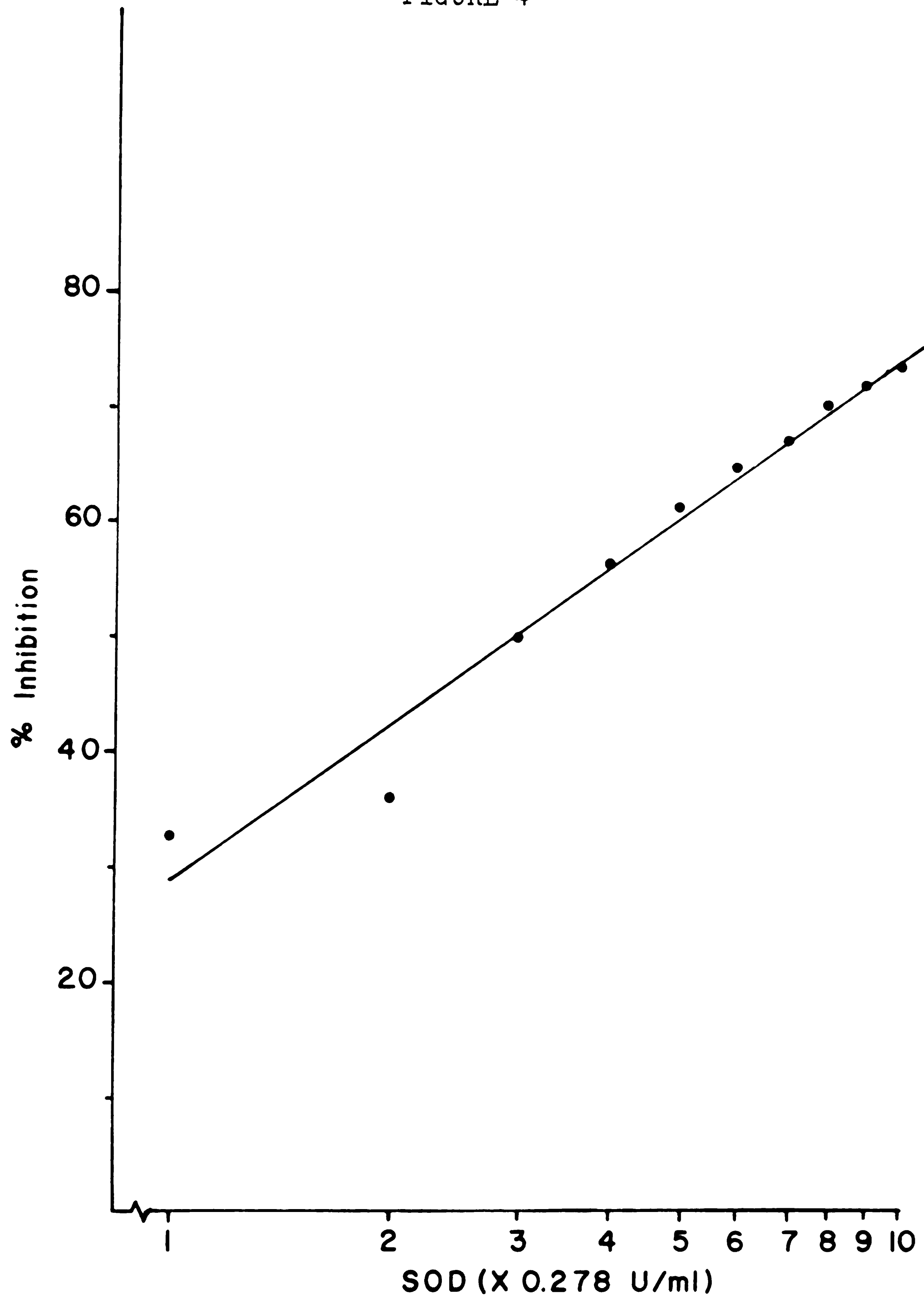


FIGURE 5.--Probit response vs. log dose BESOD. Mean data only are replotted from Figure 2.

FIGURE 5

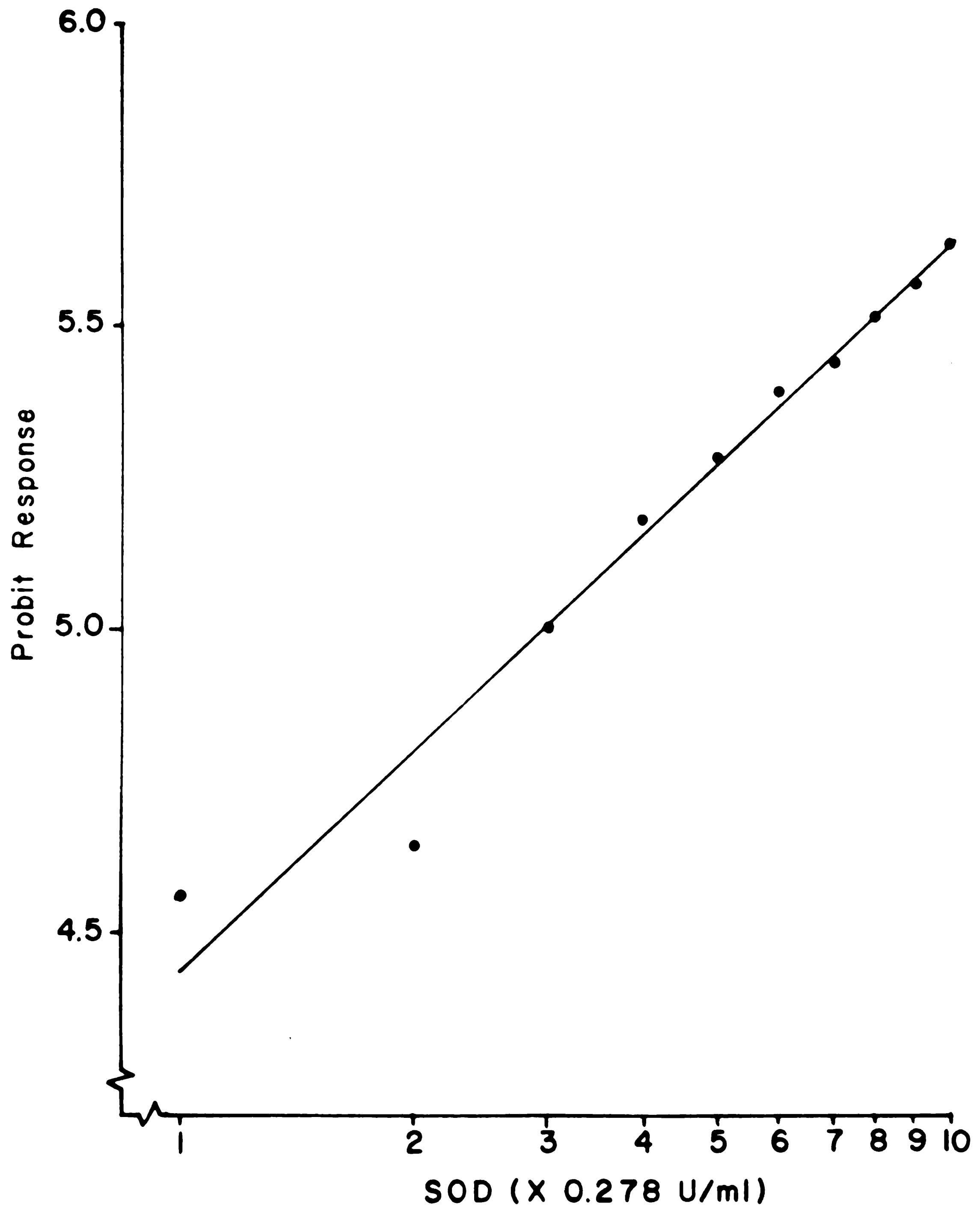
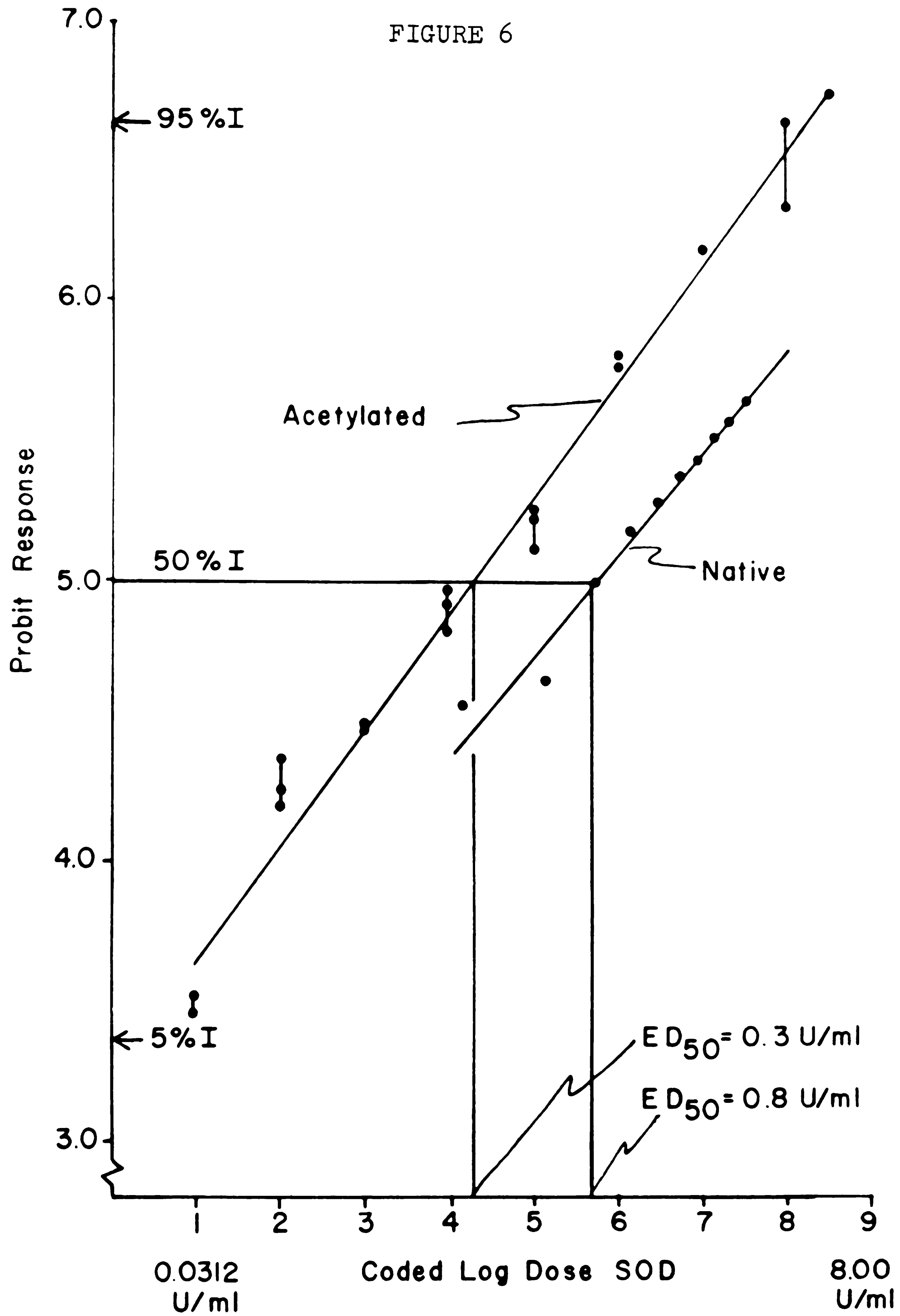


FIGURE 6.--Probit response vs. coded log dose curves for native and acetylated cytochrome c. The ED_{50} is the "effective dose" for 50% inhibition of the rate of reduction of cytochrome c. Coding of the log dose scale is described in Appendix X. Native data are replotted from Figure 2. Acetylated data are plotted as the range for four determinations. Lines are calculated from least squares regressions. Probit responses for 5, 50, and 95 percent inhibition are indicated as points of reference.

FIGURE 6



relative potency, their respective curves should be parallel in a log dose assay. By running a series of extract dosages, it is possible to determine whether the potency (a measure of the horizontal displacement of the two lines) is independent of the level of response. If this were not the case, the relative potency computed from converging curves would differ with the level of response, and the extract could not be assigned a single potency.

Hence, the assays were designed such that the assumptions of parallelism could be quantitatively tested. The crude tissue extracts are assayed in a series of four dilutions of logarithmically increasing concentrations. The doses are coded with the lowest dose being assigned a value of -1 and each successive tripling as one coded unit higher. Encoding and decoding is described in Appendix X. Data are collected at these doses for inhibited extract (IE) (i.e., 2.0 mM KCN), inhibited standard (IS), uninhibited extract (E) (i.e., 0.5 mM KCN), uninhibited standard (S), and extract containing an internal standard reference (S+E) (i.e., a 50:50 mixture of extract and standard solutions). Data collected as percent inhibition of reaction rates are transformed to probit response. The data are then paired as follows: E against S, IE against S, IE against E, IS against S, and S+E against S. The data for these pairs of lines are then subjected to a four dose parallel line analysis of

variance which tests for differences in the preparation dilutions, for positive slope, for parallelism, and for deviations from linearity as quadratic or cubic components.

A sample four-dose ANOVA table is presented in Table 4, and the corresponding pair of log dose-response curves of the analysis are plotted in Figure 7. The tests for significance of the treatment variances (i.e., the F ratios) in the last column of the ANOVA table determine the validity of the assay at the 95% probability level. The first row tests for differences in the mean responses of the two preparations; the more closely matched the dilutions, the lower the associated F ratio. The second row tests for a significant regression; i.e., a non-zero slope. This term should be highly significant for a justifiable assay. The assumption of parallelism is tested in the third row. For a valid determination, this term must not be significant, thereby indicating no significant differences in the slopes. The remaining rows test for curvature as computed with orthogonal polynomials. Significant curvature may indicate that the test doses fall outside the linear zone, too near the 'ceiling' or 'floor' of response. In this case it may be possible to omit the responses which fall outside the central linear portion and retest the data by similarly designed three- or two-dose analyses of variance. Otherwise, the dilutions should be changed and the assay repeated if possible.

FIGURE 7.--Parallel line assay of standard BESOD in the presence of 0.5 mM KCN (S) vs. 2.0 mM KCN (IS). Potency (P) is an expression of the horizontal displacement of the two lines. Both ED_{50}'s and potency ratios are expressed with 95% confidence limits.

ED_{50_S} = 10.55 (9.54 - 11.60) µl = 1 Unit SOD

$ED_{50_{IS}}$ = 56.88 (49.64 - 66.17) µl

Potency = 0.186 (0.156 - 0.220)

FIGURE 7

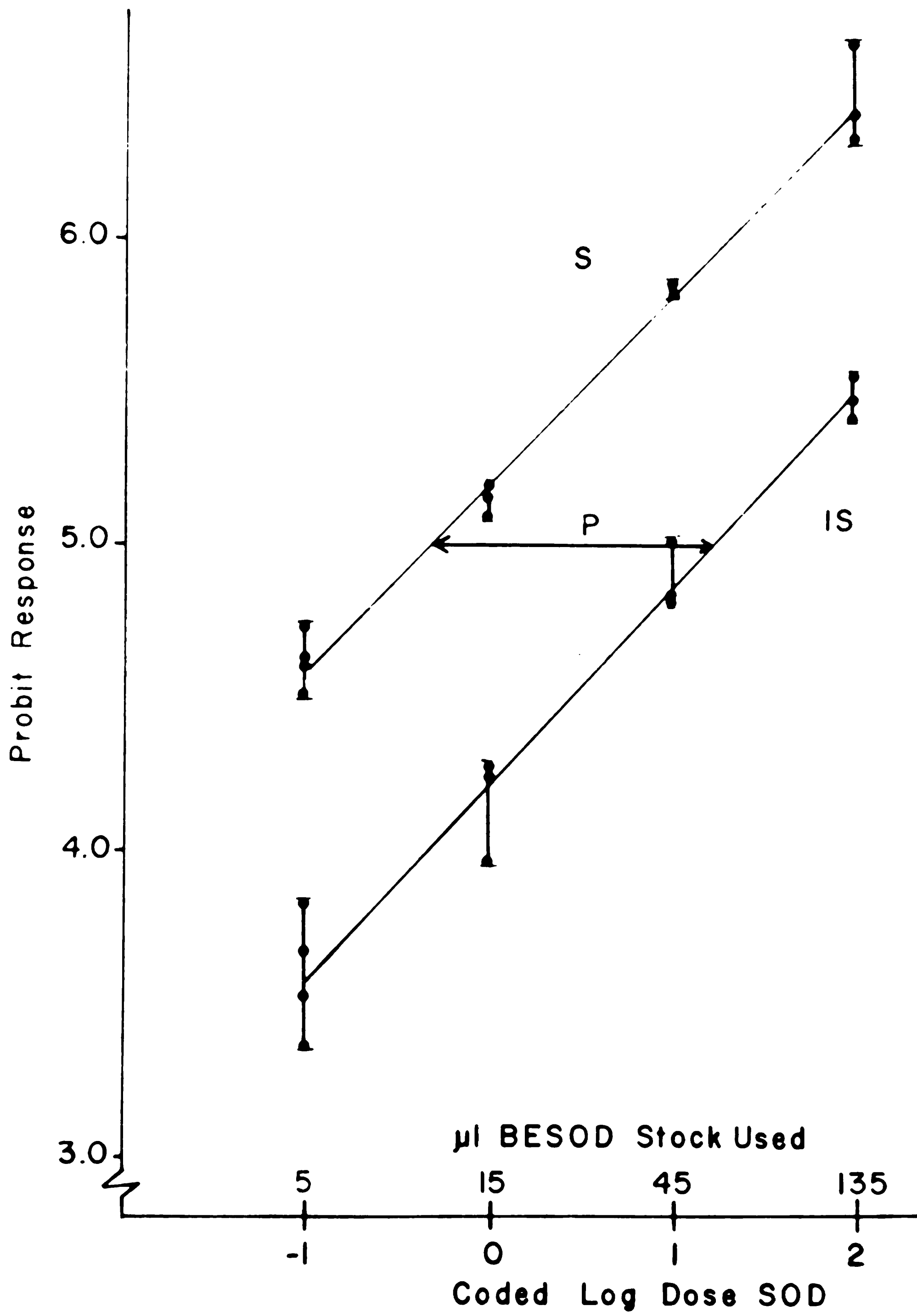


TABLE 4: Four-dose ANOVA table for data of Figure 7. S(i) and U(i) refer to corresponding coded standard and unknown dosages.

