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dissertation entitled

"Oxygen Toxicity and Adaptation to Hyperoxia:
Studies on the Retina of the
Rainbow Trout (Salmo Gairdneri)"

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Physiology

Major professor

Date 3/27/87

OXYGEN TOXICITY AND ADAPTATION TO HYPEROXIA: STUDIES ON THE RETINA OF THE RAINBOW TROUT (*SALMO GAIRDNERI*)

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology

1987

ABSTRACT

OXYGEN TOXICITY AND ADAPTATION TO HYPEROXIA:
STUDIES ON THE RETINA
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Molecular oxygen can accept electrons univalently to form toxic intermediates, i.e. superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$). The damaging effects of oxygen toxicity following hyperoxic exposure have been attributed to formation of these reactive oxygen metabolites. The retina of Salmo gairdneri is normally exposed to O_2 tensions as high as 750 mmHg. This tissue has adapted to these hyperoxic conditions and indeed is resistant to even greater oxygen tensions. There are several defense mechanisms to protect tissues against oxidative damage. One defense is to limit the concentrations of the two primary intermediates $O_2^{\cdot-}$ and H_2O_2. The superoxide dismutase enzymes (CuZnSOD and MnSOD) regulate the concentration of superoxide, while catalase and glutathione peroxidase regulate the H_2O_2 concentration. Since $^{\cdot}OH$ is believed to be formed by processes dependent on $O_2^{\cdot-}$ and H_2O_2, regulation of these two metabolites limits $^{\cdot}OH$ formation. Based on the free radical theory of oxygen toxicity, tolerance to hyperoxia is either due to a decreased propensity for generating reactive metabolites and/or the presence of efficient antioxidant defenses.

The rate of oxidant formation was estimated in retinal homogenates under normoxic and hyperoxic conditions using cyanide-insensitive respiration. Trout brain and liver tissues are sensitive to oxygen and were therefore used as internal controls for comparison to the oxygen

resistant retina. The results indicate that CN^--insensitve respiration by retinal homogenates was elevated during hyperoxic exposure. Similar results were found for the brain and liver homogenates, which indicated that oxygen resistance was not due to an inability to generate reactive oxygen metabolites. In addition, the antioxidant activities of the copper/zinc and manganese superoxide dismutases, catalase and glutathione/glutathione peroxidase enzymes were compared in these tissues. The results indicate that the efficiency of the antioxidant defenses is no greater in the retina than in the oxygen sensitive tissues. These results suggest that resistance to oxygen toxicity in the rainbow trout retina is not based on cellular properties controlling formation or removal of reactive oxygen metabolites but may be due to a predetermined genetic resistance of O_2-sensitive target sites in cells adapted to chronic hyperoxic conditions.

This dissertation is dedicated to the memory of
Dr. Jack R. Hoffert, my mentor and friend.

ACKNOWLEDGMENTS

I would like to thank the members of my guidance committee: Dr. Steve Heidemann for providing me with the opportunity to complete my dissertation work and for his support and guidance, Dr. Jacob Krier for his encouragement and willingness to help open doors which enabled me to continue this project, Dr. Bill Frantz for his availability and interest in research progress, Dr. Bob Roth for his scientific interest and keen review of this dissertation, and Dr. Steve Weiss for his willingness to contribute to my training, and for his critical insight and helpful suggestions.

I would also like to thank Dr. P.O. Fromm for his support, encouragement and interest in my career development.

A very special thanks is also extended to Esther Brenke for providing expert technical assistance, support, encouragement and friendship.

Many thanks to the members and friends of the Physiology Department, both past and present, for their encouragement, support and assistance along the way.

I am grateful for support from the National Institute of Health, Grant No. EY-00009, and funding through the Department of Physiology.

Finally, I would like to thank my wife Denise, for her patience, perseverance and dedication, and Renee and Kevin for providing me with the opportunity to escape and play.

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INTRODUCTION

The rainbow trout eye possesses a structure at the back of the retina, the choroidal rete mirabile, which, in conjunction with the choriocapillaris, has the ability to concentrate oxygen. The oxygen tension at the back of this tissue has been reported to be as high as 750 mmHg (Desrochers et al., 1985). Exposure to oxygen tensions in the range of 425-760 mmHg has been shown to be toxic to the mammalian eye (Noell, 1955a, 1955b; Yanoff et al., 1970). The teleost retina however, appears to have adapted to the hyperoxia generated at the back of the eye. In addition, the rainbow trout retina has consistently been shown to be resistant to oxygen tensions even greater than the high physiological concentration within the trout eye (Baeyens, Hoffert and Fromm, 1973, 1974; Ubels, Hoffert and Fromm, 1977; Ubels and Hoffert, 1981). The oxygen molecule itself is not very reactive, however it can accept electrons in univalent steps to form three reactive intermediates. The successive addition of single electrons to oxygen will yield the superoxide anion ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$) (Weiss, 1986). Each of these oxygen metabolites can be generated in aerobic cells and potentially can act as toxic oxidants. There are multiple sources for the formation of these reactive metabolites in vivo. Potential oxidant sources include components of the electron transport chain of the mitochondria, endoplasmic reticulum, and nuclear membranes. Various soluble enzymes and proteins are also capable of

reducing oxygen in one or two electron reduction steps (Freeman and Crapo, 1982; Weiss, 1986). All three of the reactive oxygen metabolites discussed have been implicated in oxidative injury to cells (Freeman and Crapo, 1982; Fridovich, 1983). Damage attributed to these reactive metabolites includes the inactivation of proteins via the oxidation of critical sulfhydryls, the oxidation of polyunsaturated fatty acids within membrane phospholipids and strand scission of nucleic acids (i.e., DNA and RNA).

There are several defense mechanisms to protect tissues against oxidative damage. One key defense is to limit the concentrations of the intermediates formed, i.e., $O_2^{\cdot}$ and H_2O_2. Because $O_2^{\cdot}$ and H_2O_2 are thought to play a primary role in oxidative damage, either by acting directly or via the generation of even more reactive intermediates, defenses designed to control the concentrations of these two primary oxidants are of critical importance. The superoxide dismutase enzymes strictly regulate the concentration of superoxide generated within the cell. There are two forms of the superoxide dismutase (SOD) enzyme commonly found in eukaryotic organisms. One type, a copper/zinc form of SOD, is found in the cytoplasm, whereas a manganese containing form is generally associated with mitochondria (Fridovich, 1986b). Two other antioxidant enzymes, catalase and glutathione peroxidase, provide important regulation of the H_2O_2 concentration (Chance, Sies and Boveris, 1979). It is believed that the $^{\cdot}OH$ radical is formed by processes which are dependent on $O_2^{\cdot}$ and H_2O_2 (Fridovich and Freeman, 1986). However, there are no known enzymatic regulators for specifically reducing the hydroxyl radical concentration. Thus, limiting the concentration of the reactants (i.e. $O_2^{\cdot}$ and H_2O_2) limits formation of the product ($^{\cdot}OH$).

Currently, oxygen toxicity is thought to be due to the production of reactive oxygen metabolites at a rate that is in excess of the capacity of the cellular defense mechanisms to inactivate them (Jamieson et al., 1986). Indeed, in the most extensively studied model, i.e., hyperoxic lung injury, there is evidence to indicate that production of the reactive oxygen metabolites is increased in the rat lung during hyperoxic exposure (Freeman and Crapo, 1981; Freeman, Topolosky and Crapo, 1982; Turrens, Freeman and Crapo, 1982; Turrens et al., 1982b), and synthesis of the primary antioxidants is induced (Kimball et al., 1976).

Given current views on the free radical theory of oxygen toxicity, two principal "strategies" that may account for the resistance of the trout eye to oxygen toxicity were considered: 1) a decreased propensity to generate reactive oxygen metabolites under hyperoxic conditions, and/or 2) the presence of a highly efficient antioxidant defense system within this tissue. Thus, in the present study, the rate of formation of the reactive metabolites by the trout retina was estimated in normoxic and hyperoxic conditions from a quantitative analysis of oxygen consumption in the presence of cyanide (Freeman and Crapo, 1981). In addition, the activities of the key antioxidant enzymes i.e., CuZn and Mn superoxide dismutases, catalase, and the glutathione/glutathione peroxidase system were also measured in order to determine whether the trout retina had elevated levels of these primary antioxidants. Either of these factors or a combination of both, could provide a biochemical rationale for the resistance of the trout retina to oxygen toxicity.

LITERATURE REVIEW

Oxygen Toxicity

Oxygen toxicity is a pathological condition which develops upon exposure to an elevated oxygen tension for an extended period of time. The severity of the condition is dependent upon the degree of hyperoxia and the duration of exposure. The gross manifestations of oxygen toxicity have been observed principally in the lung under hyperoxic conditions, a likely site since the lung is in direct contact with the highest partial pressures of inspired oxygen. Most susceptible is the pulmonary vasculature, especially the capillary endothelial cells (Deneke and Fanburg, 1980; Crapo, 1986).

The damaging effects of oxygen have also been demonstrated in a variety of other systems including the cardiovascular, renal, visual and central nervous systems (Haugaard, 1968; Balentine, 1982). Since the focus of this research is on the rainbow trout retina, this review will be limited to the toxic effects of oxygen on retinal tissues.

Interest in the deleterious effects of oxygen on the eye began in the early 1950's with the realization that excessive oxygen administration to premature infants caused retinal damage and eventual blindness, a condition which became known as retrolental fibroplasia (RLF) (Patz, Hoeck and De La Cruz, 1952; Patz, 1954). It was observed that this condition was due to an effect of oxygen on the immature retinal vessels. Specifically, there was an interruption of retinal vascular

development in hyperoxia, which was followed by prolific neovascularization on return to room air. In severe cases growth of the vasculature into the vitreous led to scar tissue formation and retinal detachment (Patz, 1984). These findings were confirmed experimentally in kittens, young rabbits and young mice (Ashton, Ward and Serpell, 1953; Gerschman et al., 1954b; Ashton, 1968).

In the course of studying RLF several investigators observed that oxygen was also damaging to the choroidal vasculature and visual cells in eyes from several adult animal species. Yanoff, Miller and Waldhausen (1970) noted uveal and retinal detachments in 10 of 14 eyes removed from dogs exposed to 1 atmosphere of 100% oxygen for 48 to 50 hours (PaO_2 425-664 mmHg). They discovered that the initial damage was to the choriocapillary endothelial cells, and that detachment was secondary to fluid leakage from the choroidal vasculature into the subretinal space.

The visual cells of the adult rabbit were also found to be susceptible to the effects of hyperoxia (Noell, 1955a, 1955b). In these studies adult rabbits were exposed to varying concentrations of oxygen (60-100%) at ambient pressure. Using the electroretinogram (ERG) as a measure of visual cell function, Noell noted a marked attenuation of the ERG, which occurred progressively earlier with increasing oxygen concentrations. Histological examination revealed selective degeneration of more than 70% of the photoreceptor cell population after 48 hours of 100% O_2 exposure. Irreversible damage to the visual cells was observed in animals exposed to only 55-60% O_2 at ambient pressure for a period of just 7 days.

It has also been reported that hyperbaric oxygen exposure (HBO) accelerates the onset of toxicity to the retina in vivo (Noell, 1962;

Bridges, 1966). Using the b-wave of the ERG as an index for quantifying the effects of oxygen, both investigators reported a greater reduction in amplitude of the rabbit ERG at higher pressures of 100% oxygen. Noell (1962) again reported selective visual cell layer death after exposure of rabbits to 100% O_2 at 3 atm for just 4 hours. The inner layers were unaffected histologically, but the outer nuclear layer was reduced in thickness and number of nuclei, and deterioration of the photoreceptor outer and inner segments was observed. These findings were confirmed by Bresnick (1970), who studied electron micrographs of the adult rabbit retina after the animals were exposed to 100% O_2 for 24-40 hours. The earliest ultrastructural changes observed immediately after O_2 exposure were to the inner segments and nuclei of the photoreceptor cells. Swelling of various cellular organelle membranes was extensive with marked vesiculation followed by complete disorganization and destruction of the entire visual cell.

Ubels, Hoffert and Fromm (1977) also used the ERG to investigate the toxic effects of HBO on the retina, however they performed their studies in vitro. Their design enabled them to discern whether the ERG changes were due to changes to the retinal vasculature or to the retinal cells themselves. They exposed the isolated eyecups from frogs and rats to 5 atm of pure oxygen and reported a marked attenuation of the b and c waves within 6 hours in the frog and severe attenuation of the a and b waves within 90-105 minutes in the rat, which led them to conclude that oxygen did indeed have a direct toxic effect on the retinal cells.

Much of the early work on retinal oxygen toxicity was strictly descriptive with little understanding of the mechanism of toxicity. Several possibilities had been proposed based on the generalized

damaging effects that oxygen had on cells from other tissues. These included: 1) the oxidation of sulfhydryl groups, 2) alterations to several metabolic pathways and 3) the oxidation of polyunsaturated fatty acids (Bridges, 1966). To date, the same possibilities for mechanism of damage remain under investigation (Freeman and Crapo, 1982; Weiss, 1986).

A good part of the earlier work aimed at determining the mechanism of oxygen toxicity was performed by Haugaard and associates. Decreased metabolism of various tissue slices from the rat had been demonstrated after exposure to HBO (Stadie, Riggs and Haugaard, 1945). Shortly thereafter, Haugaard (1946) was able to establish a link between inactivation of enzymes containing essential sulfhydryl groups and oxygen exposure, concluding that inactivation occurred through sulfhydryl oxidations. It was later reported that inhibition of glycolysis in rat heart homogenates occurred through reversible oxidation of sulfhydryl groups in glyceraldehyde-3-phosphate dehydrogenase after exposure to 1 atm oxygen with resultant effects on metabolism (Horn and Haugaard, 1966). Oxygen may also exert toxic effects by oxidizing nonprotein sulfhydryls essential in maintaining enzyme sulfhydryls in their reduced state and in preventing the accumulation of mixed disulfides which are able to exert deleterious effects on cell metabolism (Haugaard, 1968).

More specifically, hyperoxia has also been reported to interrupt normal cellular metabolism in retinas from several species. Hyperbaric oxygen exposure is responsible for decreased oxidative metabolism in dog retinas (Baeyens, Hoffert and Fromm, 1973), and for inhibition of lactate dehydrogenase activity in frog and dog retinas (Baeyens, Hoffert and Fromm, 1974). The effect of hyperbaric oxygenation on Na^+-K^+ ATPase

activity was also studied (Ubels and Hoffert, 1981). This enzyme contains critical sulfhydryl groups and is essential for normal retinal function. A 50% decrease in enzyme activity after exposure of rat retinas to 5 atm of pure O_2 for 4 hours was reported. In light of the effects of oxygen on the ERG they proposed that the toxicity may be due to an inability to maintain transmembrane potentials.

In addition to protein targets in the retina, the oxidation of polyunsaturated fatty acids (PUFA) had also been proposed as a mechanism for oxygen toxicity. In fact, Yagi and associates have reported that lipoperoxide levels are elevated in rabbit and chick embryo retinas after exposure to high concentrations of oxygen (Hiramitsu et al., 1976; Yagi and Ohishi, 1977). This elevation of lipoperoxide was observed prior to changes in the electroretinograms.

In summary, the gross manifestations of retinal oxygen toxicity appear to be due to: 1) an interruption of retinal vascular development in the immature retina, 2) oxidative inactivation of various enzymes, 3) oxidation of membrane-associated PUFA, 4) a decrease in cell function, as indicated by changes in enzyme activities and oxidative metabolism and 5) selective visual cell degeneration and cell death.

Chemistry of Oxygen and its Reduction Products

Molecular oxygen is a strong oxidant but exhibits limited reactivity because of its unusual electronic configuration. Oxygen in the ground state contains two unpaired electrons with parallel spins in its outermost orbitals. This configuration presents a kinetic barrier to reaction, since a change in spin state is required in order for oxygen to oxidize a two electron donor. Spin inversion however, is unlikely since it is a rather slow process in comparison to the lifetime of the

collisional complex formed between oxygen and a two electron donor. In contrast, the spin constraints do not apply to reactions with single electron donors or atoms or molecules containing unpaired electrons (Fridovich, 1974; 1977; Hill, 1979; Weiss, 1986). Because of the spin restriction, oxygen reduction can generally be categorized into two types of reactions, enzyme-catalyzed multivalent reduction (e.g., mitochondrial cytochrome oxidase) and enzyme-independent univalent reduction. The univalent reduction of oxygen is favored and commonly occurs in biological systems (Fridovich, 1977; 1978).

Molecular oxygen can accept a total of four electrons in either univalent or multivalent steps. If this reduction process were to take place in univalent steps, three reactive metabolites of oxygen would be generated (Figure 1; Hill, 1979). Successive univalent reduction of dioxygen would result in the formation of the superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$) (Fridovich, 1977; Weiss, 1986). The superoxide radical may also be protonated to form the perhydroxyl radical ($HO_2^{\bullet}$), the conjugate acid of the $O_2^{\bullet-}$ radical, with a pKa of 4.8 (Hill, 1979). The $HO_2^{\bullet}$ radical is a stronger oxidant than the $O_2^{\bullet-}$ radical but at a physiological pH of 7.4 the superoxide anion will predominate. A two electron addition to molecular oxygen results in the formation of the peroxide anion (O_2^{-2}), but at physiologic pH, it is rapidly protonated to form hydrogen peroxide (H_2O_2). Addition of the third electron to molecular oxygen results in formation of the hydroxyl radical ($^{\bullet}OH$). Finally, addition of the fourth electron results in the formation of the hydroxyl anion, which when protonated forms water. One other potentially damaging species of oxygen, singlet oxygen ($^1O_2$), is formed by the conversion of ground

state molecular oxygen to a more excited singlet oxygen state either through photochemical or thermal processes (Green and Hill, 1984). All of the above intermediate species of oxygen are oxidants capable of causing cellular damage. There is now evidence to indicate that these species are generated in vivo under a variety of pathophysiological conditions.

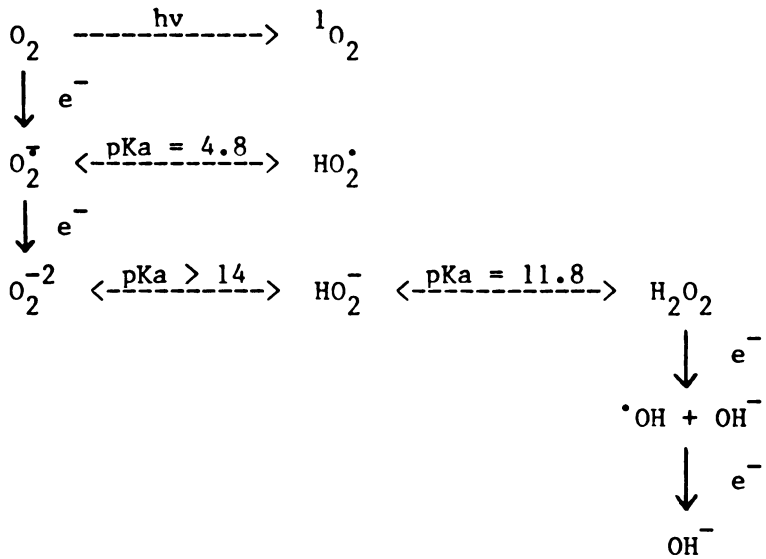

Figure 1 The excitation and reduction products of molecular oxygen.

Cellular Sources and Reactivity of Various Oxygen Metabolites

There are numerous intracellular sources of the superoxide radical, including several soluble enzymes and proteins, bound proteins associated with electron transport systems and small molecules capable of releasing superoxide through autoxidation reactions (Freeman and Crapo, 1982; Fridovich, 1983). Xanthine oxidase is the most widely studied superoxide-generating, soluble enzyme. Under normal conditions 90% of its activity is as an NAD^+-dependent dehydrogenase (type D form) which

cannot transfer electrons to molecular oxygen to form H_2O_2 or $O_2^{\cdot}$ (McCord, 1985). Proteolytic modification of the enzyme during purification or during in vivo ischemia converts it from the dehydrogenase form to the superoxide generating oxidase form (type O form) (Roy and McCord, 1983). The relative proportion of $O_2^{\cdot}$ and H_2O_2 released from the active site is dependent on the pH, oxygen concentration and substrate concentration (Fridovich, 1970; Nangano and Fridovich, 1985). Therefore under hyperoxic conditions an increased flux through the oxidase form of the enzyme would result in a greater production of the superoxide radical. Other soluble enzymes and proteins which produce $O_2^{\cdot}$ as an intermediate in their cycling reactions include aldehyde oxidase, dihydro-orotic dehydrogenase, flavoprotein dehydrogenase, tryptophan dioxygenase, hemoglobin and myoglobin (for review see Fridovich, 1978; Freeman and Crapo, 1982).