Contrast	Orthogonal Coefficients S(1)	S(2)	S(3)	S(4)	U(1)	U(2)	U(3)	U(4)	Divisor
Preparations	-1	-1	-1	-1	1	1	1	1	32
Regression	-3	-1	1	3	-3	-1	1	3	160
Parallelism	3	1	-1	-3	-3	-1	1	3	160
Quadratic	1	-1	-1	1	1	-1	-1	1	32
Difference in Quadratic	-1	1	1	-1	1	-1	-1	1	32
Cubic	-1	3	-3	1	-1	3	-3	1	160
Difference in Cubic	1	-3	3	-1	-1	3	-3	1	160

Contrast	Sum of Products	Variance Estimate	F Ratio
Preparations	-15.50000	7.50781	597.70062
Regression	50.62000	16.01490	1274.95386
Parallelism	0.98000	0.00600	0.47787
Quadratic	0.40000	0.00500	0.39804
Difference in Quadratic	-0.32000	0.00320	0.25476
Cubic	-2.15999	0.02916	2.32142
Difference in Cubic	-0.23999	0.00036	0.02866

Sample Variance = 0.01256; $F_{(1,24;0.05)} = 4.26$

If the assay proves statistically valid, the potency of the extract as compared to the standard is calculated and is expressed with its associated 95% confidence limits. Similarly, procedures are well defined (Bliss, 1952) for quantifying the dose which yielded 50% inhibition (ED_{50}) and its associated 95% confidence limits. These confidence limits provide a valuable estimate of the inherent precision of the assay.

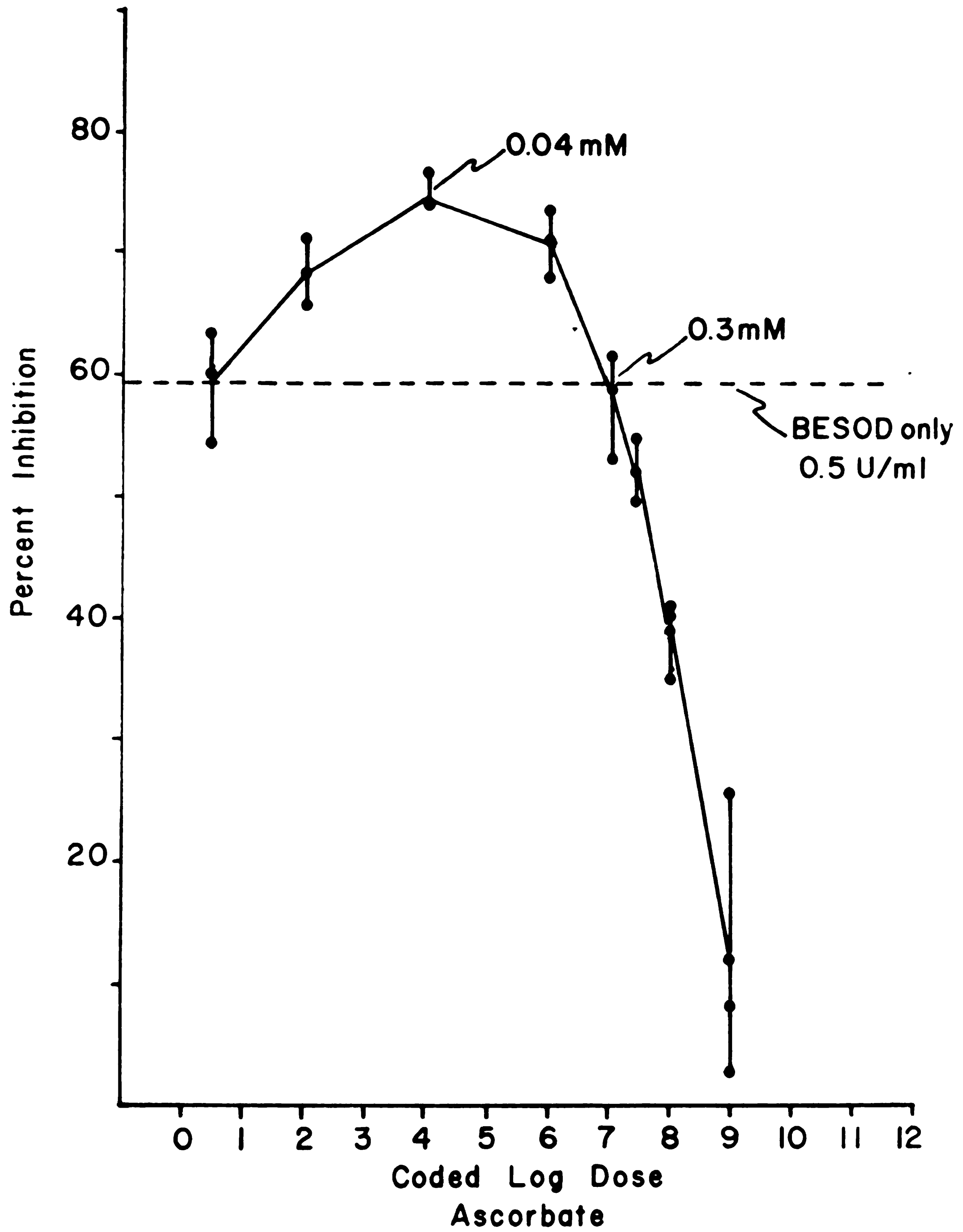
Assay Verification

To test the ability of this assay to detect the presence of interfering substances in the unknown extract, ascorbic acid was selected for its ability to react competitively with SOD and cytochrome c for the superoxide anion. Varying concentrations of ascorbate were included in the reaction mixture in the presence of constant levels of SOD. Catalase (22 μg/ml) was also included in the reaction mixture to prevent the oxidation of ascorbic acid by H_2O_2 which is formed in the xanthine oxidase reaction.

The effect of ascorbic acid upon the assay was concentration dependent (Figure 8). At low concentrations the ascorbate acted as an antioxidant as has been purported elsewhere. At approximately 40μM concentrations, the ascorbate apparently starts to participate in a competing oxidative reaction, and above 300μM the

FIGURE 8.--Effect of ascorbate on the percent inhibition of the rate of cytochrome c reduction in the presence of constant BESOD. Data are plotted as mean with the range for four replications.

FIGURE 8



ascorbate served exclusively as an oxidant in the reaction mixture, probably by enhancing the rate of superoxide anion production (see Discussion).

When the BESOD was contaminated with 1.5 mM ascorbate and then used as an unknown tissue extract and subjected to the four-dose parallel-line assay approach, the presence of ascorbate was easily detectable as a deviation from linearity (Figure 9; Table 5) as compared to the uncontaminated standard. Thus, the parallel line approach was very effective in this case.

Assay Application

This modified assay approach was next applied to the determination of SOD activity within the blood and ocular tissues of *Salmo gairdneri*. It was desired to apply the assay to crude tissue extracts without necessitating any subcellular fractionation or protein purification steps such that the final tissue activity could be expressed in terms of units of SOD activity per mg protein in the crude extract. Three samples of each fraction were prepared as described. Each sample of neural retina, retinal pigment epithelium, photoreceptor outer segment, and non-perfused choroid was comprised of the respective tissues pooled from 70 trout eyes. For each of the three perfused choroidal samples, 24 eyes were pooled. Each whole blood sample was comprised of blood collected from three trout.

Non-perfused choroidal tissues in every case displayed

FIGURE 9.--Four-dose parallel-line assay of standard BESOD vs. BESOD contaminated with 1.5 mM ascorbate and 22 μg/ml catalase. Data are plotted as means with the range for four replications.

FIGURE 9

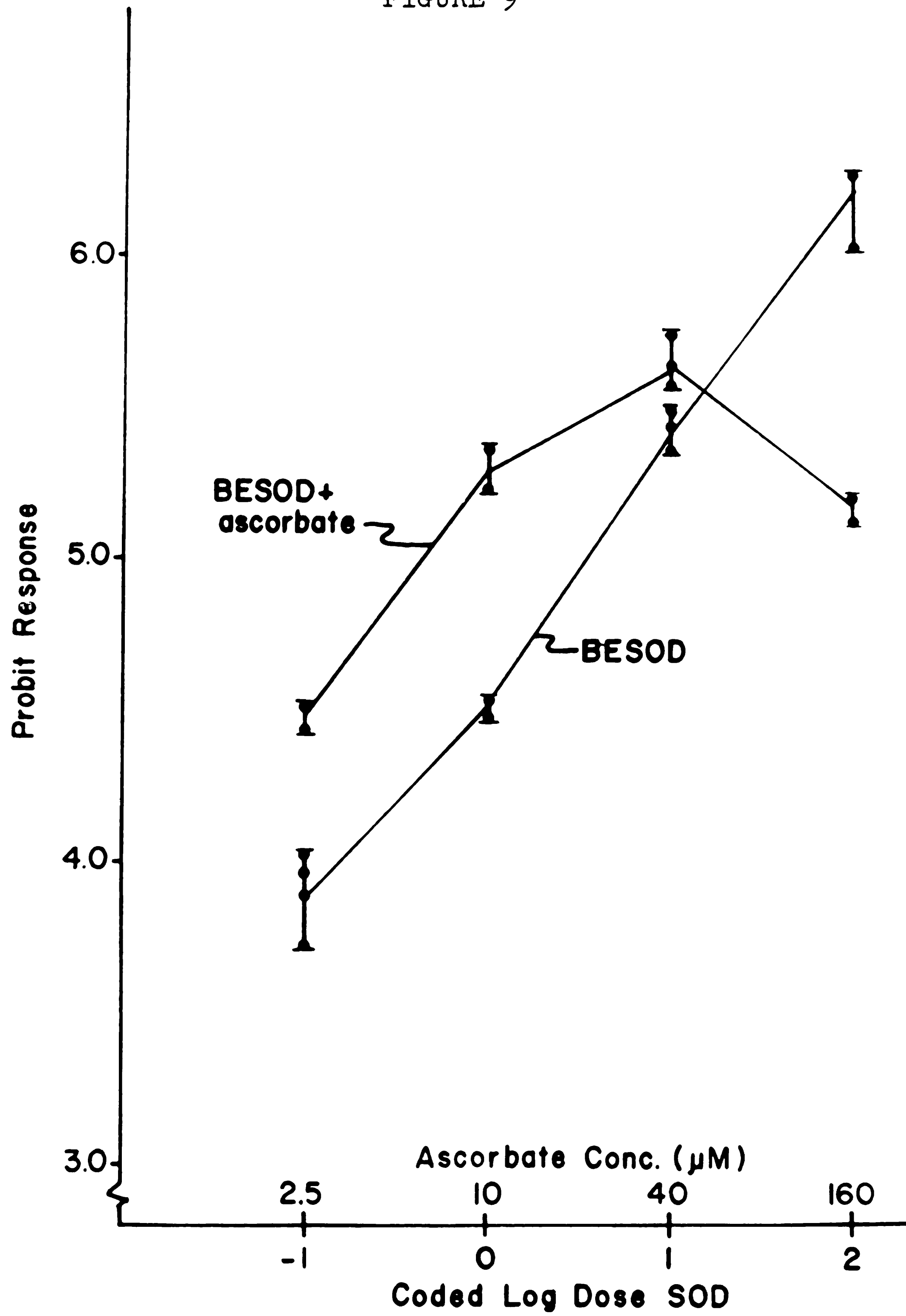


TABLE 5: Four-dose ANOVA results for data of Figure 9.

Contrast	F Ratio
Preparations	25.43972
Regression	1706.28140
Parallelism	477.46447
Quadratic	95.14580
Difference in Quadratic	163.22861
Cubic	8.36135
Difference in Cubic	0.13517

$F_{(1,24;0.05)} = 4.26$

$F_{(1,24;0.01)} = 7.82$

$F_{(1,24;0.005)} = 9.55$

$F_{(1,24;0.001)} = 14.03$

very characteristic dose-response curves with statistically significant quadratic components and lack of parallelism to the standard. A representative result is presented in Figure 10 and Table 6. If the lowest dose is removed from the data and is construed to represent the floor of response, the upper three doses yield a curvilinear response with a significantly greater slope than that of the standard. Note too that in the three-dose analysis of variance (Table 6), only nonlinearity may be tested in that four points of a line are required to detect whether the curvature is more closely cubic or quadratic. In the presence of 2.0 mM KCN the curve shifted to the right (Figure 10), but its shape remained unaltered. This shape was also evident in the internal reference recovery sample.

The non-perfused choroidal tissues contain significant amounts of blood. Whole blood assays, however, exhibited a significantly different dose-response relationship (Figure 11, Table 7). At high concentrations, in all whole blood samples analyzed, the rate of reduction was enhanced rather than further inhibited. Below this point (i.e., below about 25 mg% lyophilized whole blood) the response was linear and parallel to the standard. Inhibition by cyanide shifted the curve to the right and elminated the downturn at high doses, but significantly increased the slope of the response curve. The SOD

FIGURE 10.--Representative four-dose parallel-line assay of choroidal extract vs. BESOD. Data are plotted as means with the range for four replications.

FIGURE 10

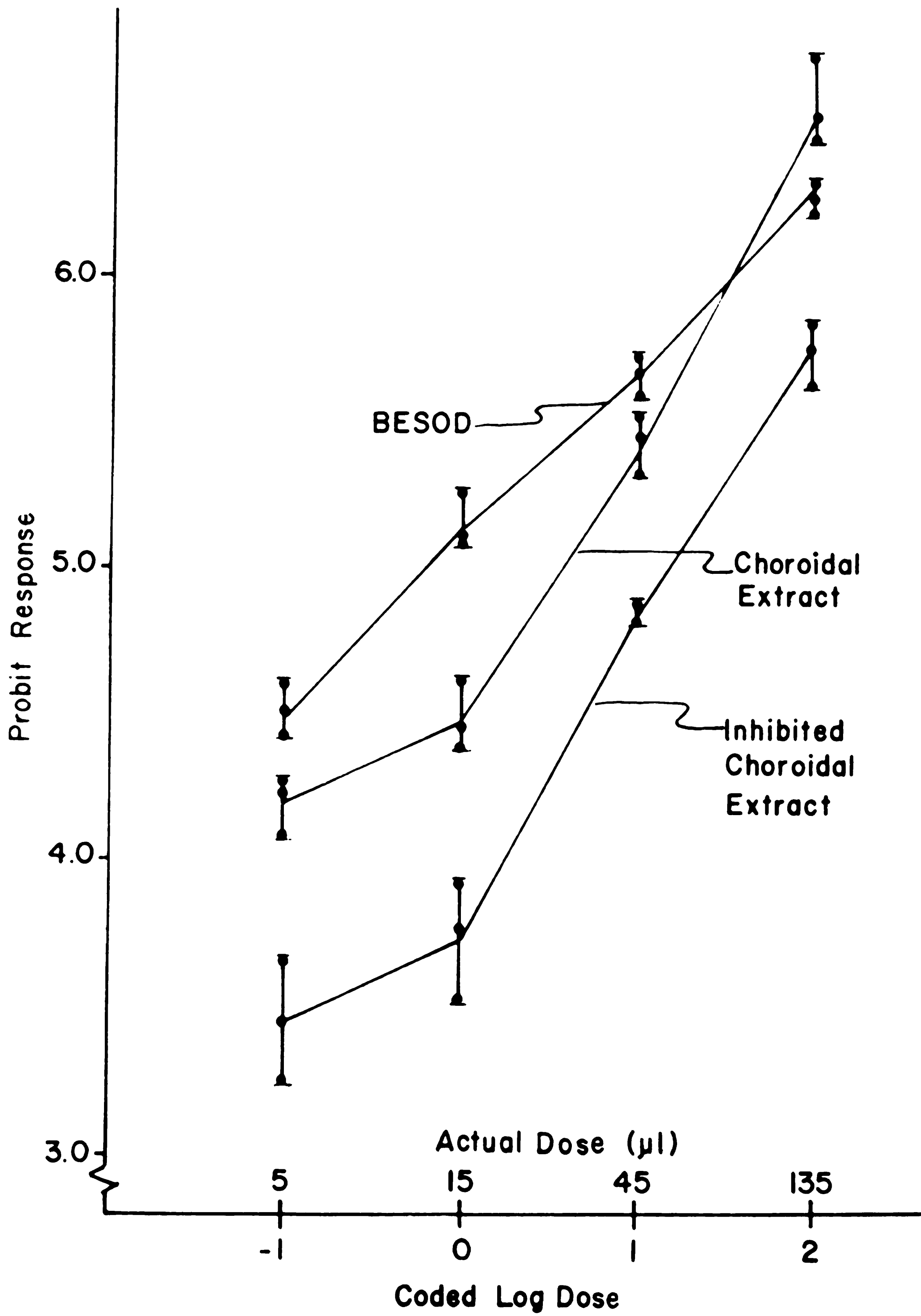


TABLE 6: Four- and Three-dose ANOVA results for data of Figure 10.

Four-dose ANOVA; Uninhibited Extract vs. BESOD:

Contrast	F Ratio
Preparations	27.50279
Regression	967.62433
Parallelism	24.77758
Quadratic	13.29022
Difference in Quadratic	12.59317
Cubic	0.00238
Difference in Cubic	1.38149

$F_{(1,24;0.05)} = 4.26$

$F_{(1,24;0.005)} = 9.55$

$F_{(1,24;0.001)} = 14.03$

Three-dose ANOVA; Uninhibited Extract vs. BESOD; lowest dosage omitted:

Contrast	F Ratio
Preparations	31.62876
Regression	1091.74036
Parallelism	80.04202
Curvature	4.59004
Difference in Curvature	0.31094

$F_{(1,18;0.05)} = 4.41$

$F_{(1,18;0.01)} = 8.29$

$F_{(1,18;0.001)} = 15.38$

FIGURE 11.--Representative four-dose parallel-line assay of whole blood extract vs. BESOD. Data are plotted as means with the range for four replications.

FIGURE 11

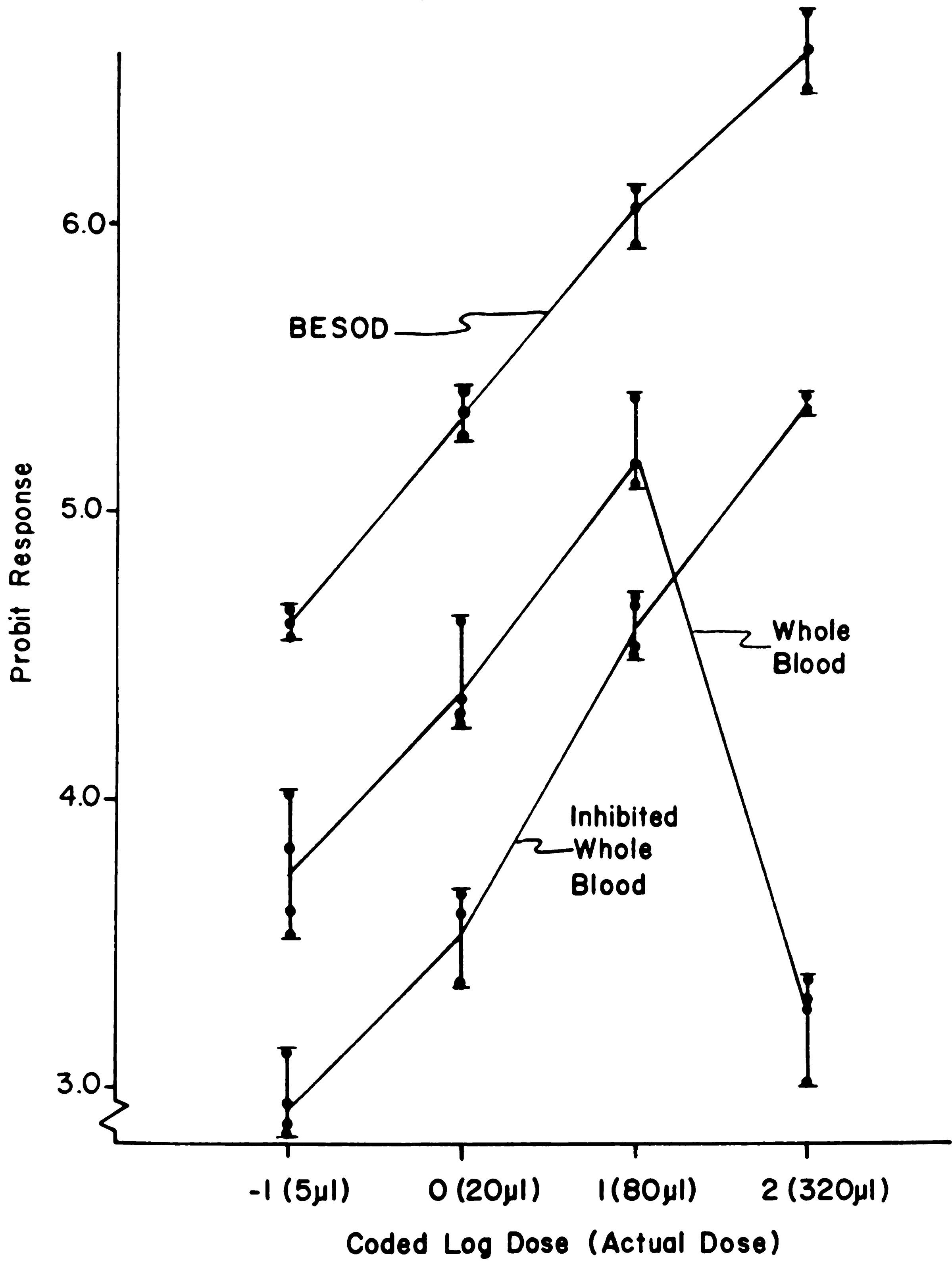


TABLE 7: Four-dose ANOVA results for data of Figure 11.

Uninhibited Whole Blood Extract vs. BESOD:

Contrast	F Ratio
Preparations	285.14737
Regression	57.13263
Parallelism	73.93367
Quadratic	44.44691
Difference in Quadratic	33.17346
Cubic	20.98105
Difference in Cubic	17.62871

Inhibited Whole Blood Extract vs. BESOD:

Contrast	F Ratio
Preparations	1325.45349
Regression	1595.49268
Parallelism	18.61973
Quadratic	0.01064
Difference in Quadratic	3.59996
Cubic	5.69247
Difference in Cubic	2.38349

$F_{(1,21;0.05)} = 4.32$

$F_{(1,21;0.01)} = 8.02$

$F_{(1,21;0.005)} = 9.83$

$F_{(1,21;0.001)} = 14.59$

activity could be quantified and expressed in terms of total SOD per δA_{HbO_2} (see Appendix VIII). At any level, 93.4% total erythrocytic activity was due to Cu-Zn SOD, the remainder due to cyanide-insensitive Mn SOD. The non-perfused choroidal extracts were similarly scanned for HbO_2, and 74% of the total activity was found attributable to erythrocytic contamination. The blood could not account for the shape of the curve, however. Perfused choroidal tissues were free of erythrocyte contamination as determined by oxyhemoglobin assay (Appendix VIII). The characteristic shape of the response curve remained the same as that of the non-perfused choroidal tissue (Figure 12, Table 8). The behavior in response to cyanide was also similar to that of the non-perfused choroidal extracts. This reconfirms that the shape of the choroidal dose-response curves could not be attributed to the presence of erythrocytic interference.

The retinal pigment epithelial tissue extracts yielded variable results (Tables 10 and 11). In two of three samples assayed, the inhibited extract dose-response curve had a significantly greater slope than that of the uninhibited extract or standard. The extract dose-response curve had a slope parallel to that of the standard in two of three cases. The conclusion which might be drawn is that the cyanide insensitive component of the extract has a significantly greater slope than that of the standard

FIGURE 12.--Representative four-dose parallel-line assay of perfused choroidal tissue extract vs. BESOD. Data are plotted as means with the range for four replications.

FIGURE 12

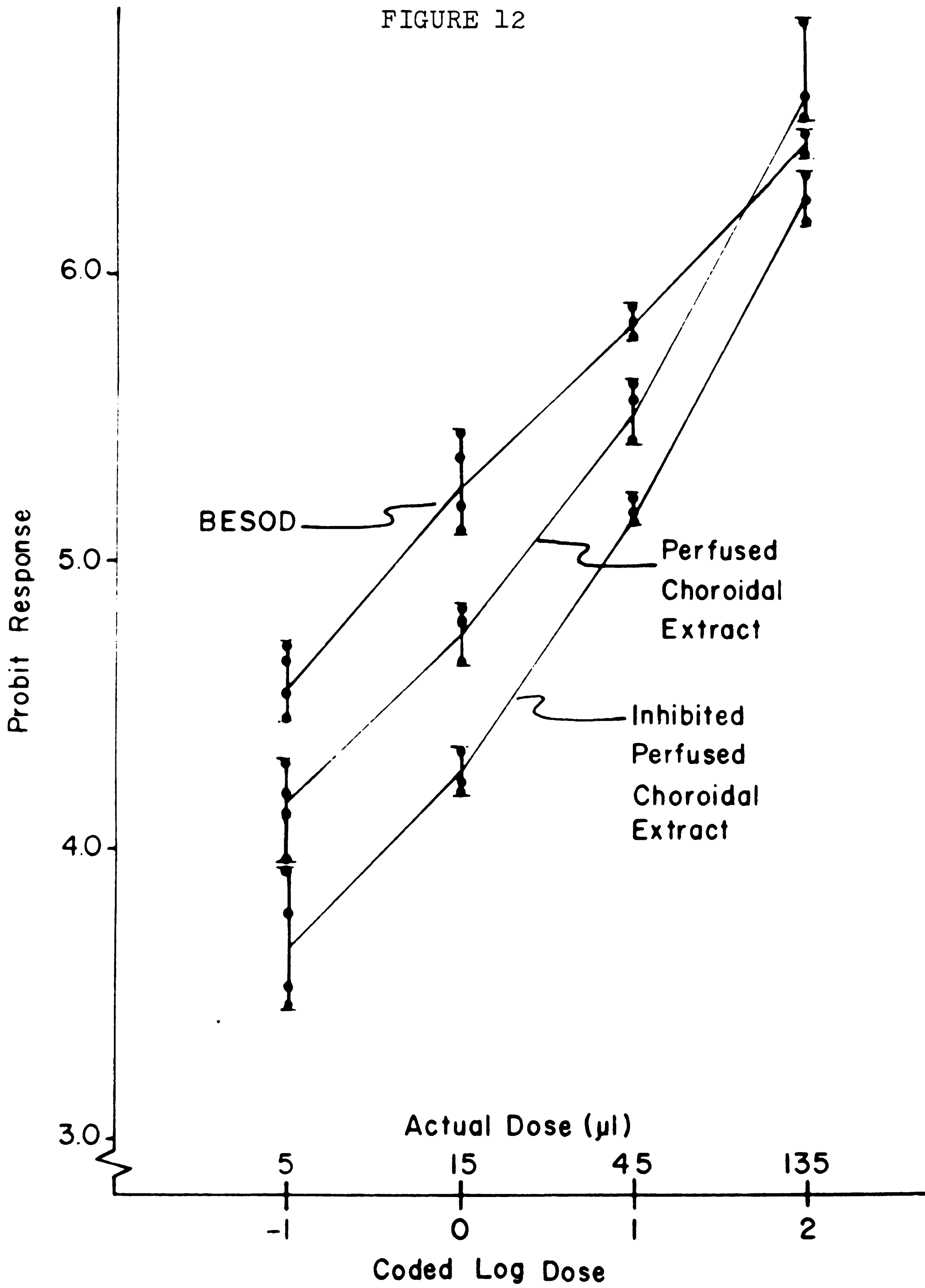


TABLE 8: Four-dose ANOVA results for data of Figure 12.

Perfused Choroidal Extract vs. BESOD:

Contrast	F Ratio
Preparations	41.68923
Regression	1302.55688
Parallelism	22.35329
Quadratic	5.09091
Difference in Quadratic	6.89200
Cubic	1.15029
Difference in Cubic	0.29099

Inhibited Perfused Choroidal Extract vs. Uninhibited Perfused Choroidal Extract:

Contrast	F Ratio
Preparations	72.40617
Regression	1515.86975
Parallelism	2.10560
Quadratic	23.87879
Difference in Quadratic	0.16318
Cubic	0.02476
Difference in Cubic	0.11034

$F_{(1,24;0.05)} = 4.26$

$F_{(1,24;0.01)} = 7.82$

$F_{(1,24;0.005)} = 9.55$

$F_{(1,24;0.001)} = 14.03$

and that when not inhibited by cyanide, this activity is largely masked by the cyanide-sensitive component. The increased slopes may be indicative of a substance which competes for the superoxide anion with a greater affinity than that of the standard bovine erythrocyte SOD.

A similar, but more definite, pattern is seen in the photoreceptor outer segments where the uninhibited extract had a significantly greater slope than did the standard, and the inhibited extract had a significantly greater slope than did the uninhibited extract (Figure 13, Table 9). This pattern was exhibited by all samples assayed. Again, the implication is that the cyanide insensitive component of SOD activity imparts a greater slope to the curve.

The retinal tissue was the only tissue which was found to possess apparent superoxide dismutase activity which paralleled that of the bovine erythrocyte standard both in the absence and in the presence of 2.0 mM KCN. This activity is tabulated in Tables 10 and 11. The neural retinal activities are the only activities which may be considered as statistically valid as defined by the restrictions of the assay approach.

In none of the extracts was any interference detected by the internal reference recovery test.

For the sake of comparison, but remaining aware that any departures from parallelism with the standards may

FIGURE 13.--Representative four-dose parallel-line assay of inhibited and uninhibited photoreceptor outer segment extract vs. BESOD. Data are plotted as means with the range for four replications.

FIGURE 13

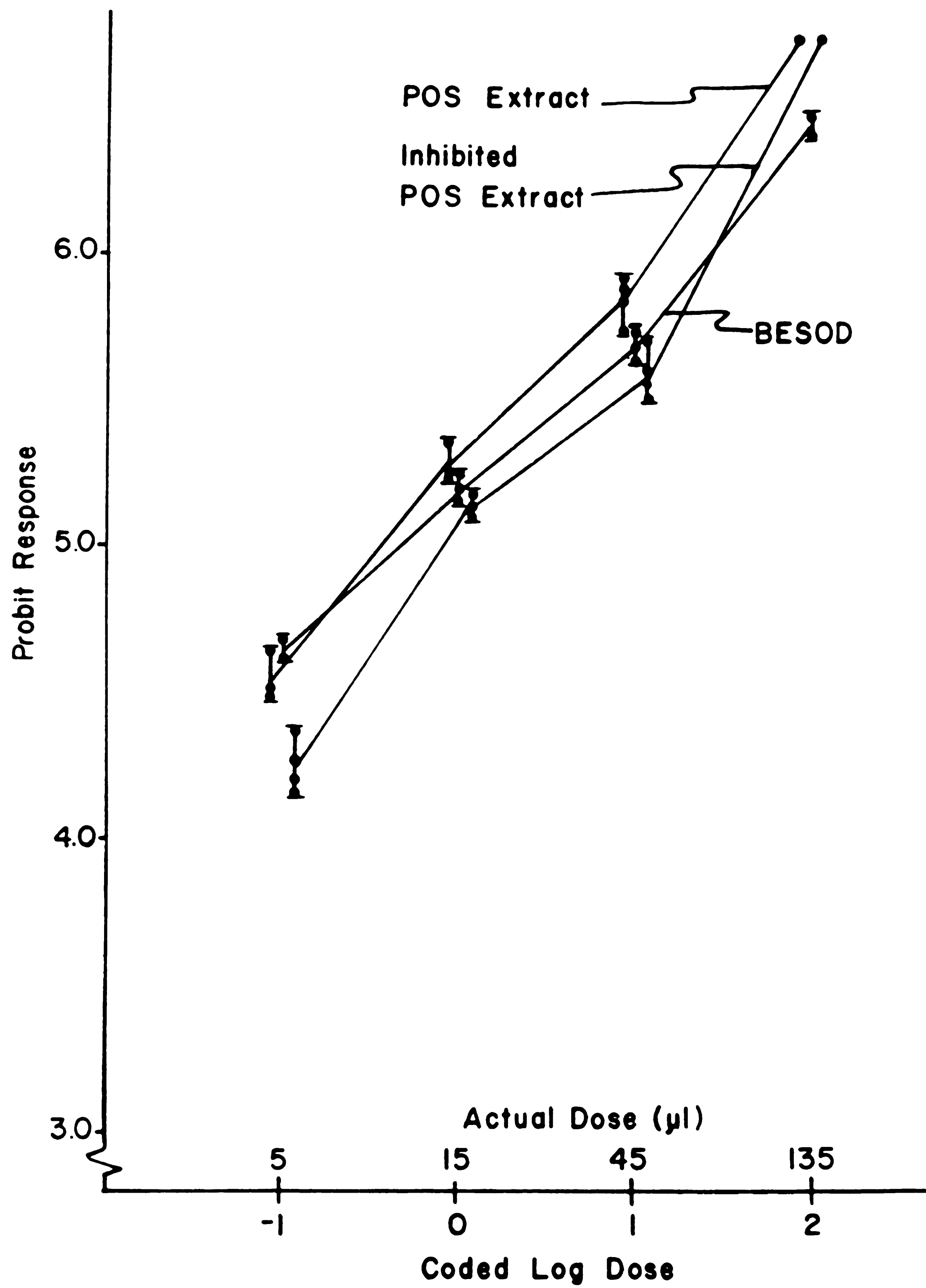


TABLE 9: Four-dose ANOVA results for data of Figure 13.

Outer Segment Extract vs. BESOD:

Contrast	F Ratio
Preparations	37.60400
Regression	6654.47607
Parallelism	59.29649
Quadratic	30.34469
Difference in Quadratic	1.00947
Cubic	28.57299
Difference in Cubic	1.14290

Inhibited Outer Segment Extract vs. Uninhibited POS Extract:

Contrast	F Ratio
Preparations	56.98240
Regression	5543.25684
Parallelism	13.83530
Quadratic	21.52020
Difference in Quadratic	1.14604
Cubic	70.71043
Difference in Cubic	11.06730

$F_{(1,23;0.05)} = 4.28$

$F_{(1,23;0.01)} = 7.88$

$F_{(1,23;0.005)} = 9.63$

$F_{(1,23;0.001)} = 14.19$

invalidate the assay, the calculated potencies and specific activities for all tissues are summarized in Tables 10 and 11. Unlike the reproducibility of the shapes of the dose-response curves, the levels of activity varied greatly. Several important trends may be seen, however. First, the cyanide-sensitive Cu-Zn SOD specific activities of the three retinal fractions were reasonably comparable, lying in the range of from 6 to 13 U/mg protein. The vast bulk of the variability occurred in the cyanide-insensitive component of the activity. Thus, abberant assay behavior both in the shape and slope of the dose-response curve, and in the variability of the levels of activity seems to arise in the cyanide-insensitive component of activity usually attributed to mitochondrial Mn SOD.

TABLE 10: Assayed Levels of SOD Activity Based Upon ED_{50}'s. Activity is expressed as Units/mg protein as defined by the acetylated cytochrome c assay.

Tissue	ED_{50_E} (95% Confidence Limits)(µl extract)	Cu-Zn SOD Activity	CN^--insensitive Activity	Total Activity
Retina	7.71 (6.01- 9.46)	7.72	8.86	16.58
	3.89 (3.57- 4.20)*	6.73	17.51	28.24
	12.67 (10.94- 14.39)*	6.99	16.17	23.16
	9.10 (8.08- 10.13)	6.18	12.52	18.70
Photoreceptor Outer Segments	32.48 (27.26- 39.56)	11.78	12.32	24.10
	7.32 (6.15- 8.48)	13.09	42.41	55.50
	10.35 (9.37- 11.36)	10.30	29.44	39.74
Retinal Pigment Epithelium	17.88 (14.87- 20.84)	7.93	51.24	59.18
	36.92 (32.04- 42.92)**	0.93	14.25	15.17
	25.81 (21.24- 31.34)	11.32	9.28	20.60
Perfused Choroid	70.10 (55.36- 94.46)	5.77	1.47	7.25
	21.65 (18.97- 24.60)	5.86	3.74	9.59
	18.62 (16.20- 21.24)	4.81	4.11	8.92
Non-perfused Choroid***	27.00 (24.53- 29.67)	3.02	1.31	4.33
	20.64 (17.14- 24.61)	3.82	1.02	4.85
	18.13 (15.18- 21.40)	4.50	2.75	7.25
Blood	59.90 (45.73- 85.37)	1.94	0.14	2.07
	118.51 (75.51-239.40)	0.20	0,16	0.37
	48.89 (33.15- 82.46)	0.11	0.96	1.07

* Duplicate Assay determinations on the same sample.
** Probable sample contamination with bovine serum albumin.
*** Erythrocyte SOD activity not deleted.

TABLE 11: Assayed Levels of SOD Activity Based Upon Potency Ratios to Standard Preparations. Activity is expressed as Units/mg protein as defined by the commercially prepared standard.

Tissue	Extract:Standard Potency Ratio (95% Conf. Limits)	Percent Cu-Zn SOD	Cu-Zn SOD Activity	Total Activity
Retina	1.08 (0.81 - 1.45)	46.58	3.59	7.71
	5.13 (5.02 - 6.59)*	38.01	8.49	22.34
	2.24 (2.09 - 2.45)*	30.19	6.30	20.86
	1.26 (1.12 - 1.44)	33.05	4.00	12.10
Photoreceptor Outer Segments	0.33 (0.24 - 0.39)	48.86	7.12	14.58
	1.37 (1.21 - 1.58)	23.58	7.41	31.44
	1.20 (1.13 - 1.28)	25.93	7.23	27.88
Retinal Pigment Epithelium	0.51 (0.41 - 0.61)	13.41	4.00	29.80
	0.35 (0.30 - 0.40)**	6.11	0.68	11.07
	0.36 (0.30 - 0.44)	54.94	5.94	10.80
Perfused Choroid	0.14 (0.06 - 0.12)	79.65	3.20	4.01
	0.68 (0.59 - 0.78)	61.06	4.87	7.97
	0.64 (0.56 - 0.74)	53.96	3.24	6.00
Non-perfused Choroid***	0.74 (0.65 - 0.82)	69.77	3.42	4.90
	0.59 (0.48 - 0.72)	78.89	2.63	3.33
	0.64 (0.54 - 0.76)	62.06	3.05	4.92
Blood	0.18 (0.12 - 0.20)	93.41	1.17	1.26
	0.11 (0.08 - 0.14)	55.85	0.15	0.27
	0.23 (0.16 - 0.32)	10.11	0.07	0.68

*Duplicate Assay determinations on the same sample.
**Probable sample contamination with bovine serum albumin.
***Erythrocyte SOD activity not deleted.