It has also been proposed that various components of the electron transport systems in mitochondria, endoplasmic reticulum and nuclear membranes are responsible for the formation of superoxide anions. Flohe et al., (1977) have presented evidence suggesting that mitochondrial H_2O_2 is primarily, if not exclusively, produced from superoxide radicals. In an attempt to identify the site of formation, electron transport chain inhibitors were used on uncoupled, intact mitochondria and on submitochondrial particles. It was soon recognized that the ubiquinone-cytochrome b_{566} region, when fully reduced, readily reacted with oxygen and was capable of producing H_2O_2. Their data as well as that of Nohl and Hegner (1978), suggested that cytochrome b_{566} was responsible for the formation of superoxide and that H_2O_2 production resulted from the dismutase reaction. The ubiquinone-cytochrome b region has been

confirmed as a superoxide generating site by several investigators using heart and lung mitochondria from bovine, rat and porcine sources (Turrens and Boveris, 1980; Turrens et al., 1982b; Turrens, Freeman and Crapo, 1982a), however they have implicated ubisemiquinone as the autoxidizable molecule responsible for the $O_2^{\cdot}$ production within this region. Several investigators have also identified the enzyme NADH dehydrogenase as a second $O_2^{\cdot}$ generating site along the mitochondrial respiratory chain (Tyler, 1975; Turrens and Boveris, 1980; Turrens et al., 1982b; Turrens, Freeman and Crapo, 1982a). Due to the rapid electron transfer rates between intramolecular redox groups, they found that it was difficult to ascertain whether $O_2^{\cdot}$ formation occurs at the enzyme itself or at the iron-sulfur clusters associated with the enzyme. In either case, at least two $O_2^{\cdot}$ generating regions have been identified along the mitochondrial electron transport chain when it is in a reduced state (Boveris and Cadenas, 1982).

The electron transport system of the endoplasmic reticulum has also been identified as a source of $O_2^{\cdot}$ formation, and its generation has been implicated in a number of hydroxylation reactions (Halliwell, 1977). In intact rat liver microsomes, as well as with purified enzyme extracts, superoxide anions are generated at the level of both NADPH-cytochrome P_{450} reductase and cytochrome P_{450} (Bartoli et al., 1977; Rosen, Finkelstein and Rauckman, 1982). Since the microsome is an artificially produced organelle it is not known whether the $O_2^{\cdot}$ radical remains bound to the active site in the complex or if it is released into the free solution in vivo, however the results do indicate that the respective enzymes can interact with oxygen to yield $O_2^{\cdot}$ (Halliwell, 1977). In addition to the electron transport sites, there are also superoxide

generating enzymes associated with the microsomal and plasma membranes (Freeman and Crapo, 1982). The NADPH oxidase associated with the plasma membrane of phagocytes is also a well studied biologic source of superoxides (Weiss and LoBuglio, 1982; Weiss, 1986).

There are also several endogenous compounds which are capable of undergoing autoxidation reactions to produce superoxide radicals. Included among these are catecholamines, reduced cytochrome c, reduced ferrodoxins, hydroquinones, leukoflavins and thiols (for references and review see Fridovich, 1978; Freeman and Crapo, 1982; Fridovich, 1983).

Whenever superoxide is generated in an aqueous system, hydrogen peroxide will also readily be formed through the dismutation reaction:

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \text{-------} > H_2O_2 + O_2 \qquad (1)$$

Over the biochemical pH range this reaction is quite rapid. The previously mentioned sites of superoxide generation would therefore also be considered sites of H_2O_2 generation. In fact, as stated, most if not all of the H_2O_2 generated by mitochondria is due to the dismutation of $O_2^{\cdot-}$. In addition to the mitochondrial and microsomal sites, H_2O_2 is generated in peroxisomes without the formation of the superoxide radical. There are several peroxisomal enzymes capable of generating H_2O_2 including; D-amino acid oxidase, fatty acyl-CoA oxidase and urate oxidase (Chance, Sies and Boveris, 1979).

The potential for $O_2^{\cdot-}$ and H_2O_2 formation within the cell is evident, but the reactivity of these two species within an aqueous environment has been questioned (Sawyer and Valentine, 1981). Both $O_2^{\cdot-}$ and H_2O_2 are unstable with respect to their dismutation products. Since the anions are stabilized by protons or cations, equilibrium reactions will be sensitive to pH, metal ions and solvent. The immediate surroundings at

the site of generation of these species will have a major impact on their stability and reactivity (Hill, 1979).

Superoxide in aqueous solution can act as a moderately effective one electron reducing agent (e.g., reducing ferricytochrome c and various transition metal ion complexes) or as a selective oxidizing agent acting on reducing substrates with readily transferable hydrogen atoms (e.g., ascorbate, a-tocopherol, hydrazines and hydroxylamines) (Fee and Valentine, 1977; Halliwell, 1979; Hill, 1979; Sawyer and Valentine, 1981). It is not, however, a highly reactive electron transfer oxidant of organic or inorganic substrates unless the peroxide anion formed can be stabilized. Furthermore, the reactivity of the superoxide radical with a given substrate is limited because of the rapidity with which it can dismutate to form oxygen and peroxide (Sawyer and Valentine, 1981). Although the view of superoxide as a dangerous species has been tempered because of its low reactivity, Fridovich (1986a), has cited several studies which have shown it to be directly responsible for various damaging effects, including the inactivation of catalase and glutathione peroxidase.

The superoxide anion can also be protonated to form the perhydroxyl radical, a much stronger oxidant with a pKa of 4.8. Normally at neutral pH only about 1 percent will exist in this form (Weiss and LoBuglio, 1982). However the generation of $O_2^{\cdot}$ in areas adjacent to membrane surfaces would favor protonation, since the polyanionic surfaces attract a high H^+ concentration, (i.e., 3 pH units lower than the bulk solvent), and since protonation of the superoxide radical would occur as movement into the hydrophobic interior of membrane anion channels took place (Freeman and Crapo, 1982; Fridovich, 1983). Therefore, to reiterate,

the environment (i.e., the pH and solvent) in which the superoxide radical is generated is extremely important in determining its reactivity.

The H_2O_2 produced from the dismutation reaction is a very powerful oxidant, but reactions with organic substrates are slow. Reactions with transition metal ions and complexes however occur much more readily and may be responsible for the formation of a more reactive species of oxygen, i.e., the hydroxyl radical (Fee and Valentine, 1977; Chance, Sies and Boveris, 1979).

Haber and Weiss had also proposed formation of the hydroxyl radical, as a result of direct interactions of the superoxide radicals with hydrogen peroxide (Willson, 1979). This reaction however has come under considerable debate (Fee and Valentine, 1977; Cohen, 1977; Willson, 1979). There is no chemical evidence to show that superoxide can directly reduce hydrogen peroxide in aqueous solutions in vitro (Fee and Valentine, 1977). However, there are numerous biological studies in which the product formed or effect observed was consistent with the formation and chemistry of the $^{\bullet}OH$ radical (Cohen, 1977).

Pure $O_2^{\bullet-}$ and H_2O_2 will not react together to form hydroxyl radical unless catalyzed by complexes of iron or some other transition metal (Fee and Valentine, 1977; Cohen, 1977). The following reactions have been proposed for hydroxyl radical production in biological systems (Czapski, 1984):

$$\text{Fe (III)} + O_2^{\bullet-} \text{ ----> } \text{Fe (II)} + O_2 \qquad (2)$$

$$\text{Fe (II)} + H_2O_2 \text{ ----> } \text{Fe (III)} + OH^- + {}^{\bullet}OH \qquad (3)$$

$$O_2^{\bullet-} + H_2O_2 \text{ ----> } O_2 + OH^- + {}^{\bullet}OH \qquad (4)$$

In these reactions, superoxide donates an electron to the ferric ion and in turn the ferrous ion then reduces H_2O_2 to form $^{\bullet}OH$. The overall net reaction has been termed the iron catalyzed Haber-Weiss reaction. The availability of free or low molecular weight chelated forms of iron within cells may be limited or non-exsistent, so this reaction remains speculative in vivo. However, in a model microsomal system, it has recently been shown that superoxide is capable of releasing iron from ferritin, thereby potentially raising the low molecular weight iron pool available for $^{\bullet}OH$ formation (Thomas, Morehouse and Aust, 1985). The availability of iron or other transition metals to act in this reaction is an area of current investigation (for reviews see Halliwell and Gutteridge, 1984; Weiss, 1986).

Hydroxyl radical formation can also occur in aqueous solution by the photolytic decomposition of H_2O_2 with ultraviolet radiation (Fee and Valentine, 1977), a reaction of considerable importance in the eye.

As the hydroxyl radical is a very powerful one electron oxidant, it is highly and indiscriminately reactive (Fee and Valentine, 1977). It will react with almost every type of molecule found in living cells, acting primarily through hydrogen atom abstractions, addition reactions (e.g. by adding to purine and pyrimidine ring structures of DNA), and electron transfer reactions (Willson, 1979; Halliwell and Gutteridge, 1984). This high reactivity however limits its lifetime as it will react with whatever biological molecule is in its vicinity, producing secondary radicals of variable reactivity (Halliwell and Gutteridge, 1984).

It is believed that singlet oxygen formation by means of photodynamic action does occur in biological systems and would be of importance

in the eye (Fridovich, 1984). Photooxidation can occur by direct oxidations, whereby a molecule is photoexcited and an electron released which can subsequently react to form superoxide; or by sensitized oxidations in which excitation of a photosensitizer molecule indirectly promotes oxidation of a second molecule (Foote, 1982). In sensitized oxidations, absorption of light promotes an electron to a higher energy orbital, which, after spin inversion, is termed a sensitizer triplet. Since the electrons in these orbitals are bound less strongly, they are readily removed by oxidizing agents. There are two basic types of photosensitized oxidation reactions (Foote, 1982). In type I reactions, the triplet sensitizer reacts directly with a substrate and allows for transfer of a hydrogen atom or an electron, such as with hydrogen abstraction from polyunsaturated fatty acids. The newly formed radicals can then undergo further reaction with oxygen or an organic molecule. In type II reactions, the sensitizer triplet interacts with oxygen via energy transfer to yield singlet molecular oxygen, which can then further react with various acceptors in solution. Two good singlet oxygen acceptor systems involve additions to olefins and dienes to form hydroperoxides and endoperoxides respectively (Foote, 1982). These types of oxidation reactions are very concentration dependent, since there is competition between substrate and oxygen for the sensitizer triplet. Low substrate concentration and high oxygen concentration would favor Type II reactions (Foote, Shook and Abakerli, 1984).

In summary, there is sufficient evidence to show that formation of the various oxygen metabolites does occur in biological systems. In addition to the above mentioned reactive oxygen metabolites, there are numerous other species (e.g., ferryl, perferryl, alkoxy and peroxy

radicals) which may be generated as a result of secondary reactions (Hill, 1979; Willson, 1979; Halliwell and Gutteridge, 1984). The potential for oxidative damage rests not only on those species with limited reactivity ($O_2^{\cdot}$ and H_2O_2) but also with the numerous secondary radicals which may be formed.

Protection Against Oxygen Toxicity

Enzymatic Scavenging of Superoxide

Biological interest in free radical chemistry grew tremendously with the discovery that the protein erythrocuprein was responsible for enzymatically scavenging the superoxide radical (McCord and Fridovich, 1969). The accumulation of superoxide radicals was prevented by catalysis of the dismutation reaction to form H_2O_2 and O_2, and the protein was therefore aptly renamed superoxide dismutase:

$$O_2^{\cdot} + O_2^{\cdot} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (5)$$

The catalyzed reaction functions at a rate of 2×10^9 $M^{-1}.sec^{-1}$ at 25 C, which is 10^4 fold faster than the spontaneous dismutation reaction (Fridovich, 1983).

There are three classes of superoxide dismutases common to respiring cells. All three catalyze the same reaction with comparable efficiency at rates which approach the diffusion limit (Fridovich, 1986b). The iron containing (FeSOD; 46 Kd) and manganese containing (MnSOD; 46 Kd) enzymes are characteristic of prokaryotes and are very similar in amino acid sequence. These enzymes are frequently dimeric containing one metal atom per subunit. The MnSOD form is also found in eukaryotes, primarily within the mitochondrial matrix, differing from prokaryotes only in that they are tetrameric. The class of SOD's containing 1 metal atom each of copper and zinc per subunit (CuZnSOD; 32 Kd) is usually

dimeric and commonly found in the cytosol of eukaryotes. A high molecular weight tetrameric form of this enzyme (135 Kd) has been found in human tissues and since it constitutes 90% of the SOD found in plasma it has been referred to as an extracellular SOD or ECSOD (Fridovich, 1983; Fridovich, 1986b). The three classes of SOD are distinguishable by their sensitivities to various reagents. The CuZnSOD form is inhibited by 5 mM cyanide while the MnSOD and FeSOD forms are resistant to it. Hydrogen peroxide inactivates CuZnSOD and FeSOD but has no effect on MnSOD. Sodium dodecyl sulfate (2%) can be added to eliminate MnSOD activity without affecting CuZnSOD, and lastly alkaline pH (pH 10.0) can be used to suppress MnSOD activity without affecting CuZnSOD activity. A measure of activities at pH 7.8 and 10.0 would allow for the calculation of both of these SOD forms (for references see Fridovich, 1986b).

The mechanism of action for all of the superoxide dismutases can be written:

$$E - Me^{n} + O_2^{\cdot-} \text{-----} > E - Me^{n-1} + O_2 \qquad (6)$$

$$E - Me^{n-1} + O_2^{\cdot-} \text{-----} > E - Me^{n} - O_2^{-2} \qquad (7)$$

$$E - Me^{n} - O_2^{-2} \xrightarrow{H^+} E - Me^{n} + HO_2^{-} \xrightarrow{H^+} H_2O_2 \qquad (8)$$

where E denotes the enzyme and Me the metal ion. In the CuZnSODs it is the copper that functions in electron transport during the catalytic cycle, while the zinc is thought to primarily play a structural role lending stability to the molecule. It has been proposed that the first superoxide anion at the active site reduces the metal ion and disrupts an imidizolate ligand bond. The released imidizolate is highly basic and would then give up that proton when a second superoxide anion entered the active site and reoxidized the active metal ion to form HO_2^- as

product. Once released from the active site the HO_2^- could then be protonated to form H_2O_2 in solution (Fridovich, 1986b).

Only a brief discussion of the enzyme has been presented here, since more extensive reviews are available in the literature (Fridovich, 1978, 1983, 1986b).

Enzymatic Scavenging of Hydrogen Peroxide

As previously mentioned, hydrogen peroxide is slow to react, but it is capable of participating in the generation of the reactive hydroxyl radical. Accumulation of hydrogen peroxide is prevented by two related enzymes, the catalases and the peroxidases. Both catalyze the reduction of H_2O_2 to water. The enzymes differ in that catalases can use H_2O_2 as the source of electrons for the reduction process, whereas peroxidases use some other reductant. Catalases can also act as peroxidases using other electron donors when the concentration of H_2O_2 is low. The respective reactions are given below:

$$H_2O_2 + H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2 \qquad (9)$$

$$H_2O_2 + H_2R \xrightarrow{\text{Peroxidase}} 2H_2O + R \qquad (10)$$

The physiological reductant for peroxidases may be glutathione, ascorbate or cytochrome c, and these would be continously renewed by NADH and NADPH (Fridovich, 1977). The peroxidase activity of catalase also requires an electron donor but the compound used in vivo is not known (Fridovich, 1977).

The catalase enzyme (240 Kd) is tetrameric with an Fe^{3+} - protoporphyrin ring at its active site. It is found in varying concentrations in tissues and is reportedly highest in liver and kidney where it is localized in peroxisomes. In other tissues it may be found in microperoxisomes or it may be distributed throughout the cytosol, e.g.

erythrocyte catalase (Chance, Sies and Boveris, 1979).

The most common peroxidase is the selenium dependent form of glutathione peroxidase (GSHPx) which has four atoms of selenium per molecule in its active site. It is a 76 Kd enzyme composed of 4 subunits. It catalyzes the reaction of hydrogen peroxide with reduced glutathione (GSH) to form oxidized glutathione and water. Although it is specific for its hydrogen donor, GSH, it is non-specific for the hydroperoxide and can react with a range of substrates from H_2O_2 to organic hydroperoxides. It is widely distributed with variable tissue concentrations, and is located in the cytosol and mitochondrial matrix but not in peroxisomes (Chance, Sies and Boveris, 1979). The complimentary distribution of these two enzymes apparently acts to effectively control the intracellular concentration of hydrogen peroxide.

Secondary Antioxidant Defenses

Although superoxide dismutases, catalase and glutathione peroxidase provide the first line of defense in the cell by preventing $O_2^{\cdot}$ and H_2O_2 from generating more reactive oxidants, other defense mechanisms have been described. Should the primary defense system become overwhelmed or if oxidant attack should occur at a site inaccessible to the primary antioxidants, then several other lines of defense against oxidative attack are available. The multilayered system of defense which has evolved to counter oxidative attack has recently been discussed (Fridovich and Freeman, 1986). As previously mentioned, glutathione peroxidase is capable of detoxifying lipid hydroperoxides (the byproducts of lipid free radical reactions) and can therefore act as a secondary defense mechanism. In addition to its role as substrate for the glutathione peroxidase enzyme, glutathione can also serve as a secondary

antioxidant. Glutathione and other thiol (SH) compounds exert a protective action against oxidative damage by undergoing non-enzymatic hydrogen abstraction to restore function to enzymes which have been oxidized. Glutathione may also serve as a nucleophile in conjugation reactions involving GSH transferase (Mannervik et al., 1983). The thiol transferases catalyze thiol interchange between glutathione and protein disulfides or low molecular weight disulfide substrates to form oxidized glutathione:

$$GSH + GS\text{-}S\text{-}R \xrightarrow{\text{Thiol Transferase}} GSSG + HS\text{-}R \qquad (11)$$

The regeneration of GSH from mixed disulfides occurs by way of conversion to glutathione disulfide, which is then reduced by glutathione reductase utilizing NADPH (Mannervik et al., 1983). Other secondary defenses include the antioxidants ascorbic acid and Vitamin E, which function as scavengers through hydrogen abstraction reactions (Nishikimi, 1975; Bielski, Richter and Chan, 1975; Tappel, 1972; Packer, Slater and Willson, 1979).

There are a number of other mechanisms utilizing numerous enzymes, small molecules, metal ions, etc., which can act to prevent, limit and repair oxidative damage to cellular constituents. The primary antioxidants discussed above, represent the most commonly studied mechanisms and provide background for this study.

A Potential Role for Oxygen Metabolites in Hyperoxic Injury

It has long been presumed that oxygen metabolite formation would be increased under hyperoxic conditions in vivo. Fridovich has demonstrated an increased rate of superoxide anion formation by the isolated enzyme xanthine oxidase with increasing oxygen tensions (Fridovich, 1970; Nangano and Fridovich, 1985). However, it is difficult to obtain

measurements of the steady state levels of the reactive oxygen metabolites in biological systems because of their very short half lives. There is now sufficient indirect evidence to show that hyperoxic exposure can indeed increase the rate of formation of superoxide and hydrogen peroxide.

Freeman and Crapo (1981), have used the method of CN^--insensitive respiration to obtain an approximate index of intracellular $O_2^{\cdot}$ and H_2O_2 production in rat lung slices, since oxygen consumption in the presence of cyanide (which inhibits mitochondrial cytochrome oxidase), is representative of the one and two electron reduction of oxygen. They found that hyperoxic conditions will increase the proportion of CN^--resistant respiration in the lung preparations relative to total oxygen consumption measured in the absence of cyanide. Cyanide insensitive respiration increased linearly as rat lung slices were incubated in various concentrations of oxygen from 5% to 95% O_2. Isolated mitochondria were also found to increase their rate of CN^--insensitive respiration from 0 nmol O_2 consumed/min/mg protein at 15% O_2 to 1.34 nmol O_2 consumed/min/mg mitochondrial protein at 85% oxygen (Freeman and Crapo, 1981). Similar results were seen when lung homogenates were subjected to measurements in hyperoxia, with CN^--insensitive respiration accounting for 7% of total respiration in air and 17% of total respiration when incubated in 80% oxygen (Freeman, Topolosky and Crapo, 1982). Thus, it was concluded that hyperoxia enhanced the cellular production of $O_2^{\cdot}$ and H_2O_2 and implied an essential role for these metabolites in hyperoxic lung damage (Freeman and Crapo, 1981; Freeman, Topolosky and Crapo, 1982).