DISCUSSION

The assay approach developed herein has the advantage over previously published approaches in that it incorporates into the design, attempts at the detection of the presence of substances which are capable of modifying the behavior of the assay reaction system. When only the curve for the standard is plotted, or when extracts are simply randomly diluted and readjusted until the 50% inhibition level is attained, there is no test for the similarity of the curve for the unknown. In assays, it is assumed that the unknown preparation differs from the standard only by a dilution factor. This assumption has been tested quantitatively in the present study by a parallel-line analysis of variance approach. An estimate of the inherent precision of the assay is often considered as important as that of the observed potency, and precision in this assay may be assessed in terms of the confidence limits as computed from the variation in response within a single assay.

In a model this approach vividly demonstrated the presence of an ascorbate contaminant. When applied to crude extracts of retinal and choroidal tissues of the rainbow trout, all tissues but the neural retinal tissues were seen to possess dose-response curves which departed significantly in shape and/or slope from that of the

standard. Thus, the concern over the presence of interfering substances appears to have been well-warranted.

In crude tissue extracts, although this approach will detect interferences, it will not shed any light on the nature of the source of interference. Certain substances in some tissue extracts may be immediately suspect, however. Erroneous activities may arise when anything in the crude extract can: 1) interfere with or enhance the generation of superoxide anions, 2) add or remove electrons from the indicating scavengers, or 3) compete with the indicator and SOD for the superoxide anion. The substances which are found in all cells which can react have been discussed in detail in the literature review.

In blood, it has been widely reported that hemoglobin in high concentrations will interfere with the assay (Bannister et al., 1977). This was found to be the case here also. The exact mechanism of this interference has not been identified, but a very complex series of reactions appears to be involved (Rotilio et al., 1977).

SOD activity has been reported in the vitreous (Crouch et al., 1978). This activity is very probably due to the presence of the acid mucopolysaccharides. The superoxide anion is known to depolymerize hyaluronic acid during inflamation of the joints in certain forms of arthritis (McCord, 1974).

In the photoreceptor outer segments, it is suspected

that upon sonification, the POS disks may be disrupted into micelles or microvessicles which remain in the supernatant after high speed centrifugation. These would be comprised of a high percentage of polyunsaturated fatty acids which may compete effectively with the SOD and acetylated cytochrome c for $O_2^{\overline{\cdot}}$.

Interestingly, the latter study utilized an ascorbate-iron sulfate reaction for the generation of superoxide anions. Similar behavior was seen at high ascorbate levels in the current study. Thus, if precautions are not taken to eliminate ascorbate from the crude extracts by dialysis or similar techniques, ascorbate may interfere by acting either as a competing oxidant, or, in the presence of trace amounts of metal, as an added source of superoxide anion.

The great variability of results seen in the absolute value of the activities is vexing and suggests that the tissue collection, fractionation and washing procedures need further refinement. The greatest variability was seen in the retinal pigment epithelial tissues. This fraction in particular is known to be especially delicate. Glockin and Potts (1962) first demonstrated that the RPE could be physically detached from Bruch's membrane with a camel hair brush without mechanical damage to the individual cells. Other workers have also used this procedure (Heller, 1975; Maraini and Gozzoli, 1975; Berman and Feeney, 1976; Berman et al., 1974; Riley et al., 1978)

accepting that suspensions obtained were composed of intact cells. Saari et al. (1977) found that when examined by light microscopy, cells were found to occur in sheets and to maintain a semblance of morphologic integrity. However, when analyzed by electron microscopy, these same cells clearly showed fragmented plasma membranes along the apical and basal cell borders, ballooned mitochondria, and an absence of cytoplasmic ground substance. These findings were further substantiated when it was observed that 97% of the binding protein for retinol was released into the first crude RPE cell wash in eyes prepared 2 to 3 hours after death. Considerable improvement in RPE preparations was obtained by Saari et al. (1977) when the procedure was started 15 min. after death and 0.32 M sucrose was used to suspend the cells. With these precautions in mind, care was taken in the present study to brush the RPE as soon after enucleation as possible (i.e., within 5 to 10 minutes). Further damage may have occurred during the glass bead wash procedure, however, particularly by trituration as the glass beads were stirred during the transfer and release procedures.

The SOD levels observed within the trout ocular tissues, though inconclusive, are suggestive of the expected antioxidant roles for SOD. The cytosolic form was reasonably constant between the neural retina, POS, and RPE. The cyanide-insensitive form was higher in the RPE

and lower in the neural retina. The RPE faces the highest oxygen tensions in the trout retina, and one would expect higher levels of inducible mitochondrial activity. There are no mitochondria in the POS, so it is suspected that the cyanide-insensitive activity was due to an interfering substance which was detectable in the ANOVA. The nature of this substance could not be determined in the present study, but as mentioned above, it is suspected that either contamination by RPE cells or by PUFA added to the supernatant as microvesicles or micelles during sonication might be responsible. If the latter proves to be the case in further investigations, one should reassess the widely quoted analysis of Hall and Hall (1975) stating that very high levels of SOD exist in the outer segments of bovine ocular tissues.

RECOMMENDATIONS

The assay as applied is very revealing and the basic approach should be preserved in future work. It should also be extended to other assay procedures for SOD which have linear log dose-probit response relationships. To establish this approach with any new tissue extract, two or three assays with 8 or 9 dosages evenly spaced over a wide range along a log dose scale should be performed such that the shape of the log dose-response curve may be ascertained. If a central segment of the curve is discovered which parallels the standard over as wide a range as possible, then three doses may be selected in this range for further parallel line assay procedures.

Several details of assay design may be modified to reduce the time involved in the assay determinations. First, the determinations at each dosage may be done in duplicate or triplicate rather than quadruplicate. This may reduce the precision of the assay, but the reproducibility of the results should also be considered prior to refining the precision. The number of dosages may be reduced as well, as mentioned above. Finally, the internal reference recovery test may be omitted after one or two determinations with the particular tissue to establish that no nonspecific interference exists.

Some investigators have expressed activity in terms

of units per mg DNA rather than protein. Protein levels would be more variable from cell type to cell type than would the DNA. This would also insure that the estimates in the case of tissues containing significant amounts of whole blood would not be underestimates due to the presence of large amounts of blood protein.

In the present study, it is sufficiently evident that the tissue fractionation procedures need refinement. The RPE procedures most particularly need to be reassessed. I am currently in communication with Dr. Elaine Berman on a new technique for the fractionation of RPE samples utilizing ultrafiltration membranes rather than the glass bead purification procedures.

Some of the variability in the results may arise in the long duration of the tissue collection and fractionation procedures (about 24 hours from first dissection to dialysis). If the assay could be performed in 1 ml cuvettes rather than 3 ml cuvettes and the dispensation of smaller volumes be accurately made, less tissue would be needed and more determinations could be made. New techniques should also be pursued to assure the total release of soluble enzymes from the tissues during homogenization as well.

It has been stressed that this assay procedure detects the presence of interfering substances, but that it says nothing of the nature of these interfering substances.

If and when just cause for concern arises as in the current studies, further characterization of the sources of interference needs to be performed prior to assigning quantitative values to the tissue SOD levels. First, species-specific differences in the SOD kinetics as detected by the assay might be checked by performing a parallel-line assay on purified trout SOD vs. the selected BESOD to assure that the purified enzyme log dose-probit response curves will be parallel. Each tissue which yields erroneous results could further be analyzed by starting with the most likely sources of interference (i.e., PUFA, nucleic acids, etc.) and trying to detect oxidation products of the proposed reactions both before and after the assay mixture incubation.

SUMMARY AND CONCLUSIONS

The picture is becoming clear that in very many cases of toxicity, disease, and aging processes, the ultimate reactions which destroy cell function involve free radicals with primary roles for the free radical species of oxygen. It also appears that as long as the cell maintains adequate antioxidant mechanisms, the foremost of which is the superoxide dismutase enzymes, it is able to continue to function.

The special significance for the involvement of free radical reactions in ocular tissues as mediating mechanisms in such phenomena as photodynamic actions, phagocytic processes, and the senescent accumulation of lipofuscin pigments, means that a thorough understanding of the sources and dispositions of oxygen free radicals within ocular tissues is of prime importance.

The study presented herein, has for the first time incorporated a careful statistical analysis into the most widely used assay technique for SOD determination, thereby allowing a sound basis for the assessment of the validity of the assay when applied to crude tissue extracts. The value of the parallel-line assay approach not only lies in its ability to point out the presence of altered reaction mechanisms, but also yields a much better feeling

for the sensitivity and precision of the assay. This approach may also be extended to other commonly used SOD assays, and thus, it has a much wider applicability than to the current assay alone.

Further work needs to be done to quantify the precise levels of activity in the trout ocular tissues. Refinements are needed especially in the tissue fractionation procedures. The results do suggest however that in the photoreceptor outer segments, retinal pigment epithelium and choroidal tissues, cyanide-insensitive activity exists which operates with a different reaction mechanism from that of the standard bovine erythrocyte SOD. The nature of the substances causing these alterations cannot presently be defined. Sulfhydryl containing proteins, polyunsaturated fatty acids, nucleic acids, and acid mucopolysaccharides all compete with SOD and the indicating scavenger for the superoxide anion in crude tissue extracts. Therefore, unless similar tests for reaction mechanism alterations have been made, the published magnitudes of SOD activity must be considered questionable.

LITERATURE CITED

LITERATURE CITED

Allen, J. and D. Hall. 1973. Superoxide Reduction as a Mechanism of Ascorbate-Stimulated Oxygen Uptake by Isolated Chloroplasts. Biochem. Biophys. Res. Commun., 52:856-862.

Allen, R.C., R.L. Stjernholm and R.H. Steele. 1972. Evidence for the Generation of an Electronic Excitation State(s) in Human Polymorphonuclear Leukocytes and Its Participation in Bactericidal Activity. Biochem. Biophys. Res. Communic., 47:679-684.

Allen, R.C., S.J. Yevich, R.W. Orth and R.H. Steele. 1974. The Superoxide Anion and Singlet Molecular Oxygen: Their Role in the Microbicidal Activity of the Polymorphonuclear Leukocyte. Biochem. Biophys. Res. Communic., 60:909-917.

Anderson, R.E., P.M. Lissandrello, M.B. Maude and M.T. Matthes. 1976. Lipids of Bovine Retinal Pigment Epithelium. Exp. Eye Res., 23:149-157.

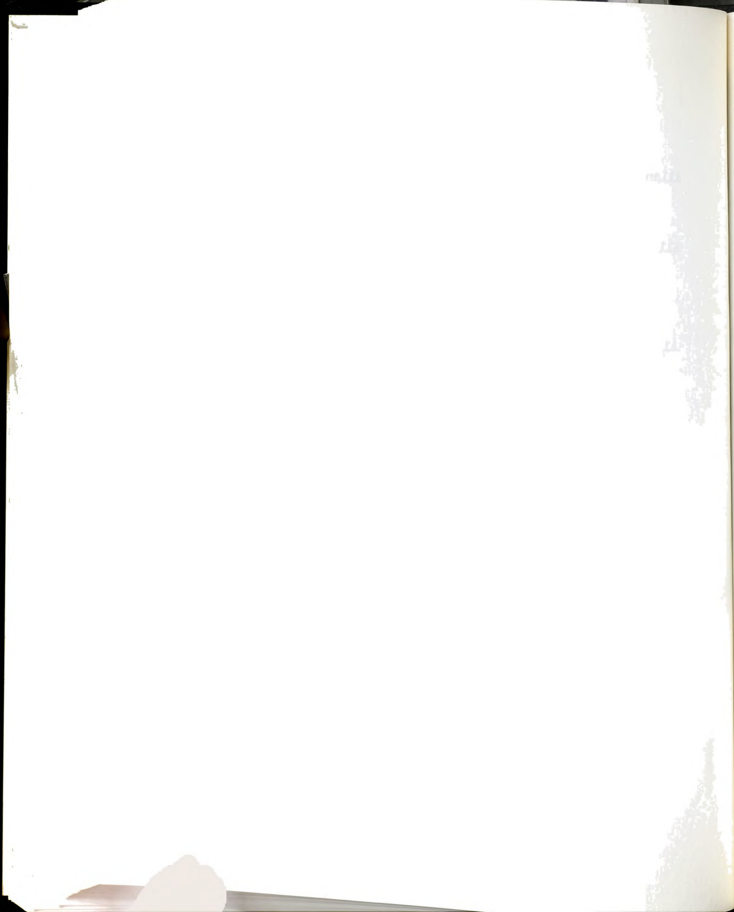
Anderson, R. and M. Maude. 1970. Phospholipids of Bovine Rod Outer Segments. Biochemistry, 9:3624-3628.

Anderson, R.E. and M. Risk. 1974. Lipids of Ocular Tissues. IX. The Phospholipids of Frog Photoreceptor Membranes. Vision Res., 14:129-131.

Armstrong, D., E. Connole, L. Feeney and E.R. Berman. 1978a. Peroxidases in the Neural Retina and Pigment Epithelium. J. Neurochem., 31:761-769.

Armstrong, D., A. Siakotos, N. Koppang, and E. Connole. 1978b. Studies on the Retina and the Pigment Epithelium in Hereditary Canine Ceroid Lipofuscinosis. 1. The Distribution of Enzymes in the Whole Retina and Pigment Epithelium. Invest. Ophthalmol. Visual Sci., 17:608-617.

Armstrong, D., D. Van Wormer, H. Neville, S. Dimmitt and F. Clingan. 1975. Thyroid Peroxidase Deficiency in Batten-Spielmeyer-Vogt Disease. Arch. Pathol., 99:430-435.



Ashton, N., B. Ward and G. Serpell. 1953. Role of Oxygen in the Genesis of Retrolental Fibroplasia. Brit. J. Ophthal., 37:513-520.

Ashton, N., B. Ward and G. Serpell, 1954. Effect of Oxygen on Developing Retinal Vessels with Particular Reference to the Problem of Retrolental Fibroplasia. Br. J. Ophthal., 38:397-432.

Azzi, A., C. Montecucco and C. Richter. 1975. The Use of Acetylated Ferricytochrome c for the Detection of Superoxide Radicals Produced in Biological Membranes. Biochem. Biophys. Res. Commun., 65: 597-603.

Babior, B.M., R.S. Kipnes and J.T. Curnutte. 1973. Biological Defense Mechanisms. The Production by Leukocytes of Superoxide, a Potential Bactericidal Agent. J. Clin. Invest., 52:741-744.

Baehner, R.L., S.K. Murrmann, J. Davis and R.B. Johnston 1975. The Role of Superoxide Anion and Hydrogen Peroxide in Phagocytosis-Associated Oxidative Metabolic Reactions. J. Clin. Invest., 56: 571-576.

Baeyens, D.A. and M.J. Meier. 1978. Effects of Hyperbaric Oxygen and Glutathione on Mammalian Liver Metabolism. Aviat. Space Environ. Med., 49:980-983.

Bannister, W.H., A. Anastasi and J.V. Bannister. 1977. "Human Erythrocyte Superoxide Dismutase (Erythrocuprein)," in <u>Superoxide and Superoxide Dismutases.</u> Edited by A.M. Michelson, J.M. McCord and I. Fridovich. pp. 107-128. Academic Press, New York.

Barber, A.A. and F. Bernheim. 1967. "Lipid Peroxidation: Its Measurement, Occurrence, and Significance in Animal Tissues," in <u>Advances in Gerontological Research</u>, Vol. 2. Edited by B.L. Strehler. pp. 355-403. Academic Press, New York.

Bartkowiak, A., W. Leyko and R. Fried. 1979. A Comparative Characterization of Cytosolic Superoxide Dismutase/(SOD) from Hog Liver and Erythrocytes. Comp. Biochem. Physiol., 62B:61-66.

Basinger, S., R. Hoffman and M. Matthes. 1976. Photoreceptor Shedding is Initiated by Light in the Frog Retina. Science, 194:1076.

Beauchamp, C. and I. Fridovich. 1971. Superoxide Dismutase: Improved Assays and an Assay Applicable to Acrylamide Gels. Anal. Biochem., 44: 276-287.

Benbough, J.E. 1969. Factors Affecting the Toxicity of Oxygen Towards Airborne Coliform Bacteria. J. Gen. Microbiol., 56:241-250.

Berman, E.R. 1964. The Biosynthesis of Mucopolysaccharides and Glycoproteins in Pigment Epithelial Cells of Bovine Retina. Biochim. Biophys. Acta, 83:371-373.

Berman, E.R. 1971. Acid Hydrolases of the Retinal Pigment Epithelium. Invest. Ophthalmol., 10:64-68.

Berman, E.R. and G. Bach. 1968. The Acid Mucopolysaccharides of Cattle Retina. Biochem. J., 108:75-88.

Berman, E.R. and L. Feeney. 1976. Clean Start for the Retinal Pigment Epithelium. Invest. Ophthalmol., 15:238-240.

Berman, E.R., H. Schwell and L. Feeney. 1974. The Retinal Pigment Epithelium. Chemical Composition and Structure. Invest. Ophthalmol., 13:675-687.

Bieri, J.G., H. Dam, I. Prange and E. Sondergaard. 1961. Effect of Dietary Selenium Dioxide, Cystine, Ethoraquin and Vitamin E on Lipid Autoxidation in Chick Tissues. Acta Physiol. Scand., 52:36-43.

Blasie, J.K. 1972. Net Electric Charge on Photo-Pigment Molecules and Frog Retinal Receptor Disk Membrane Structure. Biophys. J., 12: 205-213.

Bliss, C.I. 1952. <u>The Statistics of Bioassay with Special Reference to the Vitamins</u>. Academic Press, New York, 181p.

Block, E.R. 1977. Effect of Superoxide Dismutase and Succinate on the Development of Hyperbaric Oxygen Toxicity. Aviat. Space Environ. Med., 48: 645-648.

Bohnenkamp, W. and U. Weser. 1975. Superoxide Dismutase Micro Assay in Biological Material. Hoppe-Seyler's Z. Physiol. Chem., 356:747-754.

Boveris, A., N. Oshino and B. Chance. 1972. Cellular Production of Hydrogen Peroxide. Biochem. J., 128:617-630.

Boyer, P.D., B. Chance, L. Ernster, P. Mitchell, E. Racker and E.C. Slater. 1977. Oxidative Phosphorylation and Photophosphorylation. Ann. Rev. Biochem., 46:955-1026.

Brewer, J.M., A.J. Pesce and R.B. Ashworth. 1974. Experimental Techniques in Biochemistry. Prentice-Hall, Inc., New Jersey, 374p.

Bridges, W.Z. 1966. Electroretinographic Manifestations of Hyperbaric Oxygen. Arch. Ophthal., 75:812-817.

Buchanan, A.G. 1977. The Response of Azotobacter chroococcum to Oxygen: Superoxide-Mediated Effects. Can. J. Microbiol., 23:1548-1553.

Buchanan, A.G. and H. Lees. 1976. The Effects of pH and Temperature on the Assay of Superoxide Dismutase. Can. J. Microbiol., 22:1643-1646.

Bus, J.S. 1975. Paraquate Perinatal Toxicity and a Proposed Mechanism of Action Involving Lipid Peroxidation. Ph.D. Thesis, Michigan State University, East Lansing, Michigan.