Superoxide production under hyperoxic conditions was also

investigated in submitochondrial particles that had been washed free of superoxide dismutase (Turrens et al., 1982b). The cyanide insensitive respiration, epinephrine oxidation and cytochrome c reduction methods were used to quantitate superoxide production in lung submitochondrial particles. Superoxide production increased linearly with increasing oxygen tensions in porcine lung submitochondrial particles above an O_2 tension of zero, and in rat lung submitochondrial particles above an oxygen concentration of 40%.

Hydrogen peroxide release by mitochondria and microsomes has also been reported to be increased under hyperoxic conditions (Boveris and Chance, 1973; Chance, Sies and Boveris, 1979; Turrens, Freeman and Crapo, 1982). A four fold increase in H_2O_2 formation was noted in pigeon heart mitochondria and a 15-20 fold increase was seen in rat liver mitochondria when the pO_2 was raised to 19 atm of pure oxygen (Boveris and Chance, 1973). Hyperoxia also caused a 10-fold increase in H_2O_2 release by porcine lung mitochondria when the O_2 concentration was increased from room air to 100% oxygen and a linear increase in H_2O_2 formation by NAD(P)H supplemented microsomes was seen when oxygen concentrations were increased above 21% (Turrens, Freeman and Crapo, 1982a).

Although these studies involved indirect, in vitro measurements, it is quite clear that hyperoxia enhanced the rate of formation of the partially reduced forms of oxygen. This evidence supports the current belief that hyperoxia induced oxygen toxicity is due to the production of reactive oxygen species at a rate in excess of the capacity of the cellular defense mechanisms to inactivate them and that the susceptibility of a tissue to oxygen toxicity would vary depending on the cells defense mechanisms (Jamieson et al., 1986).

Resistance of Teleost Ocular Tissues to Hyperoxic Injury

In contrast to most tissues, the teleost retina appears to be resistant to hyperoxic injury. The choroidal rete mirabile at the back of the teleost eye operates in conjunction with the choriocapillaris to generate an oxygen tension which is far in excess of that found in arterial blood (Wittenberg and Wittenberg, 1962; Fairbanks, Hoffert and Fromm, 1969; 1974). Since the teleost retina is avascular, it is dependent on this high pO_2 to maintain normal visual function (Fonner, Hoffert and Fromm, 1973). The oxygen tension profile across the back of the rainbow trout eye has been determined (Desrochers et al., 1985). The tissue pO_2 at the choriocapillaris averaged 380 mmHg and 124 mmHg at the retinal-vitreal interface. In fact, a retinal pO_2 as high as 750 mmHg was recorded in one fish. Oxygen tensions in this range have been shown to cause oxygen toxicity in several species (Yanoff et al., 1970; Noell, 1955a, 1955b). Further, the rainbow trout has repeatedly been shown to be resistant to oxygen toxicity. In a comparative study on the influence of hyperbaric oxygen on cellular respiration (Baeyens, Hoffert and Fromm, 1973), dog retinal tissue respiration was significantly decreased after a 24 hr incubation at 2 atm oxygen, while trout retinal consumption was significantly increased. No change in respiration of frog retinal tissues was observed. Liver and brain tissue respiration was inhibited by hyperbaric oxygen in all three species after 24 hr exposures. The fact that oxygen exerted a toxic effect on trout brain tissue provided evidence that not all teleost neural tissue was equally insensitive to oxygen and that the resistance was unique to the retina. It was concluded that inhibition of respiration most likely resulted from inactivation of a critical enzyme of carbohydrate metabolism

(Baeyens, Hoffert and Fromm, 1973). A second comparative study by the same authors investigated the influence of oxygen on lactate dehydrogenase (LDH) activity (Baeyens, Hoffert and Fromm, 1974). LDH activity was determined in homogenates of retina, liver and brain from trout, frogs and dogs after exposure to hyperbaric oxygen (2 atm) for 24 hrs. All tissue preparations except trout retina exhibited lower LDH activity after hyperbaric oxygen exposure.

Ubels, Hoffert and Fromm (1977), provided additional evidence to support the hypothesis that the teleost retina was resistant to oxygen toxicity. Electroretinograms (ERG) were recorded in vitro using frog, rat and teleost retinas. Attenuation of the ERG after exposure to 5 atm oxygen was observed in frog retinas after a period of 6 hours and in rat retinas after just 90 - 105 minutes. Goldfish and trout ERG's however did not exhibit any significant changes attributable to oxygen exposure after the 6 hour period as normal function was observed in the goldfish retina for the duration of the exposure period. Ubels and Hoffert (1981) also demonstrated that trout retinal Na^+-K^+ ATPase was resistant to oxygen exposure at 15 atm pure oxygen, while Na^+-K^+ ATPase activity in the rat was inhibited by 66% at this pressure.

It appears as though the trout retina is unique in that it is functionally dependent on the high pO_2 generated at the back of the eye and is resistant to oxygen toxicity. The mechanism of this resistance is not yet understood, but in light of the numerous studies describing the toxic actions of oxygen, it must be hypothesized that the resistance is either due to an inability to generate reactive oxygen metabolites in hyperoxia and/or to an efficient antioxidant defense system within this tissue.

MATERIALS AND METHODS

Experimental Animals

Rainbow trout (Salmo gairdneri) weighing 150-450 g were obtained from Balders Fish Farming Enterprise (Baldwin, MI). They were held in fiberglass tanks at 9±1 C, with a continous flow of dechlorinated tap water which was aerated by compressed air filtered through activated charcoal. The fish were fed a maintenance diet of commercial trout pellets and exposed to a photoperiod of 16 h light - 8 h dark.

Tissue Preparation

Trout were anesthetized with tricaine methane sulfonate (MS-222; Finquel, Ayerst Laboratories, Inc., NY) and secured in an inverted position in a holding trough. They were ventilated with water passed over their gills at a rate of 4.2 L/min. An incision was made along the mid-ventral line and the heart exposed. Sodium heparin (0.1 ml, 1000 units/ml; Upjohn Co., Kalamazoo, MI) was injected directly into the ventricle and allowed to circulate prior to perfusion in order to prevent clotting. The ventricle was then cut and a cannula (PE-50) inserted through to the bulbous arteriosus. The atria were cut and Cortland's saline solution (Wolf, 1963; Appendix A) was perfused through the tissues at a rate of 6 ml/min for approximately 8 minutes. Perfusion was stopped when the tissues, particularly the liver and gills, appeared to be free of blood. Because hemoglobin is known to interfere with many of the colorimetric assays used in this study, perfusion of the tissues

with saline was required prior to enzymatic analysis. The liver, eyes and brain were immediately excised, placed in a beaker containing Cortland's saline solution and kept on ice. Brain and liver tissues were trimmed, blotted dry, weighed and then diluted with the appropriate buffers used in the respective assays. The enucleated eyes were trimmed of the extraorbital muscles and weighed. An incision was made along the corneal limbus so that the iris, cornea and lens could be lifted from the eyecup with a forceps. The vitreous adheres to the retina in the trout, but an attempt was made to remove as much of the vitreous as possible without damaging the retina. The neural retina, pigmented epithelium, perfused choroid body and any remaining vitreous were then scooped from the eyecup, weighed and diluted with the appropriate solutions used in the respective assays.

Cyanide Insensitive Oxygen Consumption Determinations

To characterize the effect of oxygen concentration on the production of partially reduced oxygen species, oxygen uptake by tissue homogenates was measured in the absence and presence of 1.0 mM potassium cyanide, with and without the addition of 1.0 mM B-NADH (Grade II from yeast, Lot #93F-7255, Sigma Chemical Co., St. Louis, MO) (Freeman and Crapo, 1981; Freeman, Topolosky and Crapo, 1982). Oxygen consumption was determined polarographically using 3.0 ml waterjacketed glass chambers (Cole-Palmer Instrument Co., Chicago, Il) fitted with Clark type oxygen electrodes (Model 5331) attached to a YSI Model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH) and the change in pO_2 recorded on a dual channel vertical-face recorder (Model 486, Cole-Palmer Instrument Co., Chicago, Il). Temperature was maintained at a constant 15 ± 1 C using a Refrigerated Bath Circulator (Neslab Instruments, Inc.,

Portsmouth, NH). A general description of the electrode and its preparation is given by Green and Hill (1984). Electrode calibration and stability were determined by immersing the oxygen probes in 3 mls of constantly stirred KCL-KH_2PO_4 incubation medium, pH 7.4 (Appendix A), equilibrated at 15 C and saturated with either room air (21% O_2) or 100% oxygen. The probes are held by a Lucite plunger that fit snugly into the glass chambers. There is a small access groove along the length of the plunger, so in order to minimize the diffusion of oxygen out of the polarograph chamber during measurements made under 100% O_2, the chambers were capped with an inverted plastic centrifuge tube which was continuously flushed with 100% O_2.

In order to obtain a sufficient amount of tissue for analysis, samples from 2 perfused fish were pooled and used in this series of experiments. After weighing, the tissues were diluted with KCl-KH_2PO_4 incubation medium (pH 7.4) containing 0.1 mM Na_2-EDTA and 5 mM glucose. Homogenates of these tissues were made using a Corning Ten Broeck tissue grinder and kept on ice. Two aliquots (2.8 ml) of the homogenates were removed and placed in separate chambers so that simultaneous recordings of total respiration and CN^--resistant respiration could be performed. Initial measurements were made in the absence of NADH at 21% O_2. Homogenates were allowed to equilibrate to temperature and become saturated with room air. A volume of 100 ul of 30 mM KCN was added to the chamber used to measure CN^--resistant respiration. Incubation medium was then added to the respective chambers to bring the volume to 3.0 mls. After determination at 21% oxygen the plungers were lifted above the level of the homogenates and 100% oxygen was flushed in the airspace between homogenate and plunger for a period of at least 15 minutes. After

saturation with 100% oxygen the electrodes were returned to the homogenates and O_2 uptake was recorded at this elevated oxygen tension. The chambers were then replaced and the procedure repeated with fresh aliquots of homogenate to which 100 ul of 30 mM NADH was added. Total respiration and CN^--insensitive respiration rates were determined using the oxygen solubility coefficient at 15 C of 0.034 ml O_2/ml fluid. Dissolved oxygen was determined using the following equation:

$$0.034 \times \text{total volume} \times FO_2 \times (DBP/760) \times 1000 = \text{ul } O_2 \qquad (12)$$

where: FO_2 is the fraction of oxygen and DBP is the dry barometric pressure. Respiration rates were expressed as nmol O_2 consumed/min/mg protein (Umbreit, Burris and Stauffer, 1964). Protein determinations were made using the Lowry method (Oyama and Eagle, 1956; Appendix B).

A Paired-t analysis was used to determine whether the addition of 1.0 mM NADH had a significant effect on baseline rates of oxygen consumption. The same test was applied to determine the effect of hyperoxic exposure on total and CN^--insensitive rates of oxygen consumption. Differences between tissue homogenates for the same set of conditions were determined using a one-way analysis of variance and the Tukey's w-procedure for multiple comparisons testing (Steel and Torrie, 1960).

Superoxide Dismutase Assay

Superoxide dismutase (SOD) was assayed on the basis of its ability to inhibit the reduction of ferricytochrome c by superoxide anion generated by the xanthine-xanthine oxidase system:

$$\text{xanthine/xanthine oxidase} \longrightarrow O_2^{\cdot} \qquad (13)$$

$$O_2^{\cdot} + \text{cyt c}^{\,Fe(III)} \longrightarrow O_2 + \text{cyt c}^{\,Fe(II)} \qquad (14)$$

$$O_2^{\cdot} + O_2^{\cdot} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (15)$$

(McCord and Fridovich, 1969). The rate of reduction of ferricytochrome

c was followed at 550 nm using a recording spectrophotometer (Beckman Model DB-G Grating Spectrophotometer, Beckman Instruments, Inc., Fullerton, CA). The xanthine oxidase concentration was adjusted to produce a rate of cytochrome c reduction of 0.025 absorbance units/min in the assay. One unit of SOD activity is defined as that amount of enzyme which inhibits the rate of cytochrome c reduction by 50%, under the specified conditions. Since there is a small amount of ferricytochrome c reduction which cannot be inhibited with SOD, the true uninhibited rate of reduction must be determined from the difference between the reduction rate in the absence of any SOD and the rate in the presence of excess amounts of SOD (e.g., 20 BESOD units in the reaction system). The degree of inhibition at each dose can likewise be determined from the difference between the rate of reduction observed and the rate in the presence of excess amounts of SOD and then expressed as percent inhibition of the true uninhibited rate of ferricytochrome c reduction. Bovine erythrocyte superoxide dismutase (BESOD), E.C. No. 1.15.1.1, was used as the purified SOD standard (Sigma Chemical Co., St. Louis, MO (Lot #16C-8030; 2900 U/mg protein; 2880 U/mg solid; assayed as per McCord and Fridovich, 1969). Preparation of the standard is described in Appendix C.

Since measurements were made in crude tissue homogenates, several controls were run in an effort to detect endogenous interfering substances. Crude homogenates may contain substances which are capable of interfering with assay reactions. For example, any substance in the crude homogenate which can react with reduced cytochrome c may alter apparent SOD activity. One of the most common problems is the presence of contaminating cytochrome oxidase or peroxidases. Either of these

enzymes can re-oxidize ferrocytochrome c and artefactually increase "apparent" SOD activity of the homogenate. These enzymes, if present, would regenerate ferricytochrome c and thus add to the 'apparent' SOD activity of the homogenate. However, sodium azide (10^{-5} M), an effective inhibitor of the cytochrome oxidases and peroxidases (Flohe and Otting, 1984), did not alter the rate of reduction of ferricytochrome c in any of the homogenates. Thus, the oxidases and peroxidases were not present in sufficient concentrations to interfere with the SOD assay.

In addition, a parallel-line analysis of variance was applied to the data in order to reveal the presence of interfering substances (Eldred and Hoffert, 1981). The applicability of these statistics is dependent on a linear response so that the slopes of the dose response curves can be compared to an SOD standard. For each tissue sample, a dose response curve was generated whereby percent inhibition was determined over at least 4 concentrations with duplicates at each concentration. This response was linear below 50-60% inhibition for the bovine erythrocytic SOD standard and for the tissue homogenates. However the slope of the individual tissue response curves was dependent upon the concentration of superoxide dismutase within the tissue. Tissue samples which were more dilute than the standard would have lower slopes and lie to the right of the standard, while samples more concentrated than the standard were shifted to the left of the standard with higher slopes. All of the lines would theoretically pass through the origin. In order to test for endogenous substances which might interfere with the response curve, this dilution effect must be eliminated without affecting the linear response of the curves. The standard and tissue homogenate dose response curves should differ at any given level of inhibition by a potency

ratio which would be independent of the level of response. This was accomplished by converting the response to log of percent inhibition and plotting the responses against a log dose (Goldstein, 1965). Since, for the most part, successive doses were increased by doubling, the values could be coded. Ascending doubled doses were coded by assigning integers values to them, beginning with the value 1 at the lowest dose (25 ul). Coded log units therefore differed by log 2. The procedure for decoding the Coded Log Dose Scale is given in Appendix C. This transformation eliminated the dilution effects, so that the response of the tissue homogenates could now be statistically compared to the response of the standard. If no endogenous interfering substances are present, then the dose response curve for the tissue homogenate should be parallel to the dose response curve for the BESOD standard. If this were not the case, the relative potency computed from converging lines would differ with the level of response, and the homogenate could not be assigned a single potency. Neither the ED_{50}'s or the potency ratios were affected by the transformation.

Tissue superoxide dismutase activity was measured in homogenates which had been stored frozen for less than one week. Tissues were initially diluted (retina, 300 mg/ml; brain, 250 mg/ml; liver, 80 mg/ml) with an ice cold phosphate/EDTA solution (pH 6.5) (Appendix A) and homogenized on ice with a Corning Ten Broeck tissue grinder. The homogenates were frozen in a dry ice/alcohol bath, stored frozen and used within one week. After thawing the homogenates for assay, they were appropriately diluted with sodium phosphate buffered saline (pH 7.8). The dilute homogenates and BESOD standard (2.9 ml distilled H_2O/vial) were then sonicated over ice for four consecutive 15 second periods at 5

second intervals using a Sonifier Cell Disruptor at a power setting of 20 watts (Model W-185-C, Heat Systems Co., Melville, L.I., NY, fitted with a Micro Tip, Branson Instruments, Inc., Danbury, CT). To disrupt cell membranes further, tissue homogenates were subjected to three cycles of freezing and thawing in a dry ice-ethanol bath and a 50 C water bath, respectively. The homogenates were then centrifuged at 13,200g for 30 min at 4 C (Sorvall, Inc., Model RC2-B, SM-24 rotor, Newtown, CT). The supernatants were then removed and recentrifuged at the above settings for an additional 20 minutes. The final supernatants were then removed and kept on ice until used in the assay. Cytosolic SOD (CuZnSOD form) was distinguished from mitochondrial SOD (MnSOD form) by the former's sensitivity to 2 mM cyanide in the assay medium (Flohe and Otting, 1984). A detailed description of the procedure and assay design is given in Appendix C.

The variation about the dose response lines was estimated using the ANOVA approach to regression analysis as described by Neter and Wasserman (1974). All lines were initially tested to ensure linear regression functions and significant positive slopes (i.e., F-tests). Comparison of the standard and sample regression lines followed from the analysis of variance. The error variances of the lines were tested for homogeneity in order that the comparison for parallelism be appropriate. For a valid assay, the crude homogenates should differ from the standards in dilution only and should be parallel.

One unit of SOD activity was defined as that amount of standard or tissue homogenate which inhibited the true rate of ferricytochrome c reduction by 50%. The volume of standard added at the 50% inhibition point was termed the $ED_{50}(S)$. The concentrations of the standard (SOD

units/ul) could then be obtained from the reciprocal of the ED_{50}. The ED_{50} for the tissue homogenates was also determined at 50% inhibition ($ED_{50}(T)$), and the potency of the tissue homogenate was expressed as the ratio of $ED_{50}(S)/ED_{50}(T)$. The potency ratio can be used to express tissue homogenate SOD activity in terms of activity of the commercially obtained BESOD standard. The 95% confidence limits about the ED_{50}'s and the potency ratios were also determined (Goldstein, 1965).

To facilitate comparisons between samples, the amount of wet tissue weight at the tissue ED_{50} was determined as well as the tissue protein concentration using the method of Lowry (Oyama and Eagle, 1956). The SOD activity could then be expressed as units/mg wet tissue weight or units/mg tissue protein. A one way analysis of variance was used to detect statistical differences in tissue SOD activities.

Cytochrome c Oxidase Assay

Cytochrome c oxidase catalyzes the electron transfer from ferrocytochrome c to dioxygen in the respiratory chain of the mitochondria. Since the MnSOD form is contained within the mitochrondria, a measure of cytochrome c oxidase (COX) activity within these organelles would provide a means of comparison for this form of SOD between tissues. Cytochrome c oxidase activity was determined using a slight modification of the method described by Rafael (1983) where COX is assayed by following the rate of oxygen uptake polarographically by means of an oxygen electrode. Oxygen concentration in the assay system was measured with a Clark-type oxygen electrode. Cytochrome c was maintained in a reduced state with the addition of ascorbate, and N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) was added to mediate electron transfer. The blank rates of autoxidation due to ascorbate and TMPD

alone and in the presence of ferrocytochrome c were determined and subtracted from the measured oxygen uptake. The uptake per unit time of oxygen dissolved in the assay solution at 25 C was used as the measure of COX catalytic activity.

The electrode and experimental setup was the same as that used in the cyanide insensitive oxygen consumption determinations. Cytochrome c oxidase and SOD activities were determined in the same tissue homogenates, so aliquots of the homogenates prepared for SOD activity were used. The homogenates were diluted 1 + 1 with a 1% Lubrol solution (type WX from Sigma, prepared in the phosphate/EDTA buffer) and kept at 0-4 C. Cytochrome c oxidase activity was calculated in terms of the rate of oxygen consumed (nmol O_2/min/mg protein) and also in terms of the standard unit, the katal (kat), and expressed as nkat/mg protein. A detailed description of the assay design and determination of activity is given in Appendix D. A one way analysis of variance was used to determine statistical differences in tissue activity and Tukey's w-procedure used for a multiple comparisons test.

Catalase Assay

The catalatic activity of catalase was assayed in crude tissue extracts using a rapid spectrophotometric variation of the technique for determining catalase activity by measuring H_2O_2 by titration with permanganate (Cohen, Dembiec and Marcus, 1970). When used on crude tissue homogenates the authors stress the importance of incorporating ethanol and Triton X-100 to increase the observable catalase levels. Ethanol acts by decomposing Complex II, an inactive complex of catalase with H_2O_2 that forms spontaneously in crude homogenates, and Triton X-100 acts either by releasing catalase from its particulate form in the

peroxisomes or by allowing free access of the substrate. A detailed description of the assay is given in Appendix E.