Carrico, R.J. and H.F. Deutsch. 1969. Isolation of Human Hepatocuprien and Cerebrocuprein: Their Identity with Erythrocuprein. J. Biol. Chem., 244:6087-6093.

Castagna, M. 1965. Blood Content of Rat Liver Homogenates. Nature, 205:905-907.

Chance, B., D. Jamieson and H. Coles. 1965. Energy-Linked Pyridine Nucleotide Reduction: Inhibitory Effects of Hyperbaric Oxygen in vitro and in vivo. Nature (London), 206:257-263.

Chance, B. and J.S. Leigh, Jr. 1977. Oxygen Intermediates and Mixed Valence States of Cytochrome Oxidase: Infrared Absorption Difference Spectra of Compounds A, B, and C of Cytochrome Oxidase and Oxygen. Proc. Natl. Acad. Sci., U.S.A., 74:4777-4780.

Chatterjee, I.B. and R.W. McKee. 1965. Lipid Peroxidation and Biosynthesis of L-Ascorbic Acid in Rat Liver Microsomes. Archs. Biochem. Biophys., 110: 254-261.

Chessin, M., R. Livingston and T.G. Truscott. 1966. Direct Evidence for the Sensitized Formation of a Metastable State of β-Carotene. Trans. Faraday Soc., 62:1519-1524.

Chio, K.S. and A.L. Tappel. 1969. Inactivation of Ribonuclease and Other Enzymes by Peroxidizing Lipids and by Malonaldehyde. Biochem., 8:2827-2832.

Chow, C.K. and A.L. Tappel. 1972. An Enzymatic Protective Mechanism Against Lipid Peroxidation Damage to Lung of Ozone-Exposed Rats. Lipids, 7:518-524.

Chow, C.K. and A.L. Tappel. 1974. Response of Glutathione Peroxidase to Dietary Selenium in Rats. J. Nutr. 104:444-451.

Chow, C.K., C.J. Dillard and A.L. Tappel. 1974. Glutathione Peroxidase System and Lysozyme in Rats Exposed to Ozone or Nitrogen Dioxide. Environ. Res., 7:311-319.

Christner, J.E., S. Nand and N.S. Mhatre. 1970. The Reversible Inhibition of β-Glucuronidase by Organic Peroxidase. Biochem. Biophys. Res. Commun., 38:1098-1104.

Christopherson, B.O. 1969. Reduction of Linolenic Acid Hydroperoxide by a Glutathione Peroxidase. Biochim. Biophys. Acta, 176:436-470.

Cohen, G. 1977. "In Defense of Haber-Weiss," in <u>Superoxide and Superoxide Dismutases</u>. Edited by A.M. Michelson, J.M. McCord and I. Fridovich. pp. 317-321. Academic Press, New York.

Cohen, G. and P. Hochstein. 1963. Glutathione Peroxidase: The Primary Agent for the Elimination of Hydrogen Peroxide in Erythrocytes. Biochem., 2:1420-1428.

Cohen, S.S. 1973. Mitochondria and Chloroplasts Revisited. American Scientist, 61:437-445.

Colowick, S., A. Lazarow, E. Racker, D.R. Schwarz, E. Stadtman and H. Waelsch. 1954. Glutathione, A Symposium. Academic Press, New York, 341p.

Cope, F.W., R.J. Sever and B.D. Polis. 1963. Reversible Free Radical Generation in the Melanin Granules of the Eye by Visible Light. Archives Biochem. Biophys., 100:171-177.

Crapo, J.D. 1975. Superoxide Dismutase and Tolerance to Pulmonary Oxygen Toxicity. Chest, Suppl., 67:39S-40S.

Crapo, J. and D. Tierney. 1973. Superoxide Dismutase and Oxygen Toxicity. Clin. Res., 21:222.

Crapo, J.D. and D.F. Tierney. 1974. Superoxide Dismutase and Pulmonary Oxygen Toxicity. Amer. J. Physiol., 226:1401-1407.

Cremona, T. and E.B. Kearney. 1964. Studies on the Respiratory Chain-Linked Reduced Nicotinamide Adenine Dinucleotide Dehydrogenases. VI. Further Purification and Properties of the Enzyme from Beef Heart. J. Biol. Chem., 239:2328-2334.

Cross, K.W. 1973. Cost of Preventing Retrolental Fibroplasia. Lancet, Vol. II for 1973, No. 7835:954-956.

Crouch, R., D.G. Priest and E.J. Duke. 1978. Superoxide Dismutase Activities of Bovine Ocular Tissues. Exp. Eye Res., 27:503-509.

Curnutte, J.T., R.S. Kipnes and B.M. Babior. 1975. Defect in Pyridine Nucleotide Dependent Superoxide Production by a Particulate Fraction from the Granulocytes of Patients with Chronic Granulomatous Disease. New Engl. J. Med., 293:628-632.

Daemen, F.J.M. 1973. Vertebrate Rod Outer Segment Membranes. Biochem. Biophys. Acta, 300:255-288.

DeChatelet, L.R., L.C. McPhail, D. Mullikin and C.E. McCall. 1975. An Isotopic Assay for NADPH Oxidase Activity and Some Characteristics of the Enzyme from Human Polymorphonuclear Leukocytes. J. Clin. Invest., 55:714-721.

Demopoulos, H.B. 1973a. The Basis of Free Radical Pathology. Fed. Proc., 32:1859-1861.

Demopoulos, H.B. 1973b. Control of Free Radicals in Biologic Systems. Fed. Proc., 32:1903-1908.

Desai, I.D. and A.L. Tappel. 1963. Damage to Proteins by Peroxidized Lipids. J. Lipid Res., 4:204-207.

Dowling, J.E. 1960. Chemistry of Visual Adaptation in the Rat. Nature, 188:114-118.

Dowty, B., J.L. Laseter, G.W. Griffin, I.R. Politzer and C.H. Walkinshaw. 1973. Reactions of Singlet Oxygen with Pine Pollen. Science, 181:669-670.

Edelstein, L.M. 1971. Melanin: A Unique Biopolymer. Pathobiol. Annual, 1:309-324.

Epel, B. and J. Neuman. 1973. The Mechanism of the Oxidation of Ascorbate and Mn^{++} by Chloroplasts: The Role of the Radical Superoxide. Biochim. Biophys. Acta, 325:520-529.

Fairbanks, M.B., J.R. Hoffert and P.O. Fromm. 1969. The Dependence of the Oxygen-Concentrating Mechanism of the Teleost Eye (Salmo gairdneri) on the Enzyme Carbonic Anhydrase. J. Gen. Physiol., 54:202-211.

Farnsworth, C.C. and E.A. Dratz. 1976. Oxidative Damage of Retinal Rod Outer Segment Membranes and the Role of Vitamin E. Biochim. Biophys. Acta, 443:556-570.

Fee, J.A. and J.S. Valentine. 1977. "Chemical and Physical Properties of Superoxide," in Superoxide and Superoxide Dismutases. Edited by A.M. Michelson, J.M. McCord and I. Fridovich. pp. 19-60. Academic Press, New York.

Feeney, L. 1978. Lipofuscin and Melanin of Human Retinal Pigment Epithelium. Fluorescence, Enzyme Cytochemical, and Ultrastructural Studies. Invest. Ophthalmol. Vis. Sci., 17:583-600.

Feeney, L. and E.R. Berman. 1976. Oxygen Toxicity: Membrane Damage by Free Radicals. Invest. Ophthalmol., 15:789-792.

Feeney, L., J.A. Grieshaber and M.J. Hogan. 1965. "Studies on Human Ocular Pigment," in The Structure of the Eye, Second Symposium. Edited by J.W. Rohen. pp. 535-548. Schattauer-Verlag, Stuttgart.

Finney, D.J. 1952. Statistical Method in Biological Assay. Hafner Publishing Co., New York, 661p.

Flohe, L., G. Loschen, A. Azzi and C. Richter. 1977. "Superoxide Radicals in Mitochondria," in Superoxide and Superoxide Dismutases. Edited by A.M. Michelson, J.M. McCord and I. Fridovich. pp. 323-334. Academic Press, New York.

Flohe, L., W.A. Gunzler and H.H. Schock. 1973. Glutathione Peroxidase: A Selenoenzyme. FEBS Letters, 32:132-133.

Fong, K., P.B. McCay and J.L. Poyer. 1973. Evidence that Peroxidation of Lysosomal Membranes is Initiated by Hydroxyl Free Radicals Produced during Flavin Enzyme Activity. J. Biol. Chem., 248:7792-7797.

Foote, C.S. 1968. Mechanisms of Photosensitized Oxidation. Science, 162:963-970.

Foote, C.S. and R.W. Denny. 1968. Chemistry of Singlet Oxygen. VII. Quenching by β-Carotene. J. Amer. Chem. Soc., 90:6233-6235.

Frank, A.L. and A.K. Christensen. 1968. Localization of Acid Phosphatase in Lipofuscin Granules and Possible Autophagic Vacuoles in Interstitial Cells of the Guinea Pig Testis. J. Cell Biol., 36:1-13.

Fridovich, I. 1972. Superoxide Radical and Superoxide Dismutase. Accounts Chem. Res., 5:321-326.

Fridovich, I. 1974a. "Superoxide Dismutase," in Molecular Mechanisms of Oxygen Activation. Edited by O. Hayaishi. pp. 453-477. Academic Press, New York.

Fridovich, I. 1974b. Superoxide Dismutases. Advan. Enzymol., 41:35-97.

Fridovich, I. 1975a. Superoxide Dismutases. Ann. Rev. Biochem., 44:147-159.

Fridovich, I. 1975b. Oxygen: Boon and Bane. Am. Scientist, 63:54-59.

Fridovich, I. 1976. "Oxygen Radicals, Hydrogen Peroxide, and Oxygen Toxicity," in _Free Radicals in Biology, Vol. I_. Edited by W.A. Pryor. pp. 239-277. Academic Press, New York.

Fridovich, I. 1977a. Oxygen is Toxic. BioScience, 27: 462-466.

Fridovich, I. 1977b. "Biological Aspects of Superoxide Radical and Superoxide Dismutases," in _Biochemical and Medical Aspects of Active Oxygen_. Edited by O. Hayaishi and K. Asada. pp. 171-181. University Park Press, Baltimore.

Fridovich, I. 1977c. "Chemical Aspects of Superoxide Radical and of Superoxide Dismutases," in _Biochemical and Medical Aspects of Active Oxygen_. Edited by O. Hayaishi and K. Asada. pp. 3-12. University Park Press, Baltimore.

Fridovich, I. 1978. The Biology of Oxygen Radicals. Science, 201:875-880.

Fridovich, I. and P. Handler. 1961. Detection of Free Radicals Generated during Enzymic Oxidations by the Initiation of Sulfite Oxidation. J. Biol. Chem., 236:1836-1840.

Fried, R. 1975. Biological Role of Xanthine Oxidase and Tetrazolium-Reductase Inhibitor. Biochimie, 57: 657-660.

Fried, R. and P. Mandel. 1975. Superoxide Dismutase of Mammalian Nervous System. J. Neurochem., 24: 433-438.

Fujimori, E. and R. Livingston. 1957. Interactions of Chlorophyll in its Triplet State with Oxygen, Carotene, Etc. Nature, 180:1036-1038.

Fujishita, S. 1962. The Organo-Iron Compound in the Digitonin Extract of the Rod Outer Segment. Japan J. Physiol., 12:460-466.

Gan, E.V., H.F. Haberman and I.A. Menon. 1974. Oxidation of NADH by Melanin and Melanoproteins. Biochim. Biophys. Acta, 370:62-69.

Gan, E.V., H.F. Haberman and I.A. Menon. 1976. Electron Transfer Properties of Melanin. Archs. Biochem. Biophys., 173:666-672.

Gershman, R., D.L. Gilbert, S.W. Nye, P. Dwyer and W.O. Fenn. 1954. Oxygen Poisoning and X-irradiation: A Mechanism in Common. Science, 119:623-626.

Glavind, J. and M. Faber. 1967. Antioxidants and Lipoperoxides in the Organs of Irradiated Rats. III. Importance of the Dose-Rate. Int. J. Rad. Biol., 12:121-126.

Glockin, V.C. and A.M. Potts. 1962. The Metabolism of Retinal Pigment Cell Epithelium. I. The _in vitro_ Incorporation of P-32 and the Effect of Diaminodiphenoxyalkane. Invest. Ophthalmol., 1: 111-117.

Gollnick, K. 1968. "Type II Photooxygenation Reactions in Solution," in _Advances in Photochemistry, Vol_. 6. Edited by W.A. Noyes, Jr., G.S. Hammond and J.N. Pitts, Jr. pp. 1-122. Wiley, New York.

Grady, F.J. and D.C. Borg. 1968. Light-Induced Free Radicals of Retinal, Retinol and Rhodopsin. Biochem., 7:675-682.

Gregory, E.M. and I. Fridovich. 1973a. The Induction of Superoxide Dismutase by Molecular Oxygen. J. Bacteriol., 114:5430548.

Gregory, E.M. and I. Fridovich. 1973b. Oxygen Toxicity and the Superoxide Dismutases. J. Bacteriol., 114:1193-1197.

Gregory, E.M., S.A. Goscin and I. Fridovich. 1974. Superoxide Dismutase and Oxygen Toxicity in a Eukaryote. J. Bacteriol., 117:456-460.

Gutman, M., T.P. Singer and H. Beinert. 1972. Relation of the Respiratory Chain-Linked Reduced Nicotinamide Adenine Dinucleotide Dehydrogenase to Energy-Coupling Site 1. Biochemistry, 11:556-562.

Haber, F. and J. Weiss. 1934. The Catalytic Decomposition of Hydrogen Peroxide by Iron Salts. Proc. Roy. Soc., A147:332-351.

Hafeman, D.G., R.A. Sunde and W.G. Hoekstra. 1974. Effect of Dietary Selenium on Erythrocyte and Liver Glutathione Peroxidase in the Rat. J. Nutr., 104: 580-587.

Hall, M.O. and D.O. Hall. 1975. Superoxide Dismutase of Bovine and Frog Outer Segments. Biochem. Biophys. Res. Commun., 67:1199-1204.

Harman, D. 1968. Free Radical Theory of Aging: Effect of Free Radical Inhibition on the Life Span of Male LAF-Mice---Second Experiment. Gerontologist, 8(Suppl.):13.

Harris, J.I. and H.M. Steinman. 1977. "Amino Acid Sequence Homologies Among Superoxide Dismutases," in Superoxide and Superoxide Dismutases. Edited by A.M. Michelson, J.M. McCord and I. Fridovich. pp. 225-230. Academic Press, New York.

Hatefi, Y. 1963. "The Pyridine Nucleotide-Cytochrome c Reductases," in The Enzymes, second edition, Vol. 7. Edicted by P.D. Boyer, H. Lardy and K. Myrback. pp. 495-515. Academic Press, New York.

Haugaard, N. 1968. Cellular Mechanisms of Oxygen Toxicity. Physiol. Rev., 48:311-373.

Hayasaka, S., S. Hara, Y. Takaku and K. Mizuno. 1977. Distribution of Acid Lipase in the Bovine Retinal Pigment Epithelium. Exp. Eye Res., 24:1-6.

Hayes, K.C. 1974. Retinal Degeneration in Monkeys Induced by Deficiencies of Vitamin E or A. Invest. Ophthalmol., 13:499-510.

Heath, H. 1962. The Distribution and Possible Function of Ascorbic Acid in the Eye. Exp. Eye Res., 1: 326-367.

Heath, H., T.C. Beck, A.C. Rutter and D.P. Greaves. 1961. Biochemical Changes in Aphakia. Vision Res., 1: 274-280.

Heath, H. and R. Fiddick. 1966. The Active Transport of Ascorbic Acid by the Rat Retina. Exp. Eye Res., 5:156-163.

Heaton, F.W. and N. Uri. 1961. The Aerobic Oxidation of Unsaturated Fatty Acids and Their Esters: Cobalt Stearate-catalyzed Oxidation of Linoleic Acid. J. Lipid Res., 2:152-160.

Heller, J. 1975. Interactions of Plasma Retinol-Binding Protein with Its Receptor. Specific Binding of Bovine and Human Retinol-Binding Protein to Pigment Epithelium Cells from Bovine Eyes. J. Biol. Chem., 250:3613-3619.

Hinkle, P.C., R.A. Butow, E. Racker and B. Chance. 1967. Partial Resolution of the Enzymes Catalyzing Oxidative Phosphorylation XV. Reverse Electron Transfer in the Flavin-Cytochrome b Region of the Respiratory Chain of Beef Heart Submitochondrial Particles. J. Biol. Chem., 242:5169-5173.

Hirs, C.H.W. 1967. Detection of Peptides by Chemical Methods. Methods in Enzymol., 11:325-329.

Hochstein, P. and L. Ernster. 1963. ADP-Activated Lipid Peroxidation Coupled to the TPNH Oxidase System of Microsomes. Biochem. Biophys. Res. Communic., 12:388-394.

Hoffert, J.R., G.E. Eldred and P.O. Fromm. 1974. Comparison of Direct and Indirect Calorimetry as a Method for Determining Total Energy Produced by the Isolated Retina of Rainbow Trout (Salmo gairdneri). Exp. Eye Res., 19:359-365.

Hogan, M.J. 1972. Role of the Retinal Pigment Epithelium in Macular Disease. Trans. Am. Acad. Ophthalmol. Otolaryngol., 76:64-80.

Hogan, M.J., J.A. Alvarado and J.E. Weddell. 1971. Histology of the Human Eye: An Atlas and Textbook. W.B. Saunders Co., Philadelphia, 687p.

Hohn, D.C. and R.I. Lehrer. 1975. NADPH-Oxidase Deficiency in X-Linked Chronic Granulomatous Disease. J. Clin. Invest., 55:707-713.

Hohorst, H., F. Kreutz and T. Bucher. 1959. Uber Metabolitgehalte und Metabolit-Konzentrationen in der Leber der Ratte. Biochem. Zeitschrift., 332:18-46.

Hollyfield, J.G. and A. Ward. 1974. Phagocytic Activity in the Retinal Pigment Epithelium of the Frog Rana pipiens. I. Uptake of Polystyrene Spheres. J. Ultrastr. Res., 46:327-338.

Holman, R.T. 1954. "Autoxidation of Fats and Related Substances," in Progress in the Chemistry of Fats and Other Lipids, Vol. 2. Edited by R.T. Holman, W.O. Lundberg and T. Malbin. pp. 51-98. Pergamon Press, London.

Horgan, V.J., J. Philpot, B.W. Porter and D.B. Roodyn. 1957. Toxicity of Autoxidized Squalene and Linoleic Acid, and of Simpler Peroxides, in Relation to Toxicity of Radiation. Biochem. J., 67:551-558.

Howes, R.M. and R.H. Steele. 1971. Microsomal Chemiluminescence Induced by NADPH and its Relation to Lipid Peroxidation. Res. Communic. Chem. Path. Pharmacol., 2:619-626.

Isherwood, F.A. 1959. "Chemistry and Biochemistry of Glutathione," in Glutathione. Edited by E.M. Crook. pp. 3-16. Cambridge Univ. Press, Cambridge, England.

Jamieson, D. and H.A.S. Van den Brenk. 1964. The Effects of Antioxidants on High Pressure Oxygen Toxicity. Biochem. Pharmacol., 13:159-164.