Liver samples were placed in cold tissue homogenizers, diluted with 10 volumes of ice cold isotonic buffer, and homogenized while in an ice water bath. Pooled retina (6 eyes) and brain samples from three fish were treated similarly but diluted with only 3 volumes of isotonic buffer. Homogenates were centrifuged for 10 min at 700g (Sorvall, Inc., Model RC2-B, SM-24 rotor, Newtown, CT) to remove cellular debris. A 3 ml aliquot of each supernatant was removed and ethanol added to a final concentration of 0.17 M (i.e. 10 ul EtOH/ml of supernatant). Samples were then incubated for 30 min in an ice water bath (0-2 C) to decompose Complex II. After incubation, 10% Triton X-100 was added to a final concentration of 1.0%. Aliquots of the tissue samples were then diluted with ice cold isotonic buffer so that they would fall along the linear portion of the standard curve.

The catalase standards were prepared from crystalline bovine liver catalase (Sigma Chemical Co., St. Louis, MO, #C-40, lot# 53F-7070; 11,000 units/mg protein). The stock standard solution was prepared with ice cold distilled water to a concentration of 0.20 mg/ml. Various dilutions were prepared from this stock catalase solution, such that a range of 5 ug to 0.5 ug of catalase were added to the reaction vessels.

All reactions were carried out in an ice water bath (0-2 C). The enzyme catalyzed decomposition of H_2O_2 was followed. The reagents were added sequentially at fixed intervals and allowed to react for exactly 3 minutes. The remaining H_2O_2 was then reacted with a standard excess of $KMnO_4$ and the absorbance of the residual $KMnO_4$ was measured at 480 nm using a Gilford single beam spectrophotometer with optics from a Beckman

DU Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA). The cuvette chamber of the spectrophotometer was maintained at 2-4 C with a refrigerated water bath to prevent the turbidity which developed when measurements were made at room temperature.

Catalase activity was expressed in terms of Bergmeyer units (units/g tissue), International units (units/g tissue) and Kat.f. (Katalasefahigkeit) units (Kat.f. $min^{-1}.mg^{-1}$ protein). See Appendix E for calculations and expression of activity.

Glutathione Peroxidase Assay

Glutathione peroxidase catalyzes the reduction of H_2O_2 and organic hydroperoxides using glutathione as the substrate. The oxidized form of glutathione (GSSG) can be cycled back to the reduced form (GSH) with the addition of exogenous glutathione reductase and NADPH, thereby maintaining substrate concentration for the enzyme. Glutathione peroxidase activity is therefore measured indirectly by following the decrease in absorbance of the reaction mixture at 340 nm as NADPH is converted to NADP (Paglia and Valentine, 1967).

Cytosolic fractions of the tissue homogenates were prepared for the determination of selenium-dependent glutathione peroxidase activity (Tappel, Chaudiere and Tappel, 1982). Tissues were diluted 1:5 (w:v) (livers 1:7) with cold 0.25 M sucrose and homogenized in an ice water bath using a glass Corning Ten Broeck homogenizer. Homogenates were centrifuged at 4 C for 15 min at 13,000g, the supernatant pipetted off and then recentrifuged for 30 min at 28,000g (Sorvall Inc., Model RC2-B, SM-24 rotor, Newtown, CT). The final supernatants were removed for assay of selenium dependent peroxidase activity using 2.2 M H_2O_2 as the substrate. A detailed description of the assay is given in Appendix F.

Activity was determined from the molar extinction coefficient for NADPH at 340 nm (6,220 $M^{-1}.cm^{-1}$; Mattenheimer, 1971) and expressed as mUnits/mg protein or nmoles.$min^{-1}.mg^{-1}$ protein. Protein determinations were made using the dye binding method of Bradford (1976). A one way ANOVA was used to determine statistical differences in tissue activity and Tukey's w-procedure used for a multiple comparisons test.

Glutathione Assay

This procedure is a spectrophotometric method for the determination of sulfhydryl content in the protein-bound (PB-SH) and nonprotein-bound (NP-SH) fractions of various tissues. The method is based on the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid), by SH groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH. The nitromercaptobenzoic acid anion has an intense yellow color which absorbs at 412 nm and is used to measure SH groups (Sedlak and Lindsay, 1968).

Tissues were homogenized in approximately 20 volumes of 0.02 M EDTA in a Ten Broeck homogenizer in an ice water bath; (i.e. tissues were diluted in order to obtain absorbance readings which fell between 0.1 and 0.8 absorbance units when 0.5 ml and 2.5 ml of homogenate were used for the estimation of total and non-protein bound sulfhydryls respectively). The homogenates were kept in an ice bath until used. A detailed description of the assay is given in Appendix G.

Sulfhydryl concentrations were determined from the molar extinction coefficient of 13,600 $M^{-1}.cm^{-1}$ at 412 nm and expressed on a per mg protein basis. Protein concentrations were determined using the method of Lowry (Oyama and Eagle, 1956). A one way analysis of variance was used to determine statistical differences in tissue activity and Tukey's w-procedure used for a multiple comparisons test.

RESULTS

Total and Cyanide Insensitive Oxygen Consumption by Trout Retina

Oxygen uptake by tissue homogenates was measured in the absence and presence of 1.0 mM potassium cyanide in order to establish the total rate of O_2 consumption as well as the cyanide resistant rate of oxygen utilization. The latter uptake rate was used as an estimate of the rate of formation of partially reduced species of oxygen (i.e., $O_2^{\cdot}$ and H_2O_2 production). Measurements were made in room air (21% O_2) and in 100% oxygen and are summarized in Table 1. Measurements were made in homogenates from the hyperoxic resistant retina and compared to values obtained from the oxygen sensitive brain and liver homogenates. Total oxygen uptake at 15 C in room air was 0.58 nmol O_2/min/mg protein in the retina, and 1.36 and 2.03 nmol O_2/min/mg protein in liver and brain respectively. Cyanide insensitive respiration accounted for 21%, 26% and 7% of total respiration in the retina, liver and brain respectively (Table 1). There was a significant increase in total oxygen consumption by the retinal homogenate after it was incubated in 100% oxygen, however no change in total consumption was observed for the other two tissue homogenates. Cyanide insensitive consumption however rose dramatically in all 3 tissue homogenates after incubation in 100% oxygen, increasing to values of 0.46, 0.62 and 0.55 nmol O_2/min/mg protein for retina, liver and brain respectively.

Total and CN^--insensitive oxygen consumption was also determined

Table 1. Total and CN^--insensitive rates of oxygen consumption. Rainbow trout tissue homogenate respiration rates with and without the addition of 1.0 mM NADH (nmol O_2 consumed/min/mg protein).

	Retina		Brain		Liver	
	without NADH	with NADH	without NADH	with NADH	without NADH	with NADH
Total O_2 Consumption Room Air	0.58^{B} ±0.17	$0.69^{B,L}$ ±0.17	2.03 ±0.50	2.32^{*} ±0.36	1.36 ±0.83	1.92^{*} ±1.03
CN^--Insensitive O_2 Consumption Room Air	0.12^{L} ±0.04	0.14^{L} ±0.04	0.14^{L} ±0.06	$0.25^{*,L}$ ±0.05	0.36 ±0.06	0.51^{*} ±0.11
Total O_2 Consumption 100% Oxygen	$0.72^{T,B}$ ±0.11	$0.75^{B,L}$ ±0.12	2.01 ±0.54	$2.60^{*,T}$ ±0.32	1.26 ±0.95	2.31 ±1.31
CN^--Insensitive O_2 Consumption 100% Oxygen	0.46^{T} ±0.07	$0.49^{T,B,L}$ ±0.10	0.55^{T} ±0.12	$0.78^{*,T}$ ±0.06	0.62^{T} ±0.18	$0.90^{*,T}$ ±0.10

$\bar{X}$ ± SD; n = 5

Retina = 76.1 ± 3.0 mg prot/g tis; Brain = 76.7 ± 6.7 mg prot/g tis; Liver = 80.0 ± 37.4 mg prot/g tis.

* - Significantly different from same homogenate without addition of 1.0 mM NADH using paired t analysis ($p < 0.05$).

T - Significantly different from respective consumption rate when measured in room air using paired t analysis ($p < 0.05$).

B - Significantly lower than brain for the same conditions using one way ANOVA ($p < 0.05$) and Tukey's w-procedure for multiple comparisons test.

L - Significantly lower than liver for the same conditions using one way ANOVA ($p < 0.05$) and Tukey's w-procedure for multiple comparisons test.

after the addition of 1.0 mM NADH as substrate for the mitochondrial electron transport chain. The addition of NADH did not significantly alter total or CN^--insensitive consumption in either 21% oxygen or 100% oxygen for the retinal homogenate. However the addition of 1.0 mM NADH to both brain and liver homogenates significantly increased both total and CN^--insensitive rates of consumption after incubation in either 21% or 100% oxygen. The lack of a statistically significant increase in total consumption at 100% oxygen for the liver homogenate was probably due to variability within this data set. In the presence of 1.0 mM NADH, CN^--insensitive consumption of oxygen in room air and 100% oxygen accounted for 20% and 66% of total respiration in retinal homogenates, 11% and 30% in brain homogenates, and 26% and 39% in liver homogenates, respectively. Therefore CN^--resistant respiration accounted for a significantly greater fraction of total consumption after hyperoxic exposure, and these CN^--resistant rates were 2-4 fold greater than those measured in room air. Although the retinal rate of CN^--insensitive consumption in the presence of substrate was less than that measured in brain and liver, the increase in CN^--insensitive consumption from room air to 100% oxygen, was not significantly different among the three tissue homogenates (Table 1; Figure 2).

Primary Antioxidants in Trout Retina

Superoxide Dismutase Activity

Retinal tissue exhibited significant SOD activity which was eliminated by boiling the samples for 15 minutes at 100 C. The calculated means for total superoxide dismutase activity in the retina of 3 rainbow trout specimens are reported in Table 2 and are expressed as tissue units/mg protein, tissue units/mg tissue (wet weight) and BESOD units/mg

Figure 2. Total and CN^--insensitive respiration in rainbow trout tissue homogenates. Retinal, brain and liver homogenates from the rainbow trout were exposed to room air (21%) and 100% oxygen. Homogenates were supplemented with 1.0 mM NADH. CN^--insensitive respiration was measured in the presence of 1.0 mM KCN. Respiration is expressed as nmol O_2 consumed/min/mg protein ($X \pm SD$). * - denotes significantly different from measurement in room air ($p < 0.05$), using a Paired-t analysis.

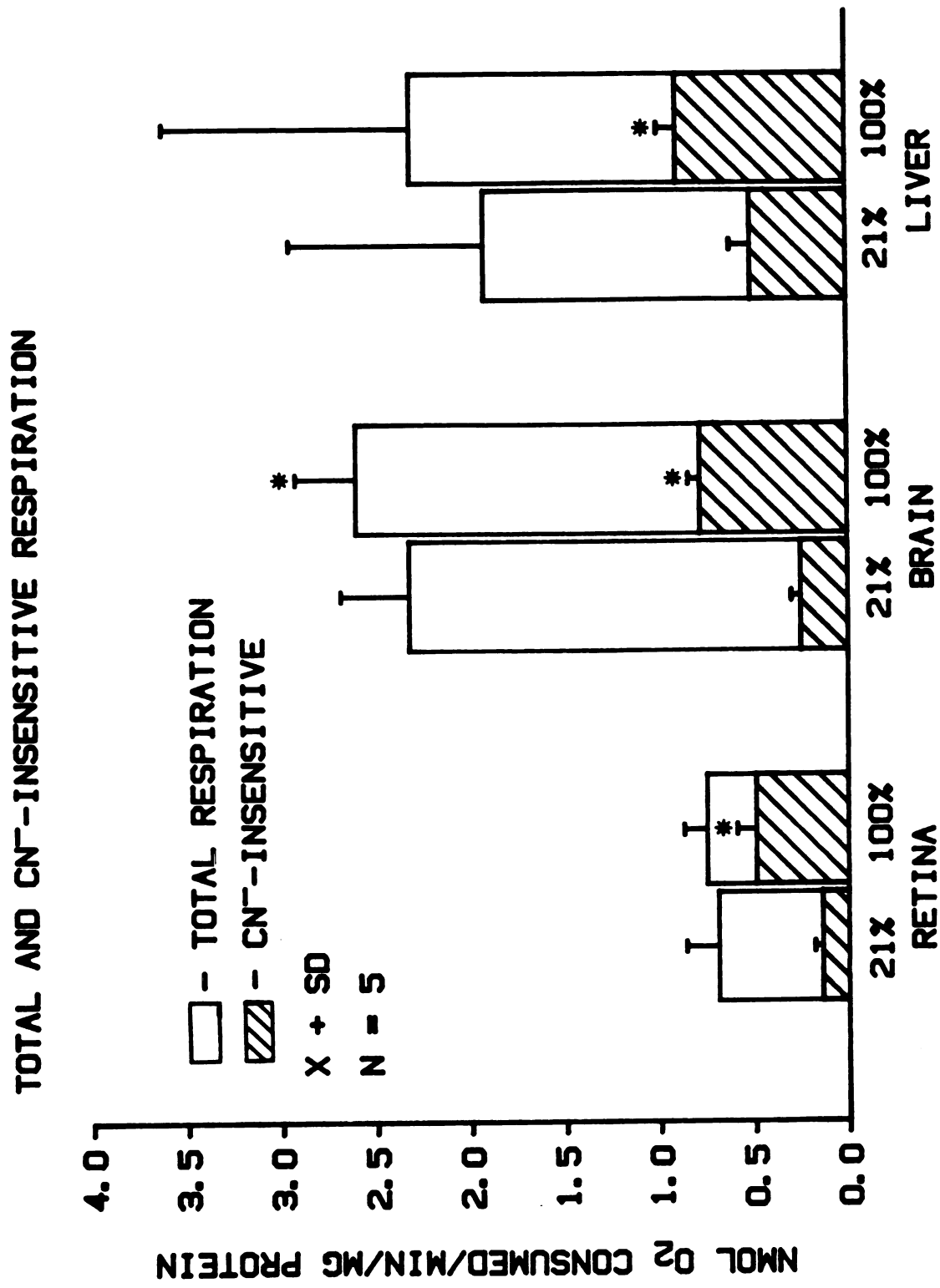
TOTAL AND CN⁻-INSENSITIVE RESPIRATION
- TOTAL RESPIRATION
- CN⁻-INSENSITIVE
X + SD
N = 5
NMOL O2 CONSUMED/MIN/MG PROTEIN
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
21% 100%
RETINA
21% 100%
BRAIN
21% 100%
LIVER

Figure 2

Table 2. Assayed levels of superoxide dismutase activity. Values are based upon ED_{50} values determined in the ferricytochrome c reduction assay.

	Retina	Brain	Liver
Total SOD Activity (Units/mg protein)	7.19 ±1.55	5.65 ±2.09	13.56* ±3.05
MnSOD Activity (Units/mg protein)	3.83 ±0.98	2.80 ±0.76	4.58 ±0.94
Total SOD Activity (Units/mg tissue)	0.16 ±0.01	0.17 ±0.03	0.89* ±0.02
MnSOD Activity (Units/mg tissue)	0.10 ±0.00	0.09 ±0.02	0.31* ±0.03
Total SOD Activity (BESOD Units/mg protein)	7.79 ±1.28	7.66 ±4.48	13.64 ±3.18
MnSOD Activity (BESOD Units/mg protein)	4.49 ±0.66	3.09 ±1.84	4.25 ±0.05

$\bar{X}$ ± SD; n = 3

* - Significantly different from the other means using a one way ANOVA and Tukey's w-procedure for comparison of means ($p < 0.05$).

protein. Superoxide dismutase activity in the retina was 7.19 units/mg protein or 0.16 units/mg wet tissue weight (Table 2). Measurement of superoxide dismutase activity was not affected by endogenous interfering substances because a parallel line analysis revealed that the dose response curve for the retinal homogenate was identical to the dose response curve of a purified SOD standard. Despite the fact that retinal tissues are exposed to hyperoxic conditions relative to brain or liver, both of these tissues expressed similar SOD activity (Table 2). Total SOD activity measured in the brain was not statistically different from that measured in the retina, however total liver activity was significantly greater than that measured in the other two tissues (Table 2).

There are two types of superoxide dismutases in aerobic cells, a CuZn form present in the cytosol and a manganous form present in the mitochondria. In order to differentiate between these two types, 1.0 mM cyanide was added to the homogenates to inactivate the CuZn form of superoxide dismutase. Significant MnSOD activity was found in the retina. Mitochondrial SOD activity (MnSOD) in the retina was 3.83 units/mg protein or 0.10 units/mg wet weight (Table 2). The MnSOD activity measured in the other two tissues however was not significantly different from that measured in the retina when expressed per mg protein (Table 2). Liver MnSOD activity was in fact significantly greater than retinal activity when expressed per mg wet weight.

All tissue dose response lines tested parallel to their respective standard lines, an indication that endogenous substances were not contributing to or altering the "apparent" SOD activity measured in this assay. The original response curves were linear below 50-60% inhibition

for the bovine erythrocytic SOD standard and for the tissue homogenates (Figure 3). When the data are expressed as percent inhibition vs volume of standard or tissue homogenate, a statistical comparison of the dose response curves cannot be made because of differences in dilution of the tissue enzyme activity. In order to eliminate the dilution effect without affecting the linear response, the data were transformed to log of percent inhibition vs coded log dose (Figure 4). The coded and uncoded ED_{50} values of the tissue homogenates, with their 95% confidence limits are reported in Table 3. The confidence limits provide a valuable estimate of the inherent precision of the assay. Total and MnSOD activity for each of the tissue homogenates, based on the ED_{50} values are also reported in Table 3. Activity is expressed as tissue units/mg protein as defined by the ferricytochrome c assay (McCord and Fridovich, 1969).

As a result of this type of statistical comparison, tissue SOD activity can also be expressed in terms of units of activity of a commercially prepared standard (Goldstein, 1965). The Homogenate : Standard potency ratios with 95% confidence limits are reported in Table 4. The assayed levels of SOD activity based upon these potency ratios are also reported in Table 4, and are expressed as BESOD units/mg protein as defined by the commercially prepared standard. Expression of the data in terms of the commercially prepared standard provides a unit of measure to which values reported in the literature can be compared. When reported in terms of BESOD units defined by the commercially prepared standard, no differences in total SOD or in MnSOD activity were detected among the three tissues.