Johnston, R.B., Jr., B.B. Keele, H.P. Misra, J.E. Lehmeyer, L.S. Webb, R.L. Baehner and K.V. Rajagopalan. 1975. The Role of Superoxide Anion Generation in Phagocytic Bactericidal Activity: Studies with Normal and Chronic Granulomatous Disease Leukocytes. J. Clin. Invest., 55:1357-1372.

Johnston, R.B., Jr., B. Keele, L. Webb, D. Kessler and K.V. Rajagopalan. 1973. Inhibition of Phagocytic Bactericidal Activity by Superoxide Dismutase: A Possible Role for Superoxide Anion in the Killing of Phagocytized Bacteria. J. Clin. Invest., 52:44a.

Johnston, R.B. and J.E. Lehmeyer. 1977. "The Involvement of Oxygen Metabolites from Phagocytic Cells in Bactericidal Activity and Inflammation," in Superoxide and Superoxide Dismutases. Edited by A.M. Michelson, J.M. McCord and I. Fridovich. pp. 291-305. Academic Press, New York.

Jolly, D.W., L.E. Mawdesley-Thomas and D. Bucke. 1972. Anaesthesia of Fish. Vet. Rec., 91:424-426.

Kagan, V., A. Shvedova, K. Novikov and Y. Kozlov. 1973. Light-Induced Free Radical Oxidation of Membrane Lipids in Photoreceptors of Frog Retina. Biochim. Biophys. Acta, 330:76-79.

Kagan, V.Ye., A.A. Shvedova, K.N. Novikov and Yu.P. Kozlov. 1975. Spontaneous and Induced Auto-oxidation of Phospholipids During Conformational Rearrangements of the Membranes of the External Segments of the Frog Retinal Rods. Biofizika, 20:1043-1048.

Kedziora, J., J. Jeske, H. Witas, G. Bartosz and W. Leyko. 1977. The Level of Superoxide Dismutase in Erythrocytes of Children with Down Syndrome (Trisomy G and Unbalanced Translocation G 21/22). Acta Biol. Med. Germ., 36:779-782.

Kellogg, E.W.,III and I. Fridovich. 1975. Superoxide, Hydrogen Peroxide, and Singlet Oxygen in Lipid Peroxidation by a Xanthine Oxidase System. J. Biol. Chem., 250:8812-8817.

Khan, A.U. 1970. Singlet Molecular Oxygen from Superoxide Anion and Sensitized Fluorescence of Organic Molecules. Science, 168:476-477.

Klebanoff, S.J. 1975. Antimicorbial Mechanisms in Neutrophilic Polymorphonuclear Leukocytes. Semin. Hematol., 12: 117-142.

Knowles, A. and H.J.A. Dartnall. 1977. "The Functioning of Visual Pigments in the Receptor," in The Eye. Vol. 2B. The Photobiology of Vision. Edited by H. Davson. pp. 425-497. Academic Press, New York.

Koppang, N. 1973/74. Canine Ceroid-Lipofuscinosis---A Model for Human Neuronal Ceroid-Lipofuscinosis and Aging. Mech. Ageing Develop., 2:421-445.

Korenbrot, J.I. 1977. Ion Transport in Membranes: Incorporation of Biological Ion-Translocating Proteins in Model Membrane Systems. Ann. Rev. Physiol., 39:19-49.

Kovacs, K. and B. Matkovics. 1975. Properties of Enzymes. IV. Non-Specific Superoxide Dismutase Activity of Iron-Porphyrin Systems. Enzyme, 20:1-5.

Kozlov, Yu.P., V.B. Ritov and V.E. Kagan. 1972. Transport of Ca^{++} and Free Radical Oxidation of Lipids of the Membranes of the Sarcoplasmic Reticulum. Proc. Acad. Sci. USSR., Biochem., 212:499-502.

Krinsky, N.I. 1958. The Enzymatic Esterification of Vitamin A. J. Biol. Chem., 232:881-894.

Krinsky, N.I. 1971. "Function," in Carotenoids. Edited by O. Isler. pp. 669-715. Halsted, New York.

Krinsky, N.I. 1974a. Singlet Excited Oxygen as a Mediator of the Antibacterial Action of Leukocytes. Science, 186:363-365.

Krinsky, N.I. 1974b. Carotenoid Chromophore Length and Protection Against Photosensitization. Photochem. Photobiol., 19:217-222.

Kritchevsky, D. and R.J. Van der Wal. 1960. Letter to the Editor. Chem. Soc., London, Proc., May, 1960:173.

Kuwabara, T. and R.A. Gorn. 1968. Retinal Damage by Visible Light. An Electron Microscopic Study. Arch. Ophthalmol., 79:69-78.

Land, E.F. and A.J. Swallow. 1971. One-Electron Reactions in Biochemical Systems as Studied by Pulse Radiolysis. IV. Oxidation of Dihydronicotinamide-Adenine Dinucleotide. Biochim. Biophys. Acta, 234:34-45.

LaVail, M.M. 1976. Rod Outer Segment Disc Shedding in Rat Retina: Relationship to Cyclic Lighting. Science, 194:1071-1074.

Lehninger, A.L. 1975. Biochemistry. Second Edition. Worth Publishers, Inc., New York, 1104p.

Livingston, R. and K.E. Owens. 1956. A Diffusion-Controlled Step in Chlorophyll-Sensitized Photochemical Autoxidations. J. Amer. Chem. Soc., 78:3301-3305.

Longuet-Higgins, H.C. 1960. On the Origin of the Free Radical Property of Melanins. Archs. Biochem. Biophys., 86:231-232.

Loomis, T.A., J.E. Jesseph and R.E. Beyer. 1952. Prolongation of the Anticoagulant Activity of Heparin with Procaine and Butacaine Conjugates. J. Pharmac. Exp. Ther., 104:87-92.

Loschen, G., A. Azzi and L. Flohe. 1973a. Mitochondrial H_2O_2 Formation at Site II. Hoppe-Seyler's Z. Physiol. Chem., 354:791-794.

Loschen, G., A. Azzi and L. Flohe. 1973b. Mitochondrial H_2O_2 Formation: Relationship with Energy Conservation. FEBS Letters, 33:84-88.

Loschen, G., A. Azzi, C. Richter and L. Flohe. 1974. Superoxide Radicals as Precursors of Mitochondrial Hydrogen Peroxide. FEBS Letters, 42:68-72.

Lumsden, J., L. Henry and D.O. Hall. 1977. "Superoxide Dismutase in Photosynthetic Organisms," in Superoxide and Superoxide Dismutases. Edited by A.M. Michelson, J.M. McCord and I. Fridovich. pp. 437-450. Academic Press, New York.

Malmstrom, B.G., L.E. Andreasson and B. Reinhammer. 1975. "Copper-Containing Oxidases and Superoxide Dismutase," in The Enzymes: Oxidation-Reduction, Vol. XII(B). Edited by P.D. Boyer. pp. 507-579. Academic Press, New York.

Mann, T. and D. Keilin. 1938. Haemocuprein and Hepatocuprein, Copper-Protein Compounds of Blood and Liver in Mammals. Proc. Roy. Soc. (London), B126:303-315.

Mapson, L.W. 1959. "Enzyme Systems Associated with the Oxidation and Reduction of Glutathione in Plant Tissues," in Glutathione. Edited by E.M. Crook. pp. 28-42. Cambridge Univ. Press, Cambridge, England.

Maraini, G. and F. Gozzoli. 1975. Binding of Retinol to Isolated Retinal Pigment Epithelium in the Presence and Absence of Retinol-Binding Protein. Invest. Ophthalmol., 14:785-787.

Markossian, K.A. and R.M. Nalbandyan. 1975. Superoxide Dismutase Does Not Inhibit the Oxidation of Cytochrome c and Cytochrome Oxidase. Biochem. Biophys. Res. Commun., 67:870-876.

Markowitz, H., G.E. Cartwright and M.M. Wintrobe. 1959. Studies on Copper Metabolism. XXVII. The Isolation and Properties of an Erythrocyte Cuproprotein (Erythrocuprein). J. Biol. Chem., 234:40-45.

Mason, H.S., D.J.E. Ingram and B. Allen. 1960. The Free Radical Properties of Melanins. Archs. Biochem. Biophys., 86:225-230.

Massey, V. 1959. The Microestimation of Succinate and the Extinction Coefficient of Cytochrome c. Biochim. Biophys. Acta, 34:255-256.

Masterson, E., P. Israel and G.J. Chader. 1978. Pentose Shunt Activity in Developing Chick Retina and Pigment Epithelium: A Switch in Biochemical Expression in Cultured Pigment Epithelial Cells. Exp. Eye Res., 27:409-416.

Matsumura, G., A. Herp and W. Pigman. 1966. Depolymerization of Hyaluronic Acid by Antioxidants and Radiations. Radiat. Res., 28:735-752.

Matthews, M. 1964. Protective Effect of β-Carotene Lethal Photo-Sensitization by Hematoporphyrin. Nature, 203:1092.

Maugh, T.H,,II. 1973. Singlet Oxygen: A Unique Microbicidal Agent in Cells. Science, 182:44-45.

May, H.E. and P.B. McCay. 1968a. Reduced Triphosphopyridine Nucleotide Oxidase-catalyzed Alterations of Membrane Phospholipids. I. Nature of the Lipid Alterations. J. Biol. Chem., 243:2288-2295.

May, H.E. and P.B. McCay. 1968b. Reduced Triphosphopyridine Nucleotide Oxidase-catalyzed Alterations of Membrane Phospholipids. II. Enzymic Properties and Stoichiometry. J. Biol. Chem., 243: 2296-2305.

McCay, P.B., H.E. May, A.E. Kitabchi, R.H. Feinberg, M.P. Carpenter, R.E. Trucco, R. Caputto. 1960. The Activation of Gulonolactone Oxidase by Hydrogen Peroxide and by O_2 at High Pressures. Biochem. Biophys. Res. Communic., 3:441-444.

McCord, J.M. 1974. Free Radicals and Inflammation: Protection of Synovial Fluid by Superoxide Dismutase. Science, 185:529-531.

McCord, J.M., C.O. Beauchamp, S. Goscin, H.P. Misra and I. Fridovich. 1973. "Superoxide and Superoxide Dismutase", in Oxidases and Related Redox Systems. Edited by T.E. King, H.S. Mason and M. Morrison. pp. 51-76. University Park Press, Baltimore.

McCord, J.M., J.A. Boyle, E.D. Day, Jr., L.J. Rizzolo and M.L. Salin. 1977b. "A Manganese-Containing Superoxide Dismutase from Human Liver," in Super oxide and superoxide Dismutases. Edited by A.M. Michelson, J.M. McCord and I. Fridovich. pp. 129-138. Academic Press, New York.

McCord, J.M., J.D. Crapo and I. Fridovich. 1977a. "Superoxide Dismutase Assays: A Review of Methodology," in Superoxide and Superoxide Dismutases. Edited by A.M. Michelson, J.M. McCord and I. Fridovich. pp. 11-17. Academic Press, New York.

McCord, J.M. and I. Fridovich. 1969. Superoxide Dismutase: An Enzymic Function for Erythrocuprein (Hemocuprein). J. Biol. Chem., 244:6049-6055.

McCord, J.M. and I. Fridovich. 1977. "Superoxide Dismutases: A History," in Superoxide and Superoxide Dismutases. Edited by A.M. Michelson, J.M. McCord and I. Fridovich. pp. 1-10. Academic Press, New York.

McCord, J.M., B.B. Keele and I. Fridovich. 1971. An Enzyme-Based Theory of Obligate Anaerobiosis: The Physiological Function of Superoxide Dismutase. Proc. Nat. Acad. Sci., USA, 68:1024-1027.

McGovern, V. and P. Russell. 1973. Mechanisms in Pigmentation. S. Karger AG, Basel. 414p.

Mead, J. 1976. "Free Radical Mechanisms of Lipid Damage and Consequences for Cellular Membranes," in Free Radicals in Biology, Vol. I. Edited by W. Pryor. pp. 51-68. Academic Press, New York.

Michelson, A.M. 1977a. "Chemical Production of Superoxide Anions by Reaction Between Riboflavin, Oxygen, and Reduced Nicotinamide Adenine Dinucleotide. Specificity of Tests for $O_2^{\cdot}$," in Superoxide and Superoxide Dismutases. Edited by A.M. Michelson, J.M. McCord and I Fridovich. pp. 85-106. Academic Press, New York.

Michelson, A.M. 1977b. "Production of Superoxide by Metal Ions," in Superoxide and Superoxide Dismutases. Edited by A.M. Michelson, J.M. McCord and I. Fridovich. pp. 77-86. Academic press, New York.

Michelson, A.M., J.M. McCord and I. Fridovich. 1977. Superoxide and Superoxide Dismutases. Academic Press, New York, 568p.

Mii, S. and D.E. Green. 1954. Studies on the Fatty Acid Oxidizing System of Animal Tissues. VIII. Reconstruction of Fatty Acid Oxidizing System with Triphenyltetrazolium as Electron Acceptor. Biochim. Biophys. Acta, 13:425-432.

Miller, S. and R.H. Steinberg. 1976. Transport of Taurine L-Methionine and 3-o-Methyl-D-glucose Across Frog Retinal Pigment Epithelium. Exp. Eye Res., 23:177-189.

Miller, S.S. and R.H. Steinberg. 1977. Passive Ionic Properties of Frog Retinal Pigment Epithelium. J. Membrane Biol., 36:337-372.

Minakami, S., F.J. Schindler and R.A. Estabrook. 1964. Hydrogen Transfer Between Reduced Diphosphopyridine Nucleotide Dehydrogenase and the Respiratory Chain. I. Effect of Sulfhydryl Inhibitors and Phospholipase. J. Biol. Chem., 239:2042-2054.

Minakami, S., K. Titani and H. Ishikura. 1958. The Structure of Cytochrome c. II. Properties of Acetylated Cytochrome c. J. Biochem., 45:341-348.

Misra, H.P. and I. Fridovich. 1971a. The Generation of Superoxide Radical during the Autoxidation of Ferredoxins. J. Biol. Chem., 246:6886-6890.

Misra, H.P. and I. Fridovich. 1971b. The Univalent Reduction of Oxygen by Reduced Flavins and Quinones. J. Biol. Chem., 247:188-192.

Misra, H.P. and I. Fridovich. 1972a. The Role of Superoxide Anion in the Autoxidation of Epinephrine and a Simple Assay for Superoxide Dismutase. J. Biol. Chem., 247:3170-3175.

Misra, H.P. and I. Fridovich. 1972b. The Purification and Properties of Superoxide Dismutase from Neurospora crassa. J. Biol. Chem., 247:3410-3414.

Misra, H.P. and I. Fridovich. 1977. Superoxide Dismutase: A Photochemical Augmentation Assay. Arch. Biochem. Biophys., 181:308-312.

Mitts, G.C. and H.D. Randall. 1958. Hemoglobin Catabolism. II. The Protection of Hemoglobin from Oxidative Breakdown in the Intact Erythrocyte. J. Biol. Chem., 232:589-598.

Moore, S. 1968. Amino Acid Analysis: Aqueous Dimethyl Sulfoxide as Solvent for the Ninhydrin Reaction. J. Biol. Chem., 243:6281-6283.

Moyer, F.H. 1969. Development, Structure and Function of the Retinal Pigmented Epithelium. U.C.L.A. Forum Med. Sci., 8:1-30.

Murray, R.L. and M.W. Dubin. 1975. The Occurrence of Actin-like Filaments in Association with Migrating Pigment Granules in Frog Retinal Pigment Epithelium. J. Cell Biol., 64:705-710.

Myers, L., Jr. 1973. Free Radical Damage of Nucleic Acids and Their Components by Ionizing Radiation. Fed. Proc., 32:1882-1894.

Nielsen, N.C., S. Fleischer and D.G. McConnell. 1970. Lipid Composition of Bovine Retinal Outer Segment Fragments. Biochim. Biophys. Acta, 211:10-19.

Nishikimi, M. 1975. Oxidation of Ascorbic Acid with Superoxide Anion Generated by the Xanthine-Xanthine Oxidase System. Biochem. Biophys. Res. Communic., 63:463-468.

Nishikimi, M. and K. Yagi. 1977. "Oxidation of Ascorbic Acid and α-Tocopherol by Superoxide," in Biochemical and Medical Aspects of Active Oxygen. Edited by O. Hayaishi and K. Asada. pp. 79-89. University Park Press, Baltimore.

Noell, W.K. 1955. Metabolic Injuries of the Visual Cell. Am. J. Ophthalmol., 40(5, Part II):60-70.

Noell, W.K. 1958. Differentiation, Metabolic Organization, and Viability of the Visual Cell. Arch. Ophthal., 60:702-733.

Noell, W.K. 1962. "Effect of High and Low Oxygen Tensions on the Visual System," in Environmental Effects on Consciousness. Edited by K.E. Schaeffer. pp. 3-18. Macmillan Co., New York.

Noell, W.K., V.S. Walker, B.S. Kang and S. Berman. 1966. Retinal Damage by Light in Rats. Invest. Ophthalmol., 5:450-473.

Noguchi, T., A.H. Cantor and M.L. Scott. 1973. Mode of Action of Selenium and Vitamin E in Prevention of Exudative Diathesis in Chicks. J. Nutr., 103:1502-1511.

Novikov, K.N., V.Ye. Kagan, A.A. Shvedova and Yu.P. Kozlov. 1975. Protein-Lipid Interactions on Peroxide Oxidation of Lipids in Photoreceptor Membrane. Biofizika, 20:1039-1042.

O'Brien, P.J. and C. Little. 1969. Intracellular Mechanisms for the Decomposition of a Lipid Peroxide. II. Decomposition of a Lipid Peroxide by Subcellular Fractions. Can. J. Biochem., 47: 493-499.

Oh, S.H., H.E. Ganther and W.G. Hoekstra. 1974. Selenium as a Component of Glutathione Peroxidase Isolated from Ovine Erythrocytes. Biochem., 13:1825-1829.

Oyama, V.I. and H. Eagle. 1956. Measurement of Cell Growth in Tissue Culture with a Phenol Reagent (Folin-Ciocalteau). Proc. Soc. Expt'l. Biol. Med., 91:305-307.

Panchenko, L.F., O.S. Brusov, A.M. Gerasimov and T.D. Loktaeva. 1975. Intramitochondrial Localization and Release of Rat Liver Superoxide Dismutase. FEBS Letters, 55:84-87.

Patriarca, P., R. Cramer, P. Dri, L. Fant, R.E. Basford and F. Rossi. 1973. NADPH oxidizing Activity in Rabbit Polymorphonuclear Leukocytes: Localization in Azurophilic Granules. Biochem. Biophys. Res. Communic., 53:830-837.

Patz, A. 1975. The Role of Oxygen in Retrolental Fibroplasia. Albrecht v. Graefes Arch. klin. exp. Ophthal., 195:77-85.

Patz, A. 1976. Current Status of Role of Oxygen in Retrolental Fibroplasia. Invest. Ophthalmol., 15:337-339.

Paul, K. 1963. "Peroxidases," in The Enzymes, 2nd. Ed., Vol.8. Edited by P.D. Boyer, H. Lardy and K. Myrback. pp. 227-274. Academic Press, New York.