Figure 5 depicts the SOD data graphically when expressed as tissue

Figure 3. Percent inhibition of ferricytochrome c reduction as an assay of superoxide dismutase activity. Dose response curves of representative data from rainbow trout tissues are plotted as linear regressions of percent inhibition of rate of reduction vs volume (ul) of BESOD standard solution or tissue homogenate. Measured ED_{50} values are: BESOD STD ED_{50}(S)=205 ul; Retina total activity ED_{50}(R1)=204 ul; and liver MnSOD activity ED_{50}(L2)=257 ul. The potency ratios for expressing activity with respect to the BESOD standard are:

Retina total:BESOD Standard Potency = 1.00

Liver MnSOD:BESOD Standard Potency = 0.80

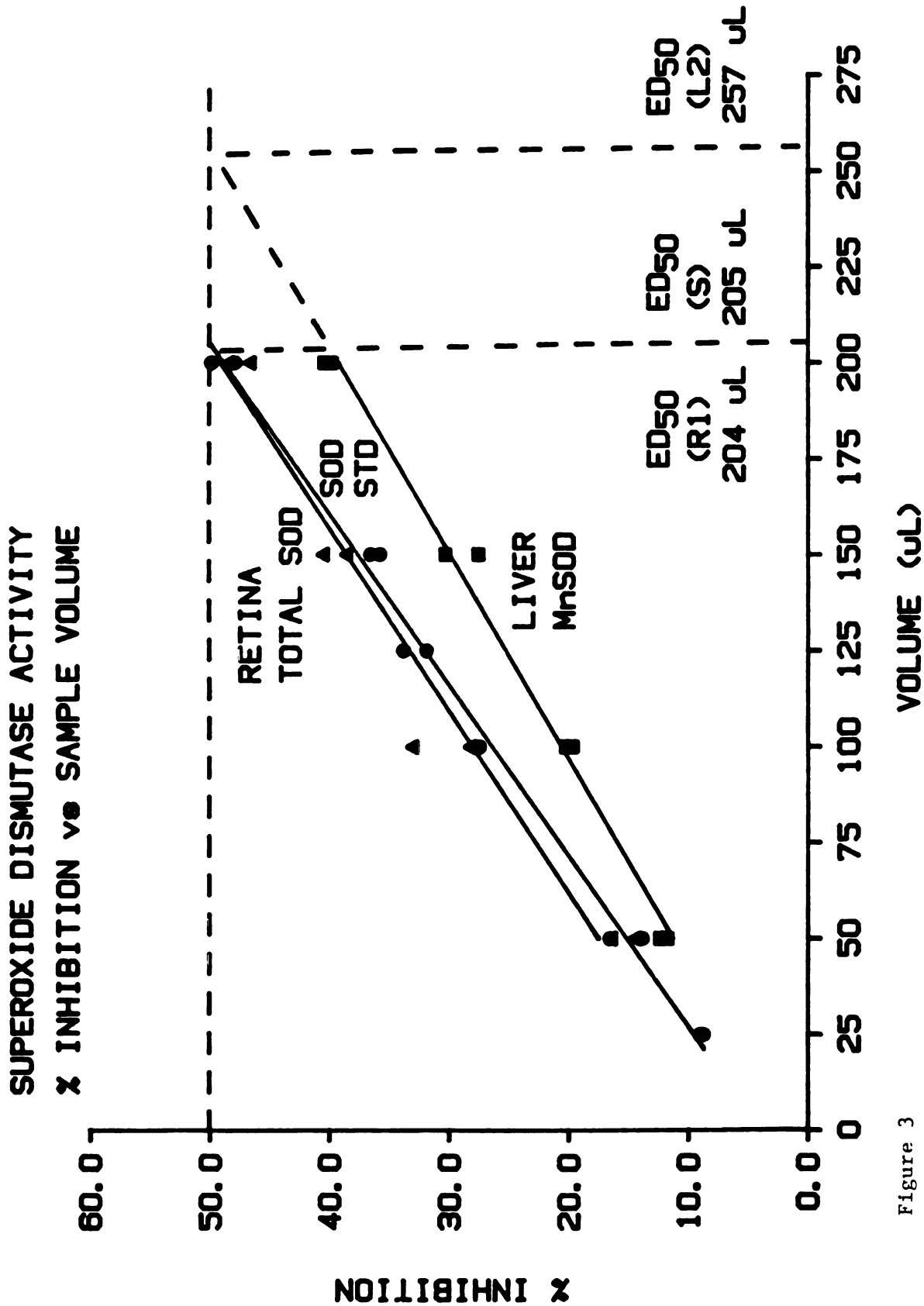
SUPEROXIDE DISMUTASE ACTIVITY
% INHIBITION vs SAMPLE VOLUME
% INHIBITION
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
25
50
75
100
125
150
175
200
225
250
275
VOLUME (uL)
RETINA
TOTAL SOD
SOD STD
LIVER
MnSOD
ED50 (R1) 204 uL
ED50 (S) 205 uL
ED50 (L2) 257 uL

Figure 3

Figure 4. Percent inhibition of ferricytochrome c reduction vs. coded log dose for the determination of superoxide dismutase activity. Data from Figure 3 are re-plotted as linear regressions of log percent inhibition vs coded log dose; where dose represents the volume (ul) of BESOD standard solution or tissue homogenates added. ED_{50} and potency values determined after decoding (see Appendix C) are: BESOD Standard ED_{50}(S) = 206 ul; Retina total activity ED_{50}(R1) = 197 uL; Liver MnSOD ED_{50}(L2) = 267 ul.

Retina total:BESOD Standard Potency = 1.05

Liver MnSOD:BESOD Standard Potency = 0.75

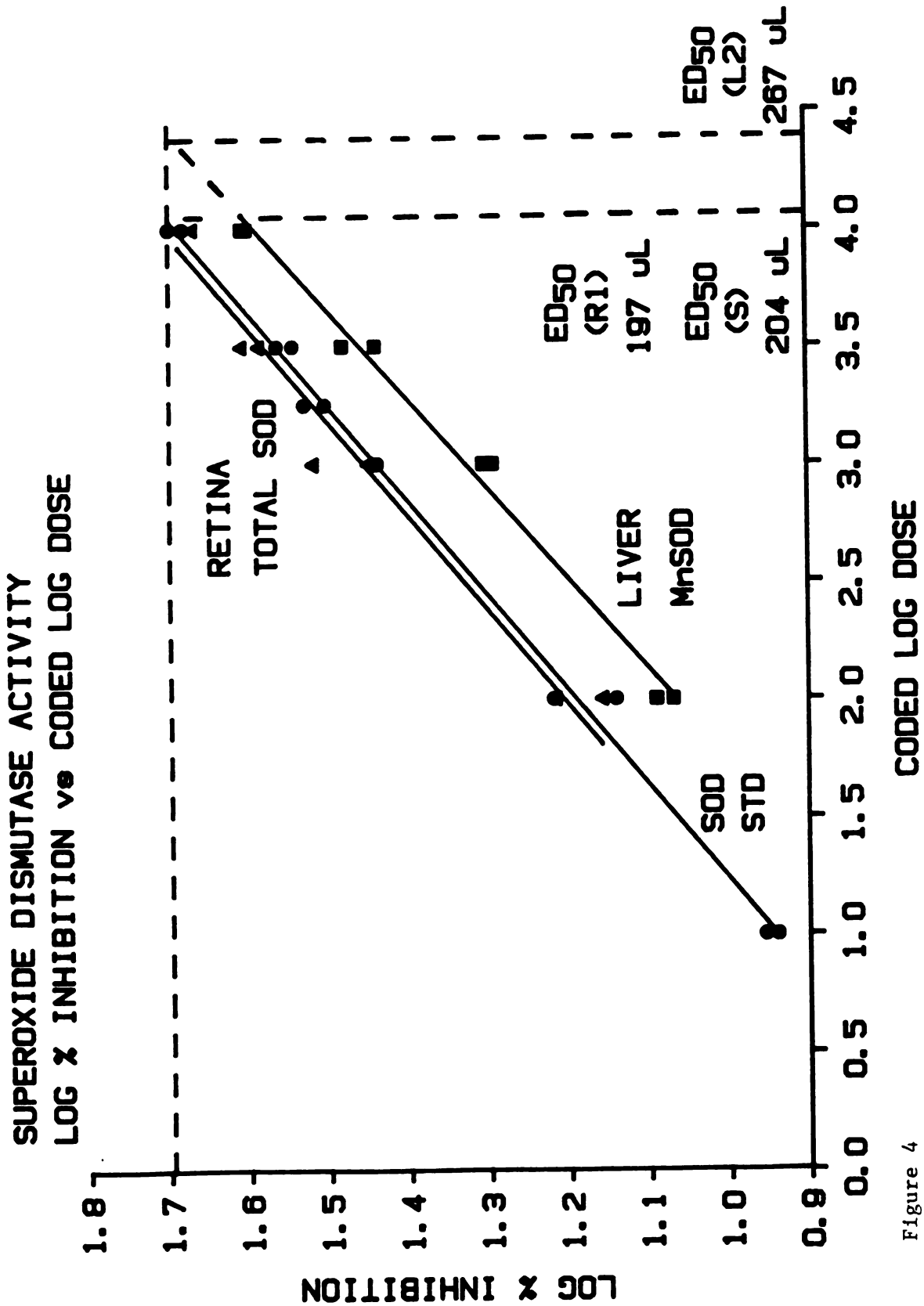
SUPEROXIDE DISMUTASE ACTIVITY
LOG % INHIBITION vs CODED LOG DOSE
LOG % INHIBITION
CODED LOG DOSE
RETINA
TOTAL SOD
LIVER
MnSOD
SOD
STD
ED50 (R1) 197 uL
ED50 (S) 204 uL
ED50 (L2) 267 uL
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5

Figure 4

Table 3. Superoxide Dismutase Activity based upon individual ED_{50} values. Values are reported as ED_{50} in coded and uncoded units (ul homogenate) with their respective 95% confidence limits. SOD Activity is expressed as Units/mg protein as defined by the ferricytochrome c assay.

Tissue Homogenate	Coded ED_{50} (95% confidence limits)	Uncoded ED_{50} (95% Confidence Limits)	SOD Activity
Retina - Total	4.171 (4.131 - 4.236)	225.2 (223.6 - 235.5)	7.79
SOD Activity	3.976 (3.795 - 4.215)	196.7 (173.5 - 232.2)	8.34
	4.042 (3.920 - 4.187)	206.0 (189.3 - 227.7)	5.44
Retina - MnSOD	3.666 (3.499 - 3.878)	158.7 (141.3 - 183.8)	3.16
Activity	3.955 (3.739 - 4.262)	193.9 (166.9 - 239.8)	4.95
	4.016 (3.891 - 4.166)	202.2 (185.5 - 224.3)	3.39
Brain - Total	3.278 (3.165 - 3.420)	121.1 (112.1 - 133.8)	4.84
SOD Activity	4.181 (4.024 - 4.377)	226.8 (203.4 - 259.8)	8.02
	4.530 (4.352 - 4.751)	288.8 (255.3 - 336.7)	4.09
Brain - MnSOD	4.588 (4.301 - 4.824)	300.7 (246.4 - 354.1)	1.95
Activity	4.146 (3.909 - 4.487)	221.2 (187.7 - 280.2)	3.43
	3.649 (3.556 - 3.754)	156.9 (147.0 - 168.6)	3.02
Liver - Total	4.133 (3.934 - 4.397)	219.3 (191.1 - 263.3)	11.37
SOD Activity	4.056 (3.879 - 4.251)	207.9 (184.0 - 238.0)	17.04
	4.113 (3.854 - 4.499)	216.2 (180.8 - 282.6)	12.26
Liver - MnSOD	4.216 (4.079 - 4.372)	232.3 (211.3 - 258.8)	4.15
Activity	4.414 (4.267 - 4.593)	266.5 (240.6 - 301.7)	5.65
	4.177 (4.023 - 4.368)	226.1 (203.2 - 258.1)	3.93

Table 4. Superoxide dismutase activity based upon potency ratios to standard preparations. Activity is expressed as Units/mg protein as defined by the commercially prepared standard.

Homogenate	Homogenate:Standard Potency Ratio (95% Confidence Limits)	SOD Activity BESOD units/mg protein
Retina - Total SOD Activity	1.07 (0.93 - 1.22)	8.30
	1.05 (0.97 - 1.13)	8.74
	1.17 (1.05 - 1.29)	6.34
Retina - MnSOD Activity	1.32 (1.07 - 1.63)	4.17
	1.06 (0.98 - 1.15)	5.25
	1.19 (1.08 - 1.32)	4.05
Brain - Total SOD Activity	2.54 (1.42 - 4.57)	12.31
	0.91 (0.85 - 0.98)	7.31
	0.82 (0.73 - 0.94)	3.38
Brain - MnSOD Activity	0.62 (0.44 - 0.87)	1.20
	0.93 (0.86 - 1.02)	3.21
	1.62 (1.31 - 1.99)	4.88
Liver - Total SOD Activity	0.93 (0.84 - 1.02)	10.52
	0.99 (0.93 - 1.06)	16.89
	1.10 (0.96 - 1.27)	13.51
Liver - MnSOD Activity	1.04 (0.82 - 1.31)	4.30
	0.75 (0.63 - 0.89)	4.26
	1.07 (1.00 - 1.14)	4.20

Figure 5. Superoxide dismutase activity of tissue homogenates from the rainbow trout. Both total and MnSOD activity is reported and is expressed as SOD units/ mg protein ($X \pm SD$; $N = 3$). * - denotes significantly different from the other two tissues using a one way analysis of variance and Tukey's w-procedure for multiple comparisons test ($p < 0.05$).

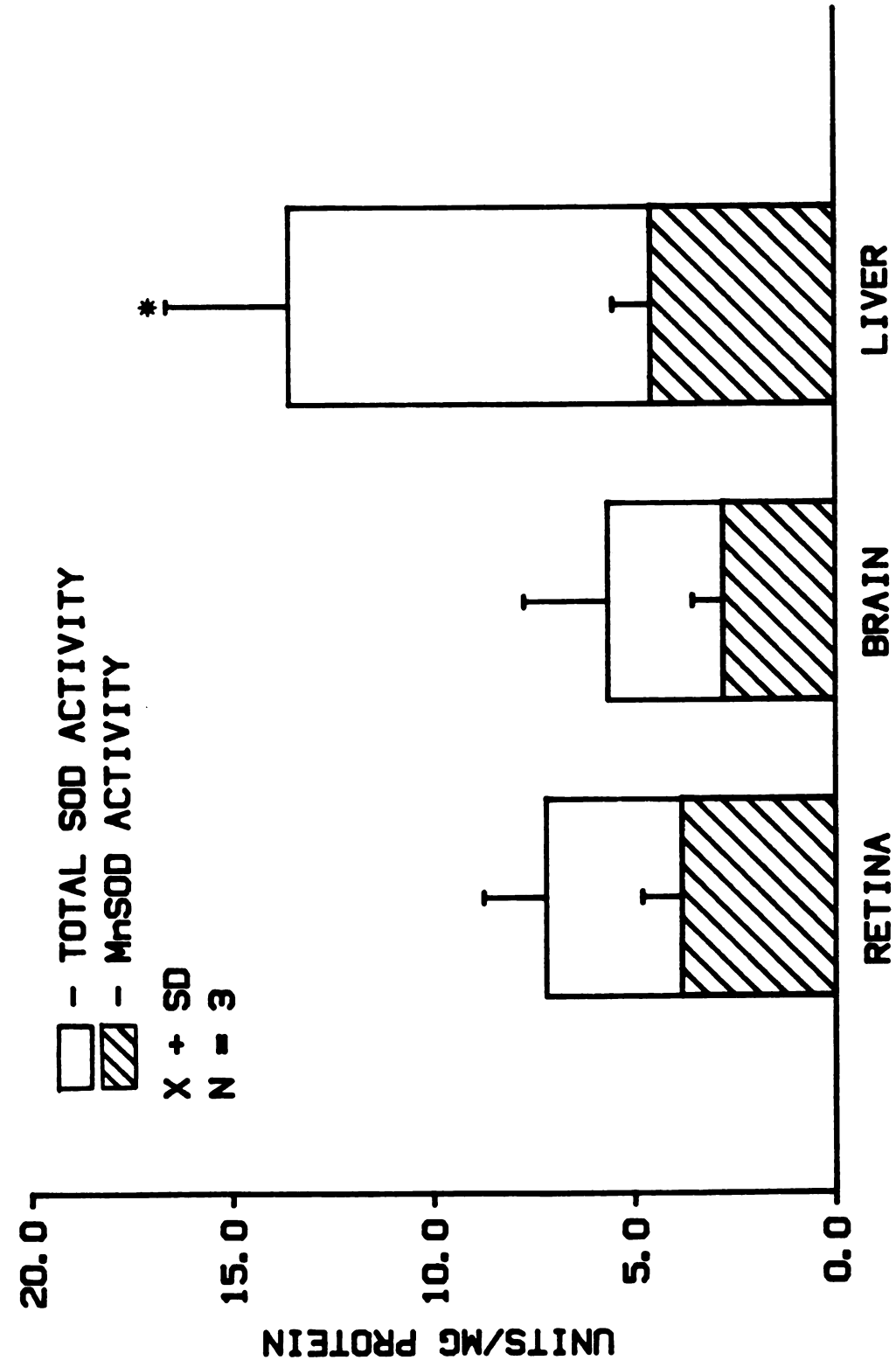
SUPEROXIDE DISMUTASE ACTIVITY
- TOTAL SOD ACTIVITY
- MnSOD ACTIVITY
X + SD
N = 3
UNITS/MG PROTEIN
20.0
15.0
10.0
5.0
0.0
*
RETINA
BRAIN
LIVER

Figure 5

units/mg protein. The CuZn superoxide dismutase activity could be determined from the difference between total SOD activity and MnSOD activity for each tissue. It is apparent from Figure 5, that MnSOD activity accounted for a larger fraction of total SOD activity in retina and brain than in liver.

Although MnSOD activity is not significantly increased in the retina, this form of the enzyme is compartmentalized to the mitochondrion. Thus, the levels of MnSOD in tissue homogenates is a reflection of both mitochondrial content and number. In order to determine if retinal mitochondria have an increased concentration of MnSOD, values were calculated on the basis of a mitochondrial marker, i.e., cytochrome oxidase activity. Cytochrome c oxidase activity was determined in the homogenates prepared for superoxide dismutase measurements. Activity is expressed as nmol O_2 consumed/min/mg protein and in the standard unit, nkat/mg protein or nkat/mg tissue (Table 5). Retinal cytochrome oxidase activity was significantly lower than that measured in brain and liver when expressed per mg protein (Figure 6), and was significantly less than that in the liver but not the brain, when expressed per mg tissue.

MnSOD activity expressed per nkat cytochrome oxidase activity was calculated to be 0.32 units/nkat cytochrome oxidase in the retina. Retinal MnSOD activity was significantly greater than that found in the brain when expressed in this fashion, however, liver MnSOD/mitochondrion was no different from that measured in the retina. The calculated means for these ratios obtained from three fish is reported for all three tissues in Table 6 and depicted in Figure 7.

Table 5. Assayed levels of cytochrome oxidase activity in selected tissues from the rainbow trout.

	Retina	Brain	Liver
Cytochrome Oxidase activity (nmol O_2/min/mg protein)	94.8* ±19.6	230.3 ±25.1	206.0 ±25.0
Cytochrome Oxidase activity (nkat/mg protein)	6.4* ±1.3	15.5 ±1.7	13.7 ±1.7
Cytochrome Oxidase activity (nkat/mg tissue)	0.32t ±0.02	0.71 ±0.05	1.14 ±0.34

$\bar{X}$ ± SD; n = 3
* = Significantly less than brain and liver values using one way ANOVA and Tukey's w-procedure ($p < 0.05$).
t = Significantly less than liver value using testing procedures above.

Table 6. Mitochondrial superoxide dismutase activity expressed per unit of cytochrome oxidase activity. Ratios were obtained from enzyme measurements in individual homogenates when expressed per mg tissue weight.

	Retina	Brain	Liver
MnSOD Activity (units/nkat Cyt Ox)	0.32 ±0.03	0.12* ±0.03	0.29 ±0.08

$\bar{X}$ ± SD; n = 3
* = Significantly different from retinal and liver values using one way ANOVA and Tukey's w-procedure ($p < 0.05$).

Figure 6. Cytochrome oxidase activity of tissue homogenates from the rainbow trout. Activity is expressed as nkat/mg protein ($X \pm SD$; $N = 3$). * - denotes significantly different from the other two tissues using a one way analysis of variance and Tukey's w-procedure for multiple comparisons test ($p < 0.05$).

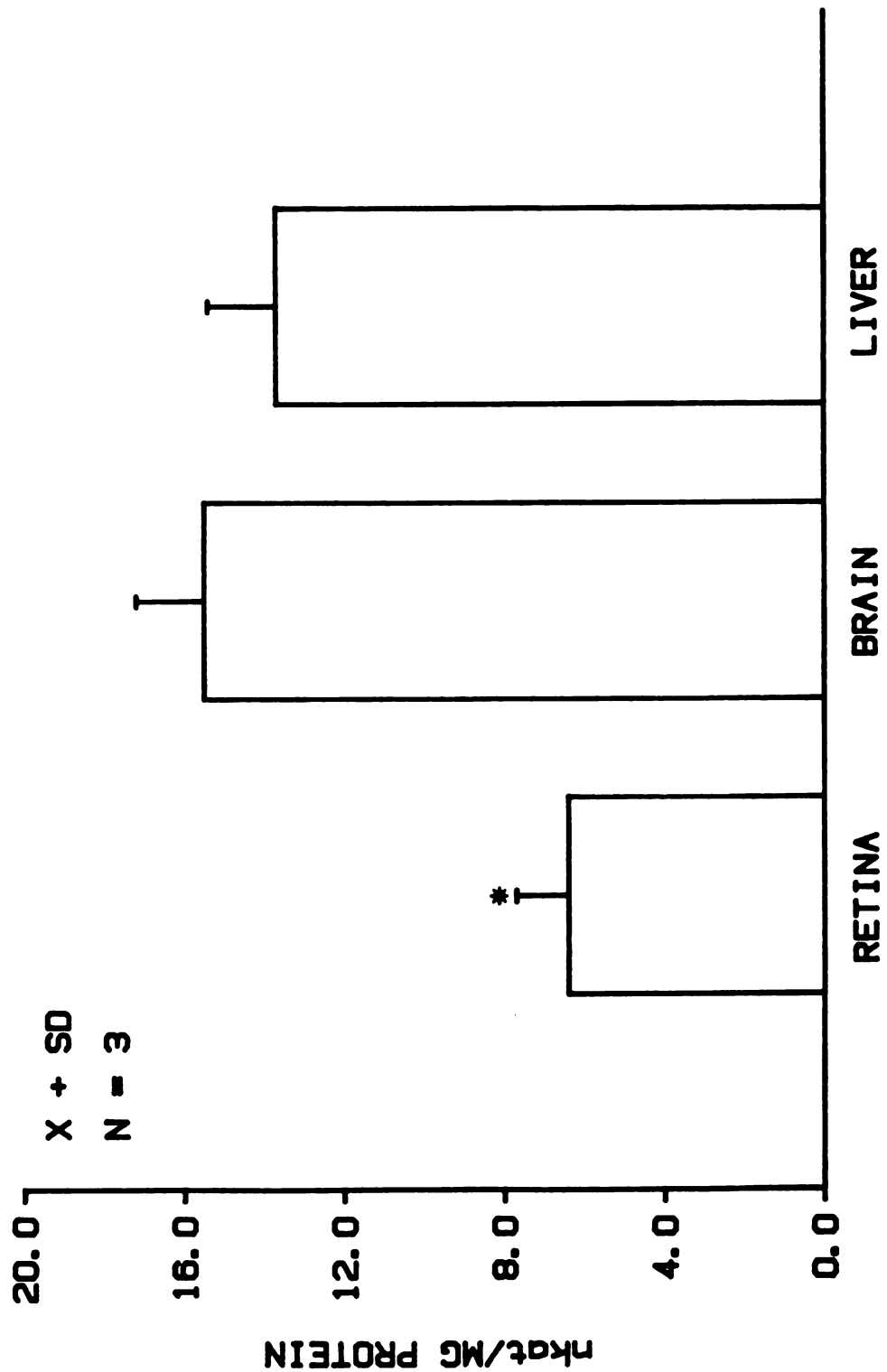
CYTOCHROME OXIDASE ACTIVITY
X + SD
N = 3
nkat/MG PROTEIN
20.0
16.0
12.0
8.0
4.0
0.0
*
RETINA
BRAIN
LIVER

Figure 6

Figure 7. Mitochondrial Superoxide Dismutase Activity from the rainbow trout tissue homogenates expressed per unit cytochrome oxidase activity. Activity is expressed as SOD units/nkat cytochrome oxidase. * - denotes significantly different from the other two tissues using a one way analysis of variance and Tukey's w-procedure for multiple comparisons test ($p < 0.05$).

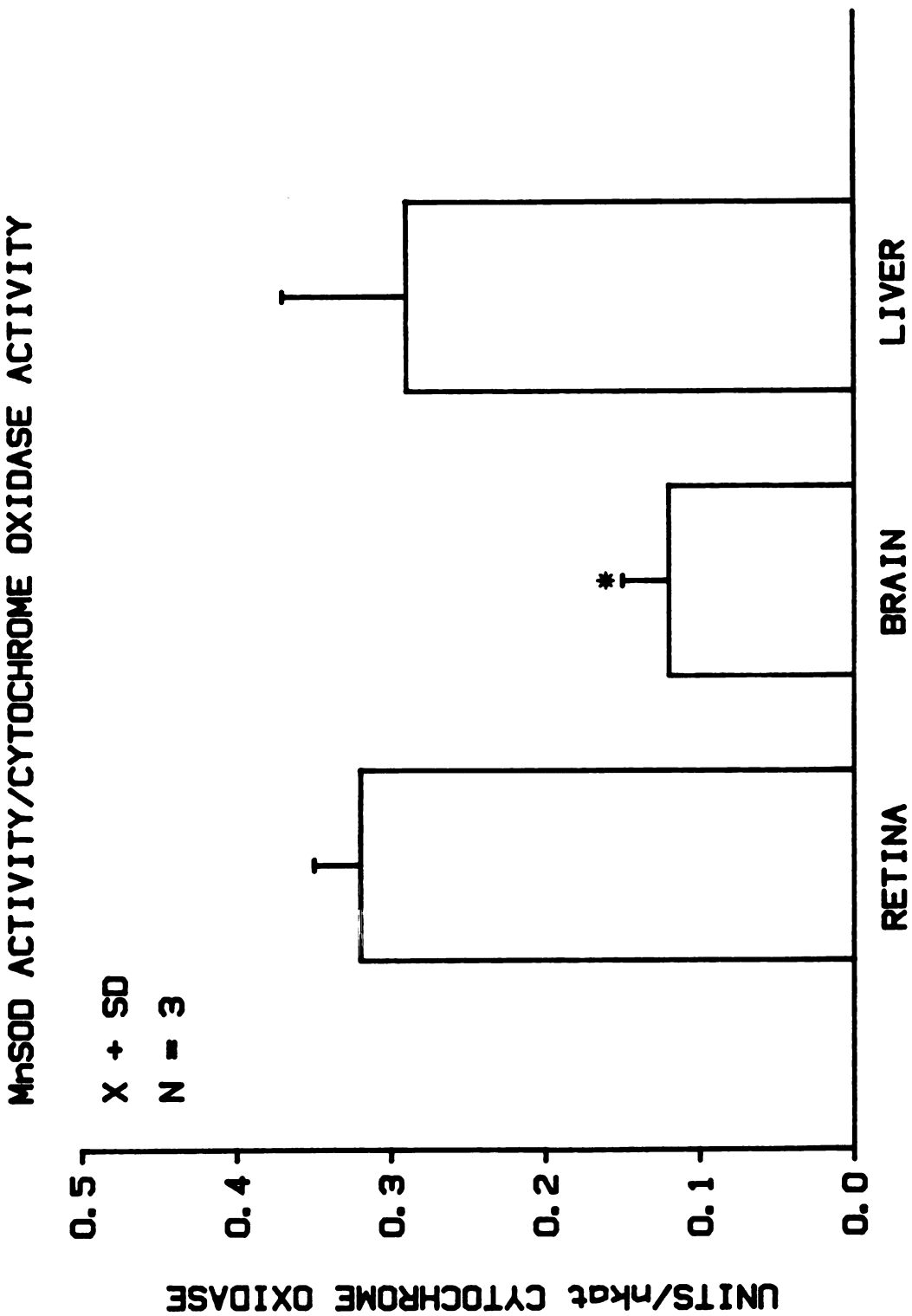
MnSOD ACTIVITY/CYTOCHROME OXIDASE ACTIVITY
X + SD
N = 3
UNITS/nkat CYTOCHROME OXIDASE
0.5
0.4
0.3
0.2
0.1
0.0
*
RETINA
BRAIN
LIVER

Figure 7

Catalase Activity

Catalase was measured in crude tissue homogenates after the incorporation of ethanol and Triton X-100. No catalase activity was detected in either retinal or brain homogenates from the rainbow trout. For liver, three dilutions of the homogenates were run in each experiment, their activities were calculated and then averaged. Trout liver tissue was very high in catalase activity. The mean liver activity when expressed in Bergmeyer units at 2 C was 1.42 ± 0.46 units/mg liver ($\bar{X} \pm$ S.D.) which corresponded to 14.53 ± 4.66 International units (units/mg tissue). Catalase activity when expressed as Kat.f. units is useful for comparisons of crude tissue homogenates. Liver activity was measured at $4.36 \times 10^{-4} \pm 0.93 \times 10^{-4}$ $min^{-1}.mg^{-1}$ protein (Figure 8). The incorporation of Triton X-100 alone into the liver homogenates accounted for a 37% increase in activity while the incorporation of both ethanol and Triton X-100 into the homogenate accounted for a 350% increase in liver catalase activity. Since a standard curve was run with each assay it was possible to estimate the equivalent amount of pure catalase to that which was measured in the homogenate. An estimate of 54.9 ± 19.6 ug of catalase was determined per mg protein in the liver homogenates.

Glutathione Peroxidase Activity and Glutathione Content

Selenium-dependent glutathione peroxidase activity was measured indirectly by following the rate of regeneration of GSH in the presence of glutathione reductase and NADPH using H_2O_2 as substrate. The change in absorbance at 340 nm was followed over time as NADPH was converted to NADP and the nmol of NADPH consumed/min/ml was determined from the extinction coefficient for NADPH at 25 C (6,220 $M^{-1}.cm^{-1}$). Protein

Figure 8. Catalase activity determined in crude tissue homogenates from the rainbow trout. Activity is expressed as Kat.f. units/min/mg protein ($X \pm SD$; $N = 8$).

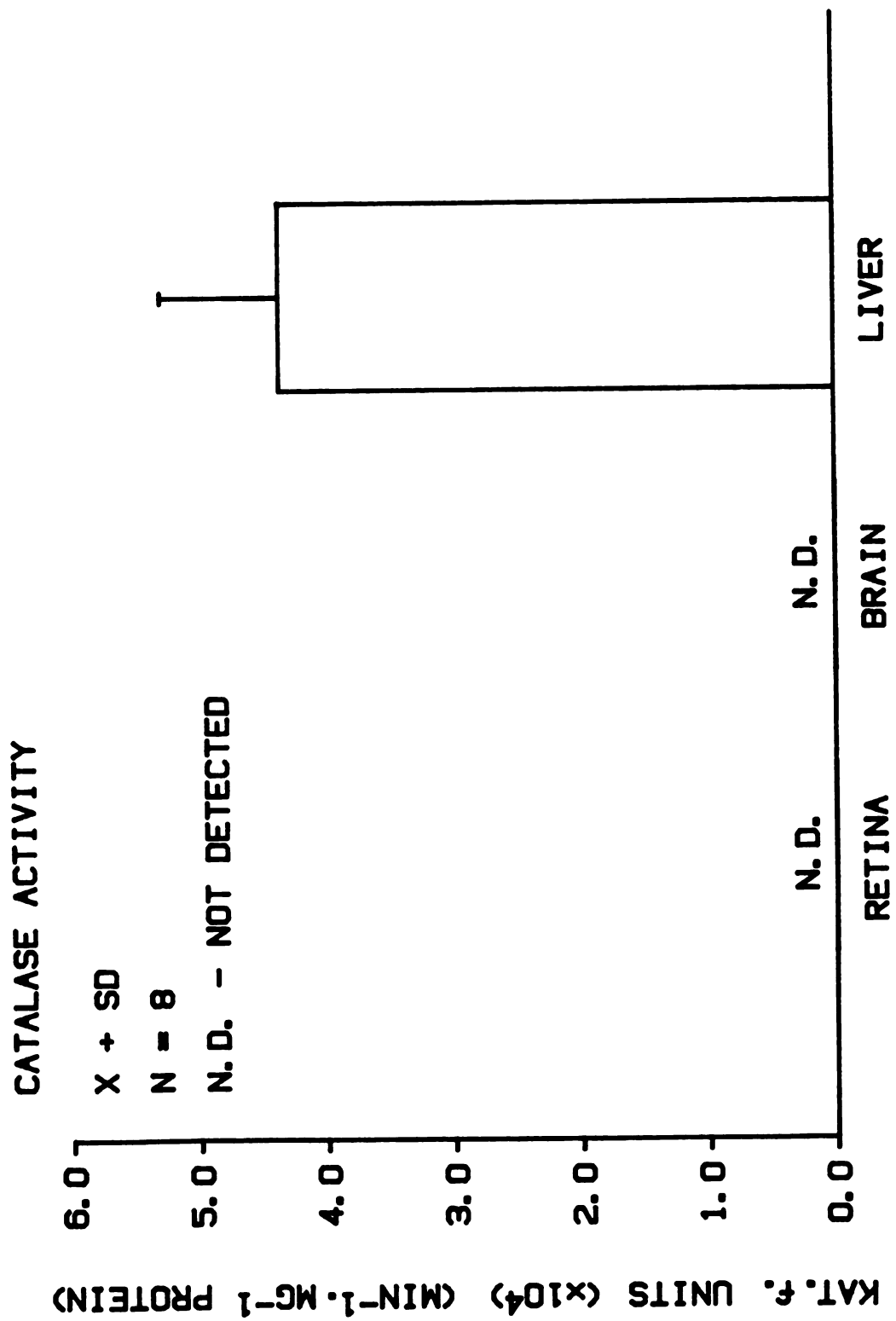
CATALASE ACTIVITY
X + SD
N = 8
N.D. - NOT DETECTED
KAT. F. UNITS (x10⁴) (MIN⁻¹.MG⁻¹ PROTEIN)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
N.D.
N.D.
RETINA
BRAIN
LIVER

Figure 8

determinations were made so that the data could be expressed as nmol NADPH consumed/min/mg protein or mUnits/mg protein. Glutathione peroxidase activity in the retina was 22.7 mUnits/mg protein. Activity in the brain was not significantly different from that found in the retina however the liver had significantly greater activity than that found in the other tissues (Table 7; Figure 9).

Glutathione content was estimated from a measure of the non-protein sulfhydryl content of the tissues. Total sulfhydryl content of the tissues was also determined. Total sulhydryl content and glutathione content were expressed as umoles SH/mg protein. Total sulfhydryl content of the retina was 0.100 uMol SH/mg protein and the non-protein sulfhydryl content (an estimate of glutathione content) of the retina was 0.014 uMol SH/mg protein. No differences in either total sulhydryl content or in glutathione content were seen among the three tissues studied (Table 7, Figure 10).

Table 7. Assayed levels of glutathione peroxidase activity and sulfhydryl content in selected tissues from the rainbow trout.

	Retina	Brain	Liver
Glutathione Peroxidase Activity (mUnits/ml)	22.7 ±8.9	14.6 ±3.6	37.8* ±4.9
Total Sulfhydryl Content (umol SH/mg protein)	0.100 ±0.040	0.115 ±0.013	0.115 ±0.013
Glutathione Content (umol SH/mg protein)	0.014 ±0.002	0.009 ±0.000	0.015 ±0.008

$\bar{X}$ ± SD; n = 5

* - Significantly greater than retina and brain values using one way ANOVA and Tukey's w-procedure ($p < 0.05$).

Figure 9. Glutathione peroxidase activity measured in tissue homogenates from the rainbow trout. Activity is expressed as mUnits/mg protein ($X \pm SD$; $N = 5$). * - denotes significantly different from the other two tissues using a one way analysis of variance and Tukey's w-procedure for multiple comparisons test ($p < 0.05$).

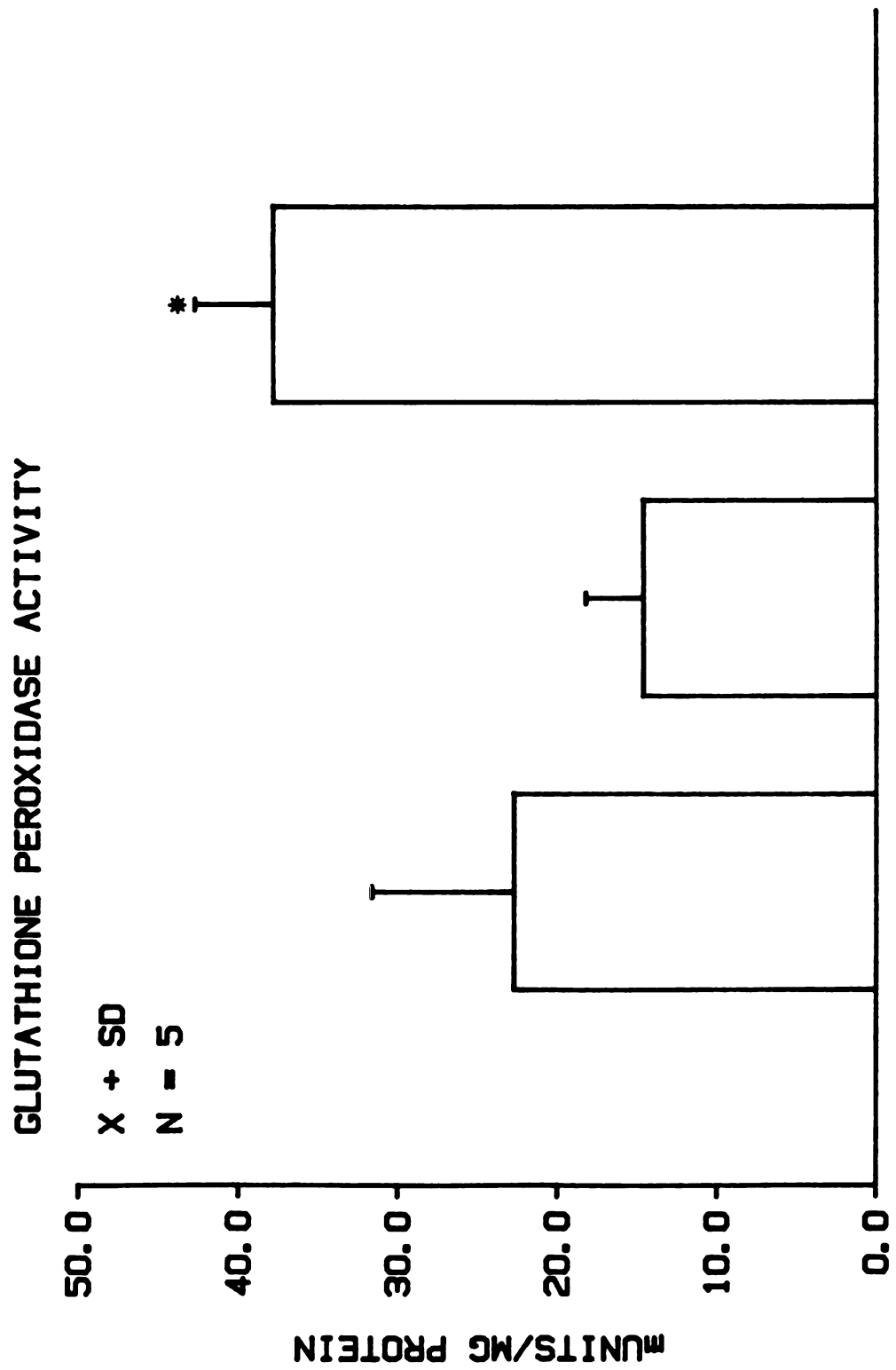
GLUTATHIONE PEROXIDASE ACTIVITY
X + SD
N = 5
*
50.0
40.0
30.0
20.0
10.0
0.0
mUNITS/MG PROTEIN
RETINA
BRAIN
LIVER

Figure 9

Figure 10. Total sulfhydryl and glutathione content of tissue homogenates from the rainbow trout. Activity is expressed as umol SH/mg protein ($X \pm SD$; $N = 5$).

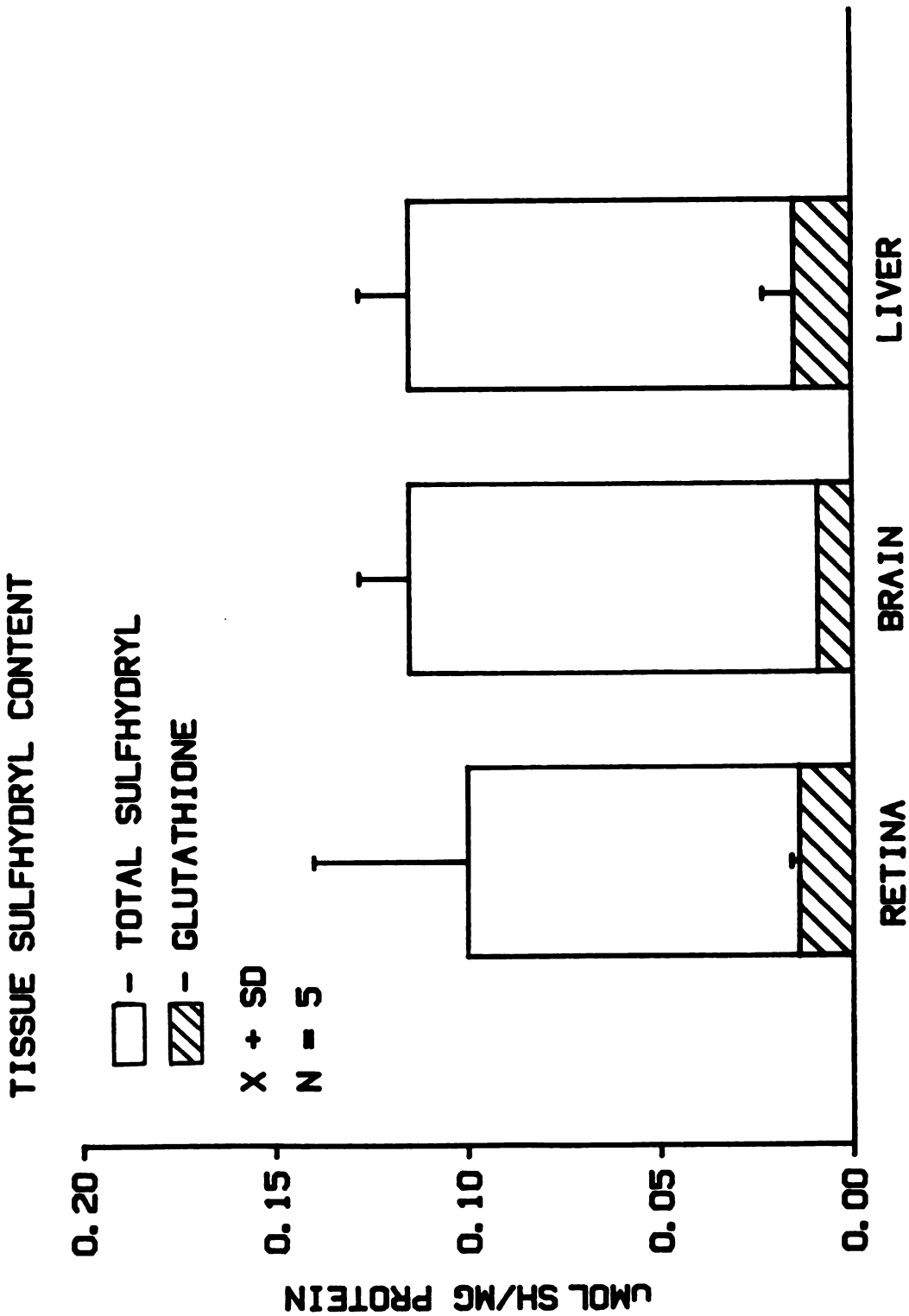
TISSUE SULFHYDRYL CONTENT
- TOTAL SULFHYDRYL
- GLUTATHIONE
X + SD
N = 5
UMOL SH/MG PROTEIN
0.20
0.15
0.10
0.05
0.00
RETINA
BRAIN
LIVER

Figure 10

DISCUSSION

According to the free radical theory of oxygen toxicity, hyperoxic tissue damage is caused by the intracellular formation of activated oxygen metabolites, i.e., superoxide ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$), at a rate that overwhelms the cellular defenses (Jamieson et al., 1986). Based on this theory, tolerance to oxygen toxicity is thought to be due to either a decreased propensity of O_2-adapted cells to generate the reactive oxygen metabolites and/or the presence of a highly efficient antioxidant defense system to limit the concentrations of the primary oxidants, i.e., the superoxide dismutase, catalase and glutathione peroxidase enzymes. In the present study these two oxidative defense "strategies" were examined in an attempt to discern the factors responsible for the resistance to oxygen toxicity observed in the rainbow trout retina.