Pederson, T.C. and S.D. Aust. 1973. The Role of Superoxide and Singlet Oxygen in Lipid Peroxidation Promoted by Xanthine Oxidase. Biochem. Biophys. Res. Communic., 52:1071-1078.

Peeters-Joris, C., A. Vandevoorde and P. Baudhuin. 1975. Subcellular Localization of Superoxide Dismutase in Rat Liver. Biochem. J., 150:31-39.

Pilz, H., J.S. O'Brien and R. Heipertz. 1976. Human Saliva Peroxidase: Microanalytical Isoelectric Fractionation and Properties in Normal Persons and in Cases with Neuronal Ceroid-Lipofuscinosis. Clin. Biochem., 9:85-88.

Porter, H. and S. Ainsworth. 1959. The Isolation of the Copper-Containing Protein Cerebrocuprein. I. From Normal Human Brain. J. Neurochem., 5:91-98.

Porter, H., M. Sweeney and E.M. Porter. 1964. Human Hepatocuprein. Isolation of a Copper Protein from the Subcellular Soluble Fraction of Adult Human Liver. Archs. Biochem. Biophys., 105:319-325.

Proctor, P., J. McGinness and P. Corry. 1974. A Hypothesis on the Preferential Destruction of Melanized Tissues. J. Theor. Biol., 48:19-22.

Pryor, W.A. 1973. Free Radical Reactions and Their Importance in Biochemical Systems. Fed. Proc., 32:1862-1869.

Pryor, W. 1976. "The Role of Free Radical Reactions in Biological Systems," in Free Radicals in Biology, Vol. I. Edited by W. Pryor. pp. 9-11. Academic Press, New York.

Puget, K. and A.M. Michelson. 1974. Iron Containing Superoxide Dismutases from Luminous Bacteria. Biochimie, 56:1255-1267.

Puget, K., F. Lavelle and A.M. Michelson. 1977. "Superoxide Dismutases from Procaryote and Eucaryote Bioluminescent Organisms," in Superoxide and Superoxide Dismutases. Edited by A.M. Michelson, J.M. McCord and I. Fridovich. pp. 139-150. Academic Press, New York.

Pullman, A. and B. Pullman. 1961. The Band Structure of Melanins. Biochim. Biophys. Acta, 54:384-385.

Rabinowitz, Y. 1964. Separation of Lymphocytes, Polymorphonuclear Leukocytes and Monocytes on Glass Columns, Including Tissue Culture Observations. Blood, 23:811-828.

Reddy, K. and A.L. Tappel. 1974. Effect of Dietary Selenium and Autoxidized Lipids on the Glutathione Peroxidase System of Gastrointestinal Tract and Other Tissues in the Rat. J. Nutr., 104:1069-1078.

Riley, M.V., B.S. Winkler, J. Benner and E.M. Yates. 1978. ATPase Activities in Retinal Pigment Epithelium and Choroid. Exp. Eye Res., 26:445-455.

Rister, M. and R.L. Baehner. 1976. The Alteration of Superoxide Dismutase, Catalase, Glutathione Peroxidase, and NAD(P)H Cytochrome c Reductase in Guinea Pig Polymorphonuclear Leukocytes and Alveolar Macrophages during Hyperoxia. J. Clin. Invest., 58:1174-1184.

Roberts, E., M. Rothstein and C.F. Baxter. 1958. Some Metabolic Studies of γ-Aminobutyric Acid. Proc. Soc. Expt'l. Biol. Med., 97:796-802.

Roos, D., J.W.T. Homan-Muller and R.S. Weening. 1976. Effect of Cytochalasin B on the Oxidative Metabolism of Human Peripheral Blood Granulocytes. Biochem. Biophys. Res. Communic., 68:43-50.

Roos, D., M.L.J. VanSchaik, R.S. Weening and R. Wever. 1977. "Superoxide Generation in Relation to Other Oxidative Reactions in Human Polymorphonuclear Leukocytes," in <u>Superoxide and Superoxide Dismutases</u>. Edited by A.M. Michelson, J.M. McCord and I. Fridovich. pp. 307-316. Academic Press, New York.

Rosen, H. and S.J. Klebanoff. 1976. Chemiluminescence and Superoxide Production by Myeloperoxidase-Deficient Leukocytes. J. Clin. Invest., 58: 50-60.

Rosenbaum, R.M., M. Wittner and M. Lenger. 1969. Mitochondrial and Other Ultrastructural Changes in Great Alveolar Cells of Oxygen-Adapted and Poisoned Rats. Lab. Invest., 20:516-528.

Rothman, H., L. Feeney and E. Berman. 1976. The Retinal Pigment Epithelium. Analytical Subcellular Fractionation with Special Reference to Acid Lipase. Exp. Eye Res., 22:519-532.

Rotilio, G., G. Falcioni, E. Fioretti and M. Brunori. 1975. Decay of Oxyperoxidase and Oxygen Radicals: A Possible Role for Myeloperoxidase. Biochem. J. 145:405-407.

Rotilio, G., E. Fioretti, G. Falcioni, and M. Brunori. 1977. "Generation of Superoxide Radical by Heme Proteins and its Biological Relevance," in Superoxide and Superoxide Dismutases. Edited by A.M. Michelson, J.M. McCord and I. Fridovich. pp. 239-244. Academic Press, New York.

Rotruck, J.T., A.L. Pope, H.E. Ganther, A.B. Swanson, D.G. Hafeman and W.G. Hoekstra. 1973. Selenium: Biochemical Role as a Component of Glutathione Peroxidase. Science, 179:588-590.

Saari, J., A. Bunt, S. Futterman and E. Berman. 1977. Localization of Cellular Retinol-Binding Protein in Bovine Retina and Retinal Pigment Epithelium, With a Consideration of the Pigment Epithelium Isolation Technique. Invest. Ophthalmol. Visual Sci., 16:797-806.

Sack, R., D.C. Borg and S. Freed. 1972. Photoinduced Free Radicals of Visual Pigments in Solutions Mobile at -196°C. Nature, 235:224-226.

Salin, M.L. and J.M. McCord. 1977. "Free Radicals in Leukocyte Metabolism and Inflammation," in Superoxide and Superoxide Dismutases. Edited by A.M. Michelson, J.M. McCord and I. Fridovich. pp. 258-270. Academic Press, New York.

Sanadi, D.R. 1977. "Metabolic Changes and Their Significance in Aging," in Handbook of the Biology of Aging. Edited by C.E. Finch and L. Hayflick. pp. 73-98. Van Nostrand Reinhold Co., New York.

Sanders, A.P. and W.D. Currie. 1971. "Chemical Protection against Oxygen Toxicity," in Underwater Physiology: Proceedings of the Fourth Symposium on Underwater Physiology. Edited by C.L. Lambertsen. pp. 25-40. Academic Press, New York.

Sanders, A.P., R.M. Gelren, R.S. Kramer and W. Currie. 1972. Protection Against the Chronic Effects of Hyperbaric Oxygen Toxicity by Succinate and Reduced Glutathione. Aerospace Med., 43:533-536.

Sanders, A.P., I.H. Hall, P.H. Cavanaugh and B. Woodhall. 1966. The Effects of Hyperbaric Oxygenation on Metabolism: I. ATP Concentrations in Rat Brain, Liver, and Kidney. Proc. Soc. Exp. Biol. Med., 121: 32-34.

Sanders, A.P., J.H. Hall and B. Woodhall. 1965. Succinate Protective Agent against Hyperbaric Oxygen Toxicity. Science, 150:1830-1831.

Sarna, T., J.S. Hyde and H.M. Swartz. 1976. Ion-Exchange in Melanin: An Electron Spin Resonance Study with Lanthanide Probes. Science, 192:1132-1134.

Sawada, Y. and I. Yamazaki. 1973. One-electron Transfer Reactions in Biochemical Systems. VIII. Kinetic Study of Superoxide Dismutase. Biochim. Biophys. Acta, 327:257-265.

Scott, M.L. 1962. Anti-oxidants, Selenium and Sulphur Amino Acids in the Vitamin E Nutrition of Chicks. Nutr. Abst. Rev., 32:1-8.

Scott, M.L. 1969. "Studies on Vitamin and Related Factors in Nutrition and Metabolism," in The Fat-Soluble Vitamin. Edited by H.J. DeLuca and J.W. Suttie. pp. 355-368. Univ. Wisconsin Press, Madison.

Shvedova, A.A., A.S. Sidorov, K.N. Novikov, I.V. Galushchenko and V.E. Kagan. 1979. Lipid Peroxidation and Electric Activity of the Retina. Vision Res., 19:49-50.

Siakotos, A.N., G. Aguirre and L. Schuster. 1978. Two Methods for the Rapid Purification of the Retinal Pigment Epithelium: Adsorption and Filtration. Exp. Eye Res., 26:13-23.

Silverman, W.A. 1977. The Lesson of Retrolental Fibroplasia. Sci. Am., 236(6):100-107.

Silverstein, S.C., R.N. Steinman and Z.A. Cohn. 1977. Endocytosis. Ann. Rev. Biochem., 46:669-722.

Simon, L.M., J. Liu, J. Theodore and E.D. Robin. 1977. Some Comparative Aspects of the Organ Distribution of Superoxide Dismutase Activity in the Fresh-water Turtle, Pseudemys scripta elegans. Comp. Biochem. Physiol., 58B:377-379.

Sinet, P.M., F. Lavelle, A.M. Michelson and H. Jerome. 1975. Superoxide Dismutase Activities of Blood Platelets in Trisomy 21. Biochem. Biophys. Res. Commun., 67:904-909.

Sinex, F.M. 1977. "The Molecular Genetics of Aging," in Handbook of the Biology of Aging. Edited by C.E. Finch and L. Hayflick. pp. 37-62. Van Nostrand Reinhold Co., New York.

Slater, E.C. 1973. The Mechanism of Action of the Respiratory Inhibitor, Antimycin. Biochim. Biophys. Acta, 301:129-154.

Slater, T.F. 1965. "More Thoughts on Ubiquinones," in Biochemistry of the Retina. Edited by C.N. Graymore. pp. 163-164. Academic Press, New York.

Slater, T.F. 1972. Free Radical Mechanisms in Tissue Injury. Pion Ltd., London, 283p.

Smith, P.J., A.L. Tappel and C.K. Chow. 1974. Glutathione Peroxidase Activity as a Function of Dietary Selenomethionine. Nature, 247:392-393.

Sorgato, M.C., L. Sartorelli, G. Loschen and A. Azzi. 1974. Oxygen Radicals and Hydrogen Peroxide in Rat Brain Mitochondria. FEBS Letters, 45:92-95.

Stauff, J., H. Schmidkunz and G. Hartmann. 1963. Weak Chemiluminescence of Oxidation Reactions. Nature, 198:281-282.

Stevens, J. and A. Autor. 1977. Oxygen-Induced Synthesis of Superoxide Dismutase and Catalase in Pulmonary Macrophages of Neonatal Rats. Lab. Invest., 37:470-478.

Streeten, B.W. 1961. The Sudanophilic Granules of the Human Retinal Pigment Epithelium. Arch. Ophthal., 66:391-398.

Swern, D. 1961. "Primary Products of Olefinic Autoxidations," in Autoxidations and Antioxidants. Edited by W.O. Lundberg. pp. 1-54. Interscience, N.Y.

Tappel, A.L. 1965. Free-radical Lipid Peroxidation Damage and Its Inhibition by Vitamin E and Selenium. Fed. Proc., 24:73-78.

Tappel, A.L. 1972. Vitamin E and Free Radical Peroxidation of Lipids. New York Acad. Sci., 203:12-28.

Tappel, A.L. 1973. Lipid Peroxidation Damage to Cell Components. Fed. Proc., 32:1870-1874.

Tappel, A.L. 1974. Selenium-Glutathione Peroxidase and Vitamin E. Amer. J. Clin. Nutr., 27:960-965.

Tappel, A.L. 1975. "Lipid Peroxidation and Fluorescent Molecular Damage to Membranes," in _Pathobiology of Cell Membranes_. Edited by B.F. Trump and A.U. Arstila. pp. 145-172. Academic Press, New York.

Tappel, A., B. Fletcher and D. Deamer. 1973. Effect of Antioxidants and Nutrients on Lipid Peroxidation Fluorescent Products and Aging Parameters in the Mouse. J. Gerontol., 28:415-424.

Thomas, J.J., Jr., E.M. Neptune, Jr., and H.C. Sudduth. 1963. Toxic Effects of Oxygen at High Pressure on the Metabolism of D-Glucose by Dispersion of Rat Brain. Biochem. J., 88:31-45.

Toda, K. and T.B. Fitzpatrick. 1972. "Ultrastructural Biochemical Studies of the Formation of Melanosomes in the Embryonic Chick Retinal Pigment Epithelium," in _Pigmentation. Its Genesis and Biological Control_. Edited by V. Riley. pp. 125-141. Appleton-Century-Crofts, New York.

Tripathi, B., G. Knight and N. Ashton. 1974. Effect of Oxygen on the Developing Retinal Vessels of the Rabbit. IV. Effect of Hyperoxia on Rabbit Retinal Vessels in Tissue Culture. Exp. Eye Res., 19:449-475.

Tyler, D.D. 1975a. A Protective Function of Superoxide Dismutase during Respiratory Chain Activity. Biochim. Biophys. Acta, 396:335-346.

Tyler, D.D. 1975b. Polarographic Assay and Intracellular Distribution of Superoxide Dismutase in Rat Liver. Biochem. J., 147:493-504.

Ubels, J.L., J.R. Hoffert and P.O. Fromm. 1977. Ocular Oxygen Toxicity: The Effect of Hyperbaric Oxygen on the *in vitro* Electroretinogram. Comp. Biochem. Physiol., 57A:29-32.

Vaish, S.P. and G. Tollin. 1971. Flash Photolysis of Flavins. V. Oxidation and Disproportionation of Flavin Radicals. J. Bioenergetics, 2:61-72.

Van Gelder, B.F. and E.C. Slater. 1962. The Extinction Coefficient of Cytochrome c. Biochim. Biophys. Acta, 58:593-595.

Van Woert, M.H. 1967. Oxidation of Reduced Nicotinamide Adenine Dinucleotide by Melanin. Life Sci., 6: 2605-2612.

Van Woert, M.H. 1968. Reduced Nicotinamide-Adenine Dinucleotide Oxidation by Melanin: Inhibition by Phenothiazines. Proc. Soc. Expt'l. Biol. Med., 129:165-171.

Varma, S.D., T.K. Ets and R.D. Richards. 1977. Protection Against Superoxide Radical in Rat Lens. Ophthal. Res., 9:421-431.

Webb, L.S., B.B. Keele and R.B. Johnston. 1974. Inhibition of Phagocytosis-Associated Chemiluminescence by Superoxide Dismutase. Infect. Immun., 9: 1051-1056.

Weiss, J. 1935. Uber das Auftreten eines metastabilen, aktiven Sauerstoffmolekuls bei sensibilisierten Photo-Oxydationen. Naturwissenschaften, 23:610.

Weiss, J. 1953. The Autoxidation of Ferrous Ions in Aqueous Solutions. Experientia, 9:61-62.

Weser, U., W. Bohnenkamp, R. Commack, H. Hartmann and G. Voelcker. 1972. An Enzymic Study on Bovine Erythrocuprein. Hoppe-Seyler's Z. Physiol. Chem., 353:1059-1068.

Weser, U. and G. Voelcker. 1972. Evidence for Accelerated H_2O_2 Formation by Erythrocuprein. FEBS Letters, 22:15-18.

Wikstrom, M.K.F. 1973. The Different Cytochrome b Components in the Respiratory Chain of Animal Mitochondria and Their Role in Electron Transport and Energy Conservation. Biochim. Biophys. Acta, 301:155-193.

Wills, E.D. and A.E. Wilkinson. 1967. The Effect of Irradiation on Lipid Peroxide Formation in Subcellular Fractions. Radiat. Res., 31: 732-747.

Wilson, T. and J.W. Hastings. 1970. "Chemical and Biological Aspects of Singlet Excited Molecular Oxygen," in Photophysiology. Edited by A.C. Giese. pp. 49-95. Academic Press, New York.

Winterbourn, C.C., R.E. Hawkins, M. Brian, and R.W. Carrell. 1975. The Estimation of Red Cell Superoxide Dismutase. J. Lab. Clin. Med., 85:337-341.

Wittenberg, J.B. and B.A. Wittenberg. 1962. Active Secretion of Oxygen into the Eye of Fish. Nature, 194:106-107.

Wolf, K. 1963. Physiological Salines for Fresh-Water Teleosts. Progr. Fish-Cult., 25:135-140.

Yagi, K. and N. Ohishi. 1977. "Mechanism of Degeneration of the Retina of Animals Exposed to a High Concentration of Oxygen," in Biochemical and Medical Aspects of Active Oxygen. Edited by O. Hayaishi and K. Asada. pp. 299-307. University Park Press, Baltimore.

Young, R.W. and D. Bok. 1969. Participation of the Retinal Pigment Epithelium in the Rod Outer Segment Renewal Process. J. Cell Biol., 42: 392-403.

Young, R.W. and D. Bok. 1970. Autoradiographic Studies on the Metabolism of the Retinal Pigment Epithelium. Invest. Ophthal., 9:524-536.

Zimmerman, J. 1977. Isolation and Purification of Melanosomal Proteins from Retinal Pigment Epithelium. 10th International Pigment Cell Conference, Cambridge, Mass., Oct. 1977, No. 155 (abst).

APPENDICES

APPENDIX I

EXSANGUINATION PERFUSATE

APPENDIX I

Exsanguination Perfusate
(Wolf, 1963)

Modified Cortland's Saline Solution:

NaCl	14.50 gm
$CaCl_2$ (anhydrous)	0.34 gm
KCl	0.76 gm
$NaH_2PO_4 \cdot H_2O$	0.82 gm
$NaHCO_3$	2.00 gm
$MgSO_4 \cdot 7H_2O$	0.46 gm
Procaine HCl	6.00 gm
Na heparin	4000 USP Units

Distilled H_2O to 2000 ml

pH 7.1 at 15°C

APPENDIX II

DIALYSIS TUBING PREPARATION

APPENDIX II

Dialysis Tubing Preparation

(Brewer et al., 1974)

Procedure:

1. Cut dialysis tubing to appropriate length. The capacity of 5/8 inch diameter tubing is 1.83 ml/cm and 20 cm is added for knots and space.
2. Simmer tubing for 1 hour in 2 L. of 50% ethanol.
3. Immerse 1 hour in another equal volume of 50% ethanol.
4. Immerse 1 hour in 10 mM $NaHCO_3$, 1 mM EDTA.
5. Repeat step 4.
6. Immerse 1 hour in distilled water.
7. Repeat step 6.
8. Store at 4^oC in distilled water containing a few ml of chloroform to discourage bacterial growth. The use of azide for this purpose should be avoided in that it is inhibitory to SOD.

APPENDIX III

LOWRY PROTEIN DETERMINATION

APPENDIX III

Lowry Protein Determination

(Oyama and Eagle, 1956)

Principle

Tyrosine and tryptophan in proteins react with Folin's phenol reagent to give a blue color which is read photometrically.