Determination of Oxidative Flux in Rainbow Trout Retinal Homogenate

Trout retina exposed to hyperoxic conditions do not exhibit any evidence of oxidative damage. In contrast, a variety of other tissues are rapidly damaged under identical conditions (Nishiki et al., 1976; Freeman and Crapo, 1981; Turrens, Freeman and Crapo, 1982). This damage is thought to be mediated by an increased generation of reactive oxygen metabolites in the presence of supraphysiologic pO_2. Indeed, in all tissues studied to date hyperoxia causes an increase in CN^--insensitive O_2 consumption. The resistance of the trout retina to hyperoxia may be

due to a decreased propensity or ability of the retinal cells to generate oxidative metabolites under these conditions. This was examined in the present study using retinal homogenates. Brain and liver tissues from the rainbow trout previously have been shown to be sensitive to oxygen (Baeyens, Hoffert and Fromm, 1973; 1974), and therefore were used in this study as internal controls for comparisons with the oxygen resistant retina. The results of the oxygen consumption measurements indicate that there was an enhanced rate of CN^--resistant respiration by the rainbow trout retina during hyperoxic exposure. These results were similar to measurements made in the brain and liver tissues and demonstrated that CN^--resistant respiration is increased comparably in all three tissues during hyperoxia. Several studies have shown that CN^--resistant respiration is a reliable indicator of the effect of pO_2 on the production of partially reduced species of oxygen (Hassan and Fridovich, 1977; Freeman and Crapo, 1981; Freeman, Topolosky and Crapo, 1982; Turrens et al., 1982b; Turrens, Freeman and Crapo, 1982a). The method however, does not provide an absolute measure of cellular $O_2^{\cdot}$ and H_2O_2 production (Freeman and Crapo, 1981). The rate of $O_2^{\cdot}$ generation is very dependent on the conditions at the time of measurement. For example an overestimation of the rate of oxidant formation may be caused by a number of factors. Superoxide production by mitochondria is enhanced when there is a decrease in ADP stores and the components of the respiratory chain are reduced (Boveris and Cadenas, 1982). This situation arises with the addition of cyanide to inhibit cytochrome oxidase and subsequently reduce the mitochondrial respiratory carriers. An estimate of the formation of partially reduced oxygen metabolites would therefore be greater in the presence of cyanide than when measured in situ

(Freeman and Crapo, 1982). In addition, only oxygen reduced to water or to an oxygen species which can covalently react with cellular constituents will be measured polarographically. Oxygen may be consumed in the oxidation of substrates which may or may not generate or release reactive intermediates, thereby overestimating their production (Freeman and Crapo, 1981). This method may also underestimate oxidant formation. Cyanide is an effective inhibitor of one electron reduction at the ubiquinone-cytochrome b region of the respiratory chain (Boveris and Cadenas, 1982). Since this is one of the two major sites of superoxide generation, inhibition would result in an underestimation of $O_2^{\cdot}$ formation. Although 1.0 mM CN^- will partially inhibit the CuZnSOD and catalase in the homogenate, the spontaneous dismutation of $O_2^{\cdot}$, and dismutation by MnSOD could all result in the regeneration of oxygen and an overall underestimation of the true rate of production of the partially reduced oxygen metabolites:

$$O_2^{\cdot} + O_2^{\cdot} + 2H^+ \xrightarrow{\text{MnSOD}} H_2O_2 + O_2 \qquad (16)$$

(Freeman and Crapo, 1981).

Thus, there are numerous pathways through which CN^--insensitive O_2 consumption can be influenced, nonetheless, the measurement currently provides the best index of the rate of formation of partially reduced forms of oxygen.

The addition of NADH as substrate for the mitochondrial electron transport chain did not have a significant effect on the rate of total O_2 consumption in retinal homogenates, probably because this tissue is heavily dependent on aerobic glycolysis to meet metabolic needs. However the addition of NADH to brain and liver homogenates significantly increased the rate of O_2 consumption in these tissues. These results

suggested that the endogenous pool of reducing equivalents may have been rate limiting in the latter two homogenates and might partly explain why total consumption was not increased on transition to hyperoxia in the absence of NADH (Table 1). The addition of NADH to brain and liver homogenates in the presence of cyanide caused an increase in consumption and was most likely due to an increase in the reduction of the cytochrome carriers. Hyperoxic exposure significantly increased the partial reduction of oxygen in all three tissues. More importantly, the change in cyanide-insensitive oxygen consumption from room air to hyperoxia was the same in all three tissues and provided evidence that the retina was indeed capable of enhancing the rate of reduction of the partially reduced oxygen metabolites. Thus, the results demonstrate that all three tissues repond similarly and that retinal tissues do not protect themselves against oxygen toxicity by a decreased propensity or ability to generate reactive oxidants in hyperoxia.

Finally, these results are consistent with those described in other tissues tolerant to hyperoxia. The hypothesis that tolerance to hyperoxic exposure may be due to a decreased production of the reactive oxygen metabolites has been similarly advanced in other models of hyperoxic damage. Rats exposed to 100% oxygen generally will die within 72 hours of exposure. However, they are tolerant to 1 atm pure oxygen if they are first exposed to 85% O_2 for a period of 7 days before subsequent challenge with 100% oxygen (Crapo and Tierney, 1974). It had been presumed that hyperoxic exposure resulted in an enhanced rate of $O_2^{\cdot}$ and H_2O_2 production, but this had not been formally tested until the studies by Freeman and Crapo (1981). They reported that CN^--resistant respiration was in fact, greater in rats pre-exposed to 85% oxygen than in

controls. Their findings led them to conclude that the increased tolerance of the adapted rats was not due to a decrease in intracellular $O_2^{\cdot}/H_2O_2$ production (Freeman and Crapo, 1981; Crapo and Tierney, 1974). Indeed, no study to date has successfully demonstrated that any tissue is able to regulate its sensitivity to hyperoxia by decreasing the generation of oxygen metabolites intracellularly (Fridovich and Freeman, 1986).

Primary Antioxidant Defenses in the Rainbow Trout Retina

If oxidants are generated within the tissue then the primary antioxidants designed to control $O_2^{\cdot}$ and H_2O_2 might be expected to play a critical role in the resistance of the rainbow trout retina to oxygen toxicity. The antioxidant activities of the CuZn and Mn superoxide dismutases, catalase, glutathione peroxidase and glutathione content of the rainbow trout retina were determined. Brain and liver tissues from the same species were also examined in an effort to compare the efficiency of the antioxidant defense system of the retina to the efficiency of the defense systems within these oxygen sensitive tissues. Superoxide dismutase is the key enzyme for regulating $O_2^{\cdot}$ in aerobic cells (Fridovich, 1986b). Indeed, all mammalian cells are known to contain at least two forms of intracellular SOD; a cytoplasmic (CuZn) form and a mitochondrial (MnSOD) form.

There are numerous assay methods for the measurement of the superoxide dismutases, each of which possesses certain advantages and disadvantages. In this study, SOD activity was measured by following the SOD inhibitable rate of ferricytochrome c reduction (McCord and Fridovich, 1969). Fridovich (1986b) has listed some of the potential problems that may be encountered with the xanthine oxidase/cytochrome c assay,

including: 1) the presence of agents capable of directly reducing cytochrome c, which if present, would increase 'apparent' SOD activity, 2) the presence of potential inhibitors of xanthine oxidase and/or 3) the presence of oxidases and peroxidases which could reoxidize ferrocytochrome c. The latter two situations would underestimate SOD activity. Each potential pitfall was directly tested for in this study. Cytochrome c was tested for contamination by SOD, but was determined to be free of SOD. The effect of oxidases and peroxidases on reduction by crude homogenates was tested for and did not appear to be present in any of the homogenates. In addition, the rate of reduction of cytochrome c was measured after the addition of boiled homogenate and no significant change in the rate was observed for any of the homogenates. This provided an indication that it was unlikely that small autoxidizable molecules in the homogenates had an effect on cytochrome c reduction. As an overall check, an indirect statistical method was used to detect the presence of any endogenous interfering substances in the homogenates (Eldred and Hoffert, 1981).

Although most investigators have determined SOD activity by simply diluting and readjusting tissue extracts until the 50% inhibition level is attained, it may not reflect true SOD activity. In most assays it is assumed that the unknown preparation differs from the standard only by a dilution factor. This should be tested in enzyme assays which rely on substrates that are not specific to the indicator. The statistical approach incorporates a parallel line analysis of variance to compare tissue response lines to those of a purified SOD standard (Goldstein, 1965). All tissue dose response lines tested in this study were parallel to their respective standards, an indication that there were no

endogenous interfering substances which were contributing to or altering the 'apparent' SOD activity.

It was determined in this study that the trout retina, like all other tissues, contains significant SOD activity. It is difficult however to compare the values reported here to others reported in the literature since the specific activity of SOD is expressed in a number of ways, e.g., per mg protein, per mg DNA, per whole tissue, or per number of cells. Thus, internal controls were selected, i.e., tissues from the rainbow trout which were not normally exposed to hyperoxic conditions and had been shown to be sensitive to oxygen toxicity. From measurements of SOD-inhibitable ferricytochrome c reduction it was shown that the retina did not contain more total SOD activity than that measured in the oxygen sensitive trout tissues. Despite the fact that total SOD activity was not highest in the retina, most evidence suggests that oxidative flux is greatest at the mitochondrial level and that mitochondrial SOD (MnSOD) plays a disproportionate role in oxidative defense (Stevens and Autor, 1977; Crapo et al., 1980; Forman and Fisher, 1981; Freeman et al., 1986). Thus, manganous SOD activity was measured in the retina and compared to activity in the brain and liver. Because the number of mitochondria differs between the tissues, MnSOD activity expressed per mg protein does not reflect the mitochondrial content of MnSOD. Therefore activity was also expressed per unit cytochrome oxidase. Cytochrome oxidase has previously been used as a mitochondrial marker enzyme (Freeman and Crapo, 1981; Freeman et al., 1986). When MnSOD activity was expressed per unit cytochrome oxidase, there was no indication that MnSOD/mitochondria was greater in the retina relative to the other tissues. Thus, total SOD content as well as MnSOD content of

the oxygen resistant trout retina is no different than that measured in the oxygen sensitive brain and liver tissues from this species. Taken together, the data indicate that regulation of $O_2^{\cdot}$ is not solely responsible for the resistance of the retina to hyperoxia.

With sufficient superoxide dismutase to handle the superoxide flux the resistance to oxygen toxicity in the trout retina might be due to a more efficient defense mechanism to handle the byproduct of the dismutation reaction, i.e., H_2O_2 (see equation 1). The two primary antioxidant enzymes which scavenge H_2O_2 are catalase and the glutathione/gluthathione peroxidase redox system. Catalase decomposes H_2O_2 into H_2O and O_2. During the catalytic cycle, the active heme site undergoes successive divalent oxidation and reduction reactions with H_2O_2. Catalase is present in a variety of aerobic cells although its concentration can vary significantly among tissues. The enzyme may be found in the cytosol but most often it is localized within peroxisomes or microperoxisomes (Chance, Sies and Boveris, 1979). However, catalase activity could not be detected in retina or brain homogenates from the rainbow trout. Indeed, neither the presence of Triton X-100 to release catalase from peroxisomes nor the addition of ethanol to decompose the inactive form of catalase unmasked catalase activity. These findings, however, are consistent with the fact that mammalian retina and brain tissue are known to exhibit only low catalase activity (Bhuyan and Bhuyan, 1977; Cohen, Dembiac and Marcus, 1970). No further attempt was made to quantitate catalase levels in these tissues, since liver activity was within the normal expected range (Cohen et al., 1970).

Although there is no evidence that catalase plays an important role in retinal defense, H_2O_2 concentration may still be limited by

glutathione peroxidase. Glutathione peroxidase can reduce H_2O_2 by catalyzing the reduction of H_2O_2 to H_2O as reduced glutathione is oxidized to form its corresponding disulfide. Glutathione peroxidase is distributed throughout the cytosol, and indeed it reacts more readily with H_2O_2 at low substrate concentrations than does catalase, suggesting that it may play a more important role in cellular decomposition of H_2O_2 (Fridovich and Freeman, 1986). For this reason, selenium dependent glutathione peroxidase activity was also determined. Selenium dependent glutathione peroxidase activity was detected in all three tissues but retinal glutathione peroxidase was not greater than that found in the other tissues. Thus, both catalase and glutathione peroxidase activities in the trout retina were not significantly greater than that measured in the other two oxidant sensitive tissues and therefore do not appear to play a significant role in the protection of the trout retina against oxidative damage. The glutathione S-transferases have also reportedly served as antioxidants in rat lung as selenium independent glutathione peroxidases (Jenkinson et al., 1983), however, it has been reported that no GSH peroxidase activity of this kind could be detected in trout tissues by differential assay of GSH peroxidase with different substrates (Bell, Cowey and Youngson, 1984).

Total sulfhydryl and non-protein sulfhydryl content was also measured in the trout retina and compared to brain and liver. The concentration of soluble protein and non-protein sulfhydryl groups were determined with Ellman's reagent (Sedlack and Lindsay, 1968). Reduced glutathione reportedly accounts for over 90% of the non-protein sulfhydryls in the retina (Winkler and Giblin, 1983), therefore values of non-protein sulfhydryls obtained with Ellman's reagent are reported as

glutathione. In addition to its role as substrate for the glutathione peroxidase enzyme, glutathione as well as other thiol (SH) compounds may provide protection against oxidative injury. These substrates act to repair oxidative damage by undergoing non-enzymatic hydrogen abstraction to restore function to oxidized proteins (Mannervik et al., 1983). However, no differences in either total sulfhydryl content or glutathione content were measured in any of the tissues studied. Thus, glutathione as substrate for glutathione peroxidase or as a secondary antioxidant does not appear to contribute significantly to the oxidative resistance observed in the trout retina.

Although it is difficult to make comparisons to values reported in the literature for the previously stated reasons, some relative comparisons could be drawn, i.e., superoxide dismutase and glutathione peroxidase activities were highest in the liver and very low in brain tissues from the carp (Mazeaud, Maral and Michelson, 1979). High SOD and catalase activities have also been reported in the liver of mackerel (Aksnes and Njaa, 1981) and in livers of several freshwater fish species (Wdzieczak et al., 1982; Desrochers and Hoffert, 1983). The measure of glutathione peroxidase activity in the trout liver in the present study compares favorably with some published values in rat and trout (Lawrence and Burk, 1976; Smith and Shrift, 1979), but it is considerably higher than values obtained from rainbow trout liver by Tappel et al., (1982). The glutathione content of the trout retina is also comparable to the level determined in the rat retina (Winkler and Giblin, 1983). Taken together the results of the present study indicate that the primary antioxidants do not appear to play a significant role in the protection of the trout retina against oxygen toxicity.

A Rationale for the Adaptive Response of Tissues to Acute vs Chronic Hyperoxic Exposure

In all models of acute hyperoxia, one or all of the key antioxidant enzymes (i.e., CuZn and Mn superoxide dismutase, catalase and glutathione peroxidase) have been induced in a relatively short period of time (Jamieson et al., 1986). The effect of acute hyperoxic exposure on tissue antioxidant activity has primarily been studied in the lungs since, in vivo, pulmonary epithelial and lung capillary endothelial cells would be exposed to the same concentration of oxygen as that which is inspired. Due to the saturation of hemoglobin in air at 1 atm and the steep pO_2 gradients along the vascular supply and within the tissue, most tissues are not exposed to high oxygen tensions, even in hyperoxic situations (Crapo and Tierney, 1974; Chance, Sies and Boveris, 1979). Crapo and Tierney (1974) first investigated the induction of antioxidants as a possible mechanism for tolerance to oxygen toxicity. They noted that SOD activity changed in parallel to oxygen tolerance and remained elevated for the duration of the tolerant state. Several investigators have reported the induction of a variety of antioxidants in lung tissue after hyperoxic exposure in rats (Kimball et al., 1976; Crapo, Sjostrom and Drew, 1978; Crapo et al., 1980; Jenkinson et al., 1983). Significant increases in the primary antioxidants CuZn and Mn superoxide dismutase, catalase and glutathione peroxidase (GSHPx), as well as reduced glutathione and total non-protein sulfhydryl content have all been shown to occur in lung tissue after 5-14 days of exposure to 85-90% oxygen. It is thought that induction of these antioxidant defense systems is responsible for the partial protection of the tissues observed with subsequent hyperoxic challenge. Although a causal

relationship between induction of the enzymes and hyperoxic exposure is not yet clearly established, there is sufficient evidence to indicate that a number of the antioxidant enzymes do increase their activities after exposure to 85-90% oxygen for at least 5 days.

The results presented here appear to contradict the free radical theory of hyperoxic damage, i.e. current theories on the biochemical basis for resistance to hyperoxia all point to a central role for the primary antioxidants. A critical difference appears to be related to the type of oxygen exposure to which the cells are exposed, i.e., acute vs. chronic hyperoxic exposure. Tissues exposed to normoxic conditions (i.e., 20% oxygen) will undergo a rapid induction of one or more of their antioxidant defense enzymes when exposed to elevated O_2 tensions (Crapo and Tierney, 1974; Kimball et al., 1976; Forman and Fisher, 1981; Freeman et al., 1986). However, the increased level of antioxidant defense will only yield a partial protection against oxidative damage when subsequently challenged with hyperoxia, i.e., tolerance to acute hyperoxic challenge is limited and oxidative damage is still observed (Crapo et al., 1980; Freeman et al., 1986). In contrast, the rainbow trout retina is a unique tissue that is completely resistant to hyperoxic damage, yet does not rely on the primary antioxidant defenses. In contrast to all other tissues acutely exposed to hyperoxia, the rainbow trout retina is adapted to chronic hyperoxic exposure. Thus, the resistance of a particular tissue to oxidative injury may be dependent upon the means by which adaptation was attained, i.e. acute induction of antioxidant defenses vs an inherent genetic adaptation to hyperoxia. If so, one would predict that other tissues chronically adapted to hyperoxia would exhibit similar resistance despite non-induced levels of the

primary antioxidants. Interestingly, recent evidence obtained in the teleost swimbladder and in a HeLa cell line adapted to 80% oxygen, support the contention that a genetic resistance to chronic hyperoxic exposure is afforded by unique, yet undefined, mechanisms. Superoxide dismutase, catalase and glutathione peroxidase have each been measured in the fish swimbladder, a tissue chronically exposed to extremely high oxygen tensions (Matkovics et al., 1977; Mazeaud, Maral and Michelson, 1979; Morris and Albright, 1981; 1984). Interestingly, catalase and peroxidase activities in the swimbladders of the carp and toadfish were not significantly greater than the other tissues studied. In addition, no correlation was seen between average percent oxygen contained in the swimbladder and catalase or glutathione peroxidase activities from six marine species studied (Morris and Albright, 1984). Superoxide dismutase activity was also not significantly greater in the swimbladder of the carp when compared to other tissues (Matkovics et al., 1977; Mazeaud et al., 1979). However, swimbladder SOD was observed to be greater than gill, kidney and heart tissues in the toadfish (Morris and Albright, 1981). Morris and Albright (1981), postulated that the low SOD activity measured in the carp may be due to the lower oxygen tension in the physostomatous swimbladder of that species. However, in their study (Morris and Albright, 1981), SOD activity in the liver, a tissue which had been shown to have the highest superoxide dismutase activity of the tissues studied, was not reported (Matkovics et al., 1977; Mazeaud et al., 1979). In addition, their measure of SOD activity in the gas gland of the toadfish swimbladder was not corrected for contamination by blood, which they reported to have the highest superoxide dismutase activity (Morris and Albright, 1981). In a later study these authors do

report a positive correlation between average percent oxygen measured in the swimbladder and superoxide dismutase activity for six marine fish (Morris and Albright, 1984). However, activity was not measured in any of the other tissues and SOD activity did not change within any one species of fish as swimbladder oxygen tension was increased over a period of 4 days. They concluded that SOD may play a role in the adaptation of swimbladders to high levels of oxygen, but since it was not induced with increasing oxygen concentrations it appears that defense mechanisms other than the induction of the primary antioxidants play a key role in protecting the swimbladder against the rapid increases in oxygen concentration that occur during inflation (Morris and Albright, 1984).