Reagents

A. Lowry A

1.	Sodium carbonate (anhydrous)	60.0 g
2.	Sodium hydroxide (pellets)	12.0 g
3.	Sodium or potassium tartrate	0.6 g
4.	Distilled H_2O to make	3000 ml

B. Lowry B

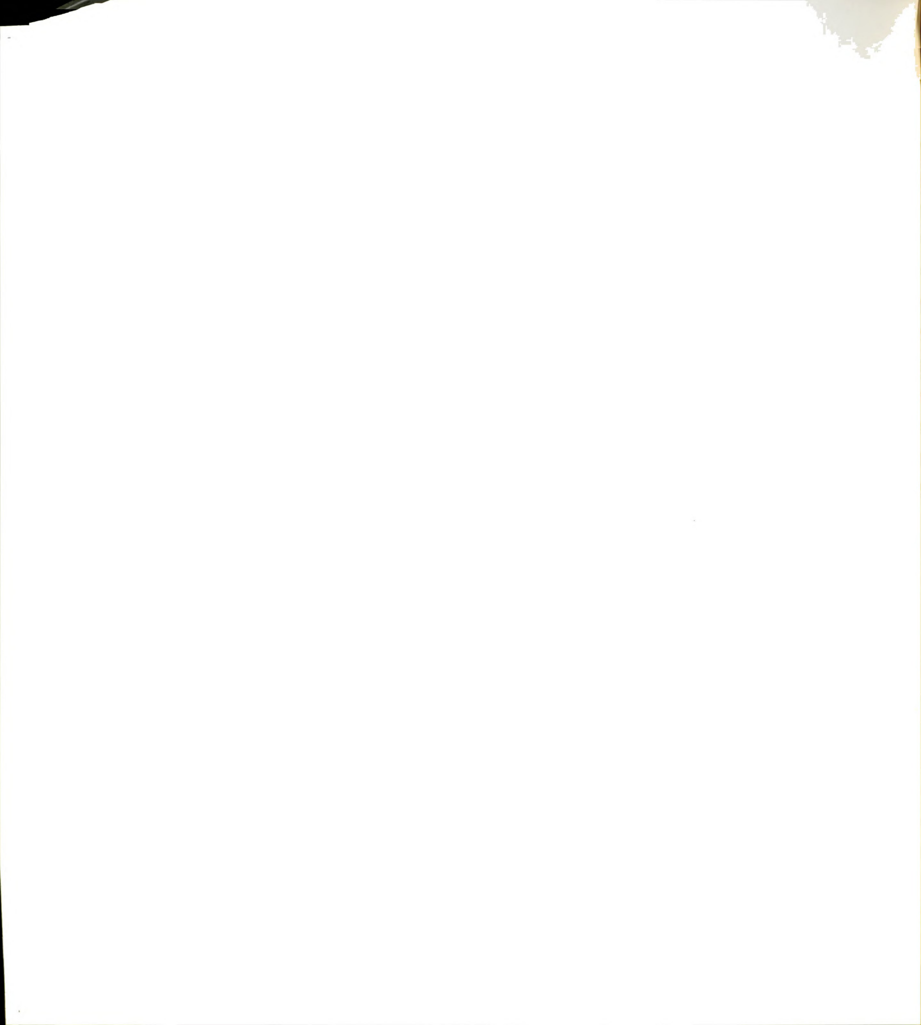
Copper sulfate solution ($CuSO_4 \cdot 5H_2O$)	0.5 g%

C. Lowry C (prepared fresh daily)

1.	Lowry A	50 parts
2.	Lowry B	1 part

D. Phenol reagent according to Folin Ciocalteu

1.	Phenol Reagent-concentrate (Central Scientific Co.)	1 part
2.	Distilled H_2O	1 part



E. Protein Standard 8.0 g% (Dade Reagents Inc., Miami, Fla.)

   1. Dilute with 300 ml distilled H_2O to give 800μg/ml

Concentrations of protein standards used for determination of standard curve: 0, 20, 40, 60, 80 and 120 μg/ml.

Procedure

1. 1 ml of protein solution (standard or unknown) added to 5 ml of Lowry C.
2. Incubate 20 min at room temperature (20-22°C)
3. 0.5 ml phenol reagent jetted in for rapid mixing.
4. Incubate ½ hr at room temperature, mix occasionally.
5. Read at 660 mμ.

APPENDIX IV

SUPEROXIDE DISMUTASE DETERMINATION

APPENDIX IV

Superoxide Dismutase Determination

Principle

Superoxide anion ($O_2^{\overline{\cdot}}$) is generated by a xanthine oxidase-catalyzed reaction. Acetylated cytochrome c (ACc) is reduced by the $O_2^{\overline{\cdot}}$ to yield a product which absorbs at 550 nm. In the presence of superoxide dismutase, $O_2^{\overline{\cdot}}$ is removed from the reaction and the rate of ACc reduction is inhibited.

Reagents

A. Stock Dispenser Solution

1.	Xanthine	13.1 mg
2.	EDTA	27.9 mg
3.	KCN (for 0.5 mM mixture)	24.3 mg
	or	
	KCN (for 2.0 mM mixture)	97.6 mg
4.	KH_2PO_4	222.5 mg
5.	Na_2HPO_4	1.6929 g
6.	Distilled H_2O to make	250 ml

B. Stock Cytochrome Solution

1. Desire 65 ml of 60 μM ACc:

   X ml ACc = (65 ml) (60 μM ACc)/(* μM ACc)

   * See Appendix VII

2. Distilled H_2O to make 65 ml.

C. Stock Xanthine Oxidase Solution (Prepare just prior to assay)

1. 100-150 µl xanthine oxidase (13.2 U/ml; No. X-1875, Sigma Chemical Co., St. Louis, Mo.). The concentration is adjusted to yield an initial uninhibited reaction rate of 0.025 to 0.045 OD units/min.

2. Distilled H_2O to make 50 ml

D. Stock Tissue Extract Solution

1. Lyophilized extract approx. 60 mg

2. Distilled H_2O to make 4 ml

E. Stock Standard SOD Solution

1. One preprepared vial of lyophilized standard*

2. Distilled water to 5 ml

*Standard is prepared in advance as follows:

BESOD (#S-8254; Sigma Chemical Co.) 4.9 mg

Distilled H_2O to make 250 ml

This solution is divided into 5 ml aliquots which are frozen, lyophilized, and stored dessicated at $0^{o}C$ until used.

Procedure

1. Prepare a reference cuvette as follows and place in the reference well of the spectrophotometer:

Dispenser Stock Solution 1.0 ml

Cytochrome c Stock Solution 0.5 ml

Extract or Standard appropriate dosage

Distilled H_2O to bring volume to 3.0 ml

2. Sample cuvette for determination is prepared as follows:

Dispenser Stock 1.0 ml

Cytochrome c Stock 0.5 ml

Extract or Standard   appropriate dosage

Distilled H_2O to bring volume to 2.0 ml

3. Place sample cuvette into the sample well of the spectrophotometer, turn on the recorder chart drive (chart speed 1"/min) and turn on the spectrophotometer.

4. Start the reaction by injecting 1.0 ml of xanthine oxidase stock solution into the sample cuvette.

5. Read the change in absorbance per minute at 550 nm and record the time.

Final Assay Reaction Mixture Composition:

10 μM acetylated ferricytochrome c

100 μM xanthine

50 mM phosphate buffer, pH 7.8 at 25°C

0.1 mM EDTA

0.5 mM or 2.0 mM KCN

0.015-0.020 units xanthine oxidase

sample dosage

Expression of SOD Activity

The unit of SOD activity may be defined as the amount of enzyme inhibiting the rate of reduction of acetylated ferricytochrome c by 50% (i.e., ED_{50} ± 95% confidence limits); OR

The activity may be expressed in terms of potency ratios with associated 95% confidence limits in relation to standards of known and verifiable activity.

Assay Design

1. For each determination, note the time to account for the decay of xanthine oxidase with time (McCord and Fridovich, 1969). In the dilutions described and at room temperature, the control reduction rate declined at a rate of $(-0.65 \pm 0.29)\times10^{-4}$ OD units/min/min ($\bar{x} \pm$ SD) in 97 determinations.

2. Start by determining the basal rate of cytochrome c reduction in triplicate using the 0.5 mM KCN dispenser solution.

3. For a single dose determine each of the following reduction rates in quadruplicate:

Extract in 0.5 mM KCN

Extract in 2.0 mM KCN

Standard in 0.5 mM KCN

Standard in 2.0 mM KCN

Standard + Extract in 0.5 mM KCN

4. Halfway through these readings, determine the basal reduction rates in triplicate this time using the 2.0 mM KCN dispenser solution.

5. At the end, determine in triplicate the basal rates using the 0.5 mM KCN dispenser solution.

6. Make fresh xanthine oxidase stock solution and repeat steps 1-5 for the next dose.

Isozyme Determination:

1. Determine by what percentage the standard was inhibited by 2.0 mM KCN using the figures of the relative potency of inhibited standard to uninhibited standard:

$$\% \text{ Inhibition} = (1\text{-Potency}) \times 100 = A$$

2. Similarly calculate the percentage inhibition of the extract:

$$\% \text{ Inhibition} = (1\text{-Potency}) \times 100 = B$$

3. If the purified Cu-Zn BESOD was inhibited A% (approx. 79.19 $\pm$ 4.60 (19) percent), then that percentage inhibition observed in the extract must represent a similar proportion of Cu-Zn SOD inhibitability:

$$\frac{A \text{ \%I Std.}}{100 \text{ \% Cu-Zn SOD}} = \frac{B \text{ \%I Ext.}}{C \text{ \% Cu-Zn SOD}}$$

All uninhibited activity above and beyond this must be due to the cyanide-insensitive mitochondrial form.

C% Cu-Zn SOD activity in the extract = B/A x 100.

APPENDIX V

CYTOCHROME C ACETYLATION

APPENDIX V

Cytochrome c Acetylation

1. Over ice dissolve 50 mg cytochrome c (#C-2506; Type III; from horse heart, M.W. = 12,384; Sigma Chemical Co., St. Louis, Mo.) in 5 ml of saturated sodium acetate.

2. Under stirring add 10 times excess acetic anhydride with respect to the lysine groups on cytochrome c and allow reaction to proceed 30 min. The number of lysine residues in horse cytochrome c is approximately 18 plus 2 α-amino terminal residues (Minakami et al., 1958). Thus, the appropriate amount of acetic anhydride is 20 x 10 = 200 moles of reagent per mole of cytochrome c.

3. Dialyze in 5/8 inch dialysis tubing at $0^{o}C$ for 12 hours against 1 liter distilled water with 4 changes of water.

4. Store at $-20^{o}C$.

APPENDIX VI

DETERMINATION OF EXTENT OF ACETYLATION

APPENDIX VI

Determination of Extent of Acetylation

Ninhydrin Peptide Assay: (Hirs, 1967)

1. Prepare 30 tubes as follows:

3 tubes	50 µl leucine
3 tubes each	25 µl, 50 µl, 75 µl, 100 µl acetylated cytochrome c (ACc)
3 tubes each	25 µl, 50 µl, 75 µl, 100 µl native cytochrome c (NCc)
3 tubes	50 µl distilled H_2O

2. Add 0.5 ml ninhydrin reagent to each tube and shake to mix.
3. Cover with a loosely fitting culture tube cap.
4. Heat exactly 15 min. in a covered poiling water bath.
5. Cool in a basin of cold water about 10 min.
6. Remove caps and add 2.5 ml of 50% ethanol to each tube.
7. Shake vigorously for 45 sec.
8. Measure OD at 570 nm against distilled water.

Results: (Azzi et al., 1975)

1. Absorbance of the blank should not exceed 0.05
2. Molar absorptivity of the colored product, the anion of diketohydrindylidene-diketohydrindamine, at 570 nm is very nearly 2×10^4 M^{-1} cm^{-1}.

3. The percent acetylation is determined as:

$$\% \text{ acetylation} = 100 \times \left(1 - \frac{S_{ACc}}{S_{NCc}}\right)$$

where S is the slope of A_{570} vs. concentration.

Storage of the Ninhydrin Reagent:

1. Ninhydrin reagent solution for the determination of primary and secondary amines and amino acids (Moore, 1968) was obtained from Sigma Chemical Co., St. Louis, Mo. (#N-1632).

2. This is to be stored dark at 0-5°C under nitrogen.

3. A 125 ml aspirator bottle was painted black with the exception of a narrow vertical viewing port and was fitted with a silicone rubber stopper through which are inserted a vent and a feed tube, the latter of which reaches almost to the bottom of the bottle.

4. The feed tube is closed with a three-way stopcock (K-75, Pharmaseal Inc., Toa Alta, Puerto Rico) carrying one connector to a plastic tube used to drain the reagent bottle and the other to a nitrogen tank.

5. The tubulation of the aspirator bottle leads to an automatic dispensing pipet (Fisher Scientific Co., Livonia, Mi.; #313-689-130A) of 1 ml capacity and 0.01 ml divisions.

6. The vent tube is connected to a constant pressure

nitrogen reservoir of 2 liter capacity. This is made from two 2 liter aspirator bottles, the tubulations of which are connected by a short length of plastic tubing. One bottle is fitted with a silicone rubber stopper through which a single tube passes to the vent tube of the ninhydrin storage bottle. The other bottle remains open. Sufficient light mineral oil is added to approximately half fill both bottles of the reservoir. Nitrogen is passed into the storage bottle through the feed tube and the oil in the closed half of the reservoir is displaced into the open half. Excess nitrogen excapes to the atmosphere through the open bottle. When first assembled, the unit is thoroughly flushed for 2 hours to be certain that only negligible quantities of oxygen remain.

7. The transfer of the ninhydrin reagent from its packaged bottle into the storage bottle was performed in a glove bag (Model X-17-17, Instruments for Research and Industry, Cheltenham, PA) which had been purged with nitrogen.

8. The storage bottle and nitrogen reservoir were stored at 0-5°C.

APPENDIX VII

ACETYLATED CYTOCHROME C CONCENTRATION DETERMINATION

APPENDIX VII

Acetylated Cytochrome c Concentration Determination

1. Dilute 300 μl of acetylated cytochrome c to 3 ml in a cuvette.

2. Add a few grains of dithionite to the cuvette to reduce the acetylated ferricytochrom c.

3. The concentration of acetylated ferrocytochrome c is determined at 550 nm using the extinction co-efficient, $E_{mM} = 27.7$.

APPENDIX VIII

OXYHEMOGLOBIN DETERMINATION

AND

ERYTHROCYTE CONTAMINATION

APPENDIX VIII

Oxyhemoglobin Determination and Erythrocyte Contamination

(Hohorst et al., 1959)

1. Reconstituted tissues containing erythrocytic contamination are diluted and scanned spectrophotometrically between 620 nm and 520 nm at a chart speed of one inch per minute as follows:

Blank:

1.0 ml 0.5 mM Dispenser Solution (See Appendix IV)

2.0 ml Distilled H_2O

Sample:

1.0 ml 0.5 mM Dispenser Solution

1.5 ml Distilled H_2O

Sample + H_2O to make 500 μl

2. Record A_{578}, A_{560}, and A_{540}.

3. Calculate

$$\delta A_{HbO_2} = (A_{578} - A_{560}) + \{(A_{540} - A_{578}) \cdot 0.47\}$$

4. Determine:

$a = \delta A_{HbO_2} /$ mg lyophilized whole blood

$b = \delta A_{HbO_2} /$ mg lyophilized tissue extract

5. Predetermined:

c = Units SOD/ mg lyophilized whole blood

d = Units SOD/ mg lyophilized tissue extract

6. Calculate:

Units erythrocytic SOD/Unit total extract SOD = $(c)(b)/(a \cdot d)$

APPENDIX IX

CONTAMINATION OF CYTOCHROME C BY SUPEROXIDE DISMUTASE

APPENDIX IX

Contamination of Cytochrome c by Superoxide Dismutase

Contamination of cytochrome c by SOD is detectable as an increase in the basal rate of cytochrome c reduction in the presence of 2.0 mM KCN over the basal rate measured in the presence of 0.5 mM KCN.

If assayed activities are expressed on the basis of potency ratios in relation to simultaneously assayed standardized SOD solutions, this contamination need not be quantified in that both sample and standard will be affected equally.

If it is desired to express the assayed tissue activity in terms of ED_{50}'s under the prescribed assay conditions, then the contaminating SOD activity must be quantified and subtracted from the results as follows:

1. All contaminating SOD is taken to be Cu-Zn SOD (McCord and Fridovich, 1977b).

2. Calculate:

$X\% = \{100 - (0.5\text{mM basal rate}/2.0\text{ mM basal rate}) \times 100\}$

3. Thus, the 2.0 mM KCN revealed that the contaminating SOD caused an X% reduction in the observed rate of reduction, but it is known that 2.0 mM KCN only inhibits Cu-Zn SOD by about 80%, thus the actual 0.5 mM rate

observed represents 1.25 · X% inhibition of the rate if no contaminant were present.

4. The number of units of Cu-Zn SOD which cause a 1.25X% reduction in reaction rate may then be calculated from the log dose-probit response relationship derived for the standard BESOD.

APPENDIX X

INTERNAL REFERENCE RECOVERY TEST

APPENDIX X

Internal Reference Recovery Test

Principle

To test for the masking of activity by a non-specific binding substance in the extract, a known amount of standard BESOD is added to an equal amount of extract. All added activity should be recoverable in the assay of combined extract and standard. Having determined the ED_{50}'s for both standard and extract separately, the ED_{50} assayed for the combined sample is predictable.

Encoding and Decoding in the Coded Log Dose Scale:

A. To Decode:

1. For the present discussion, let A be the lowest dose and coded 0 on the arbitrary coded log dose scale.
2. If the successive doses are doublings, triplings, or quadruplings, let B equal 2, 3, or 4 respectively and the coded doses will differ by log B units.
3. Any point on the arbitrary log scale can be decoded by multiplying by log B and adding log A.

B. To Encode:

1. Let C be the uncoded number to be encoded.

2. Code = (log C - log A)/log B.

<u>Predicting the ED_{50} of the Combined Standard and Extract Sample</u>:

1. The following formulations are derived to apply only to parallel response assays.

2. Calculate a regression for the extract coded log dose vs. probit response line. It will have the form $y = mx + b$.

3. The response measured at ED_{50_S} μl of standard solution is 5.00. This is the same response seen with ED_{50_E} μl of extract . If an additional ED_{50_S} μl of extract are added, then the expected response at $(ED_{50_E} + ED_{50_S})$ μl will be:

$$y = m\{\text{encoded}(ED_{50_E} + ED_{50_S})\} + b$$

4. Thus, at $2(ED_{50_S})$ μl of combined sample and standard the response will be y probits. This gives one point on the S+E dose-response line with a slope of m, again assuming parallelism. The $ED_{50_{S+E}}$ should lie at the intersection of this line with a probit of 5.00:

$$X = \frac{\log(2ED_{50_S}) - \log A}{\log B} - \frac{m\left\{\frac{\log(ED_{50_E} + ED_{50_S}) - \log A}{\log B}\right\} + b - 5.00}{m}$$

where X is the predicted $ED_{50_{S+E}}$.

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