Additional evidence that primary antioxidants do not play a role in cells genetically adapted to chronic hyperoxia is demonstrated by recent studies with an oxygen adapted HeLa cell line. Joenje et al., (1985), successfully selected a substrain of HeLa cells capable of withstanding increasing oxygen tensions. Indeed, they report that the hyperoxia-adapted HeLa cell line proliferates in 80% oxygen yet does not exhibit any of the hyperoxia induced increases in superoxide dismutase, catalase or glutathione peroxidase enzyme changes normally associated with acute hyperoxic exposure (Joenje et al., 1985). In their study, comparisons were drawn between the HeLa cell line adapted to 80% oxygen and the parent cell line maintained in 20% oxygen. Under hyperoxic conditions, 20% O_2-adapted and 80% O_2-adapted cells exhibited similar CN^--resistant respiration rates, suggesting that the increased O_2 tolerance was not due to a decreased cellular production of activated oxygen species in hyperoxia (Joenje et al., 1985). The cellular levels of CuZnSOD, MnSOD,

catalase, glutathione peroxidase and glucose-6-phosphate dehydrogenase activities were normal or slightly below normal in the 80% O_2-adapted cells, leading the authors to conclude that these enzymes were of no significance for the increased O_2 tolerance observed (Joenje et al., 1985). They concluded that the increased tolerance was due to a genetically determined increased resistance of oxygen sensitive cellular targets.

In summary, the biochemical basis for insensitivity to hyperoxic exposure appears to be dependent upon the oxygen tension to which the cells are normally exposed. In cells exposed to a normoxic environment and challenged with hyperoxia, the threat of oxidative damage causes an induction of the primary antioxidants, which then enables the cells to tolerate subsequent hyperoxic challenge. However, in cells adapted to chronic hyperoxia, the resistance to oxidative damage is independent of the primary antioxidant defense system. Cells adapted to chronic hyperoxia do not exhibit a decreased ability to generate reactive oxygen metabolites or enhanced antioxidant activities. Nonetheless, these cell types are completely resistant to oxidative injury. The biochemical basis for this resistance has not been determined but may be due to a genetic modification of key oxygen sensitive target sites. In the present study, it may be postulated that the lipid and/or protein composition of the rainbow trout retina has been altered such that oxidation of the lipid and/or protein molecules by oxidants is unlikely. Indeed, single amino acid substitutions in proteins or enzymes can drastically decrease their sensitivity to oxidative damage (Janoff, George-Nascimento and Rosenberg, 1986). Recent evidence also suggests that hyperoxia may interfere with DNA synthesis and specifically damage

enzymes important in nucleic acid metabolism. Oxidative damage may occur at the level of thymidine kinase, which, when inactivated would impair the phosphorylation of thymidine to form nucleotides which are incorporated into the DNA molecule (Junod et al., 1985; Junod, Petersen and Jornot, 1987). Taken together, the genetic resistance to oxidative injury of trout retinal cells, may be related to an inherent resistance of key oxygen sensitive target sites. The identification and analysis of key O_2-sensitive regulatory enzymes like thymidine kinase in the teleost retinal cells, may provide specific insights into the basis of the resistance of the rainbow trout retina to hyperoxia.

SUMMARY AND CONCLUSIONS

Although the rainbow trout retina is continually exposed to an elevated oxygen tension, it exhibits a resistance to oxygen toxicity. The mechanism for this resistance was investigated in the present study. Using the method of CN^--insensitive O_2 respiration for an estimate of oxidant formation, it was determined that the rainbow trout retina can generate reactive oxygen species and that formation of these metabolites is not decreased under hyperoxic conditions in this tissue. In addition, the primary antioxidants, superoxide dismutase, catalase and glutathione/glutathione peroxidase do not exhibit greater antioxidant activity in the trout retina when compared to the oxygen sensitive liver or brain tissues. These results are consistent with recent studies which report no correlation between resistance to chronic oxidative stress and an induction of the antioxidant defense systems. Clearly the mechanism for resistance to oxygen toxicity remains to be determined in this model, but may be related to a predetermined genetic resistance of O_2-sensitive target sites in cells which function in chronic hyperoxic conditions. Thus, the free radical theory of "antioxidant induction to protect against hyperoxic exposure" requires modification, and should exclude those specialized tissues in which chronic exposure to hyperoxic conditions is the norm.

APPENDICES

Appendix A

Solutions

Cortland's Saline Solution (pH 7.6)	mmol/L	g/L
NaCl	124.06	7.249
$CaCl_2.2H_2O$	1.56	0.229
KCl	5.10	0.380
$NaH_2PO_4.H_2O$	2.97	0.410
$NaHCO_3$	11.97	1.000
$MgSO_4.7H_2O$	0.93	0.229
Glucose	5.55	1.000

Oxygen Consumption Media (pH 7.4)	mmol/L	g/L
KCl	105.0	7.828
KH_2PO_4	20.0	2.722
Na_2-EDTA	0.1	0.037
Glucose	5.0	0.901

Phosphate/EDTA 50mmol/L (pH 6.5)	mmol/L	g/L
KH_2PO_4	---	4.533
K_2HPO_4	---	2.908
Na_2-EDTA	1.0	0.372
Adjust pH with 2.0 M KOH		

Appendix B

Lowry Protein Determination

Principle

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

Reference

Oyama, V.I. and H. Eagle., Proc. Soc. Expt'l. Biol. Med., 91:305-307, 1956.

Reagents

A.	Lowry A	
	1. Sodium carbonate (anhydrous)	60.0 g
	2. Sodium hydroxide	12.0 g
	3. Sodium potassium tartrate	0.6 g
	4. Distilled water to make:	3000.0 ml
B.	Lowry B	
	1. Copper sulfate solution ($CuSO_4.5H_2O$)	0.5 g%
C.	Lowry C	
	1. Lowry A	50.0 parts
	2. Lowry B	1.0 part
D.	Phenol reagent according to Folin & Ciocalteu (2.0 N)	
	1. Phenol reagent (Sigma Chemical Co.)	1.0 part
	2. Distilled water	1.0 part
E.	Human albumin protein standard 8.0 g% (Lot # PRS-426, Dade Reagents, Inc., Miami, Fl)	
	1. Dilute 3 ml vial with 300 ml distilled water to give 800 ug/ml.	

Procedure

1. Concentrations of protein standards used for determination of standard curve: 0, 20, 40, 60, 80, 120 and 150 ug/ml.
2. Volume of protein solution (standard or unknown) diluted to 1 ml with distilled water.
3. Add 5 ml of Lowry C to protein solution.
4. Incubate 20 min at room temperature.
5. Add 0.5 ml phenol reagent and vortex rapidily.
6. Incubate 30 min at room temperature, mix occassionally.
7. Read at 660 nm on spectrophotometer.

Appendix C

Superoxide Dismutase Assay

Principle

Superoxide dismutase (SOD) is assayed on the basis of its ability to inhibit the rate of reduction of ferricytochrome c by superoxide anions generated by the xanthine - xanthine oxidase system. Manganese SOD is distinguished from the Cu,Zn SOD form by the latter's sensitivity to 2.0 mM potassium cyanide.

Reference

McCord, J.M. and I. Fridovich., J. Biol. Chem., 244:6049-6055, 1969.

Reagents

A. Stock Dispenser Solution

1.	Xanthine	13.1 mg
2.	Na_2-EDTA	55.8 mg
3.	Na_2HPO_4	9.680 g
4.	KH_2PO_4	0.928 g
5.	Distilled water to make:	500 ml

B. Stock Dispenser Solution with 6mM KCN for measurement of MnSOD activity

1.	KCN	39.1 mg
2.	Stock Dispenser Solution (A. above) to make:	100 ml

C. Stock Ferricytochrome c Solution, 0.06 mM (Type III from Horse Heart, MW 12,384; Sigma Chemical Co., St. Louis, Mo.); (R. DiGuilio, personal communication)

1. This protein is contaminated with H_2O and reduced Cytochrome c which absorbs at 550 nm, therefore the concentration must be adjusted spectrophotometrically. The extinction

coefficient (E_M) for reduced-oxidized cytochrome c is 21,000 $M^{-1}.cm^{-1}$ at 550 nm. Any spectrophotometric measurements should be performed on stock diluted 1:10.

2. About 10-30% of commercial cytochrome c (cyt c) is in the reduced form, the remainder is oxidized. Therefore to make 250 ml of 0.06 mM cyt c (ox), a greater amount of the dry protein must be dissolved. Assume only 70% is oxidized, so for 250 ml of 0.06 mM cyt c (ox), one must dissolve 265.4 mg instead of 185.8 mg of dry cyt c.
3. To calculate the exact ratio of cyt c (red) to cyt c (ox) and to adjust cyt c (ox) to 0.06 mM, proceed as follows:
   a) Dilute 265.4 mg/250 ml dH_2O 1:10 and measure A_{550}. Add a few grains sodium dithionite, remeasure the A_{550} and subtract the "minus dithionite" A_{550}.

$$\begin{array}{l} A_{550}\ (+DT) = 0.1615 = \text{reduced + oxidized} \\ A_{550}\ (-DT) = 0.0250 = \text{reduced} \\ \hline 0.1365 \end{array}$$

   0.1365 x 10 (dil. factor) = 1.365, since E_M = 21,000, 1.365/21.0 = 0.065 mM cyt c (ox).

   b) To make the 250 ml cyt c stock solution 0.06 mM dilute it appropriately (i.e. $C_1V_1 = C_2V_2$). If the A_{550} change of cyt c stock $\pm$ dithionite is less than 1.26, add more dry cyt c and repeat concentration determination.
   c) Recheck the concentration of the dilute cytochrome c stock as in 3a) above and then divide the stock into 12-15 ml splits. Freeze the cytochrome c in a dry ice - ethanol bath and store at 0 C.

C. Stock Xanthine Oxidase Solution (Make fresh stock prior to running the dose response curve for each tissue.)
   1. 38 ul xanthine oxidase (from cow milk, Lot #10641720-84, 20 Units/ml; Boehringer Mannheim, Indianapolis, IN). The concentration should be adjusted to yield an initial uninhibited reaction rate of 0.025 to 0.030 OD units/min. Since there is a small amount of ferricytochrome c reduction which cannot be inhibited with SOD, this initial rate must be

determined from the difference between the rate of reduction in the absence of any SOD and the rate in the presence of maximal amounts of SOD (e.g., 20 BESOD units in the reaction system).

2. Distilled H_2O to make 50 ml.
3. Stock should be kept on ice during assay.

D. Stock Standard SOD Solution

1. One preprepared vial of lyophilized standard*
2. Distilled water 2.9 ml

*Standard is prepared in advance as follows:

BESOD (#S-8254; Sigma Chemical Co.)	10.1 mg
Distilled H_2O to make:	500 ml

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

3. Working solution for dose response curve was made by diluting 0.5 ml of stock to 10 mls with distilled water (5 units/ml).

E. Stock Tissue Homogenate Solution

1. See description of tissue homogenate preparation in text.

Procedure

1. Prepare a reference cuvette as follows and place in the reference well of the thermostatically controlled (25 C) spectrophotometer:

Appropriate 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:

Appropriate Dispenser Stock Solution	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 and mix with glass rod.
5. Record the change in absorbance per minute at 550 nm. (Eldred, 1979).

Final Assay Reaction Mixture Composition

10 uM	ferricytochrome c
50 uM	xanthine
50 mM	phosphate buffer, pH 7.8 at 25 C
0.1 mM	EDTA
2.0 mM	KCN (when appropriate)
	sample dosage

Assay Design

1. Determine the uninhibited rate of ferricytochrome c reduction by measuring the basal rate in the absence of SOD in triplicate.
2. Determine the rate of ferricytochrome c reduction in the presence of excess SOD (20 BESOD units) in duplicate. The difference in these rates is a measure of the true initial rate of reduction and should be on the order of 0.025-0.030 OD units /min.
3. Generate a dose response curve using at least four doses between 10 and 60% inhibition of the uninhibited rate of reduction, for the BESOD standard, running duplicates at each dose.
4. Generate a tissue dose response curve to measure total SOD activity as was done for the standard.
5. Repeat Steps 1 & 4 this time using the Stock Dispenser Solution containing 6.0 mM KCN in order to measure Mn-SOD activity. Use the average rate of reduction obtained from Step 2 to obtain the true initial rate of reduction.
6. Make fresh Xanthine Oxidase Stock Solution and repeat steps 1-5 for the next tissue.

Decoding in the Coded Log Dose Scale

1. For the present discussion, let A be the lowest dose used and coded 1 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 subtracting the value 1, then multiplying by log B and adding log A.

Expression of SOD Activity

The unit of SOD activity may be defined as the amount of enzyme inhibiting the rate of reduction of ferricytochrome c by 50% (i.e., $ED_{50} \pm$ 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.

Appendix D

Cytochrome c Oxidase Assay

Principle

Cytochrome c oxidase (COX) catalyzes the electron transfer from ferrocytochrome c to dioxygen in the respiratory chain of mitochondria. The catalytic activity of cytochrome c oxidase is determined by following the rate of oxygen uptake polarographically by means of an oxygen electrode. Cytochrome c is kept reduced by ascorbate, with TMPD mediating the electron transport. The blank rate of oxygen consumption, due to the low autoxidation rates of ascorbate and TMPD alone and in the presence of cytochrome c, is subtracted from the measured oxygen uptake.

Reference

Rafael, J., In: Methods of Enzymatic Analysis, Third Edition, edited by H.U. Bergmeyer. New York: Academic Press, 1983, p. 266-273.

Reagents

A. Phosphate/EDTA solution (phosphate buffer, 50 mmol/L, pH 6.5; EDTA 1 mmol/L)

1.	KH_2PO_4	2.266 g
2.	K_2HPO_4	1.454 g
3.	Na_2-EDTA	0.186 g
4.	Distilled water	450 ml
5.	Adjust pH to 6.5 with 2.0 M KOH	
6.	Adjust volume with distilled water to make:	500 ml

B. Ascorbate (0.1 M)

1.	L-Ascorbic acid (Sigma Chemical Co.)	0.176 g
2.	Phosphate/EDTA solution	9 ml

3. Adjust pH to 6.0 with 2 M KOH (no higher, turns yellow)
4. Adjust volume with Phosphate/EDTA (A) to make: 10 ml
5. Freeze 400 ul aliquots in dry ice - ethanol bath and store frozen until ready for use.

C. N,N,N',N'-Tetramethyl-p-phenylenediamine, (50 mM)
1. TMPD dihydrochloride (Eastman Kodak) 0.118 g
2. Place in cold flask on ice
3. Add ice cold distilled water to make: 10 ml
4. Rapidly freeze 100 ul aliquots in dry ice-ethanol bath and store frozen and protected from light. If solution turns darker than a very faint purple, discard and make fresh solution.

D. Reduced cytochrome c (0.7 mM; Type III from Horse Heart, MW 12,384; Sigma Chemical Co.)
1. See below for purification procedure

E. Tris/Acetate Solution (50 mM; pH 7.5)
1. Trizma base (Sigma Chemical Co.) 6.055 g
2. Distilled water 900 ml
3. Adjust pH to 7.5 using glacial acetic acid
4. Adjust volume with distilled water to make: 1.0 L

F. Tissue Homogenate preparation see text

Purification of Cytochrome c

Only 10-30% of the commercially available cytochrome c is in the reduced form, the remainder is in the oxidized form. Therefore the cytochrome c must be reduced with sodium dithionite and then desalted before the correct concentration can be adjusted spectrophotometrically.

A. Preparation of column
1. Clean and dry a 7 mm ID x 300 mm column.
2. Prepare bottom plug using an 18 guage needle by cutting the barrel end at the needle edge and shaving it to fit inner hole of an invertible rubber stopper.
3. Wrap parafilm around the joint. The opening should be level with the stopper bottom, and the stopper should fit snugly into the tube. Attach PE 160 tubing to needle end and clamp.

4. Pack glass wool around the bottom plug and make sure no air bubbles are trapped in the parafilm or glass wool by tamping with a steel rod.

B. Packing and testing column

1. Prepare sephadex G-25 (coarse) by weighing out 4 grams and adding 40 mls of the buffer to be used.
2. Heat at 100 C for 2-3 hrs and cool overnight at 10 C.
3. After cooling, remove air bubbles from sephadex by evacuating with vaccum filter flask for 10-15 minutes with intermittent swirling.
4. Attach column to support and check that it is perfectly vertical.
5. Add buffer (4-6 mls) to column and let 2-3 mls drain out then close outlet.
6. Gently add gel slurry to column with pasteur pipette and allow to settle. If gel begins to pack before completely poured, mix top layer. Fill column to bed height and allow it to pack. Gel should pack as one continous column.
7. Run 100-150 ml of buffer through column to stabilize column, then mark bed level on outside.
8. To test column for leakage and to obtain separation volumes run 1 test separation of Blue Dextran 2000 and Bromphenol blue (Cooper, 1977).

C. Running cytochrome c sample

1. Run 100-150 ml of Tris/acetate solution through column to prepare column for cytochrome c desalting.
2. Dilute cytochrome c sample with distilled water and add a few grains of sodium dithionite to fully reduce the cytochrome c (e.g., 0.1305 g of cytochrome c diluted with 0.3 ml water). Store concentrated sample on ice.
3. Allow the Tris/acetate solution to drain until it just reaches the bed surface.
4. Place the 0.3 ml cytochrome c solution unto the column with a drawn out pasteur pipette and allow it to drain just below the bed surface.

5. Layer 1.0 ml of Tris/acetate solution on top of column and allow it to run into the column so that all of the sample is into the column.
6. Add Tris/acetate solution to top of column, connect reservoir containing Tris/acetate solution and elute at 1 ml/min.
7. Cytochrome c will chromatograph in three layers, the initial front will be comprised of the polymer form and should be discarded. The next layer, the darkest band, is the purified cytochrome c and should be collected and saved on ice. The last layer contains the de-aminated cytochrome c and also should be discarded.
8. The column should be washed with buffer and preserved with 0.02% sodium azide.

D. Determination of Cytochrome c concentration

1. The chromatographed cytochrome c should be diluted to 3 ml and then its concentration determined spectrophotometrically.
2. Reduced cytochrome c absorbs at 550 nm with an extinction coefficient of 21,000 $M^{-1}.cm^{-1}$. The spectrophotometric measurements should be performed on stock diluted 1:10. The final concentration should be adjusted to 0.7 mM and then 400 ul aliquots should be frozen in a dry ice - ethanol bath and kept frozen until used.

Procedure

Pipette the following reagents successively into the waterjacketed (25 C) electrode chamber; the smaller volumes (<0.2 ml) are injected into the chambers through the access groove using a 2" needle attached to a Hamilton syringe:

1. Phosphate/EDTA solution 2.400 ml
2. Wait for 3-5 min for final equilibration with air. Mixing should be stopped during the following additions to prevent the introduction of air bubbles.
3. Ascorbate solution 0.200 ml
4. TMPD solution 0.040 ml
5. Record oxygen consumption for 2-3 min until the assay blank remains constant.

6. Cytochrome c solution 0.120 ml
7. Record oxygen consumption for 2-3 min until the assay blank remains constant.
8. Homogenized sample containing 1% Lubrol (1:1) 0.040 ml
9. Oxygen consumption is monitored on the recorder.

Final Assay Reaction Mixture Composition

50 mM Phosphate buffer, pH 6.5
1 mM EDTA
7 mM Ascorbic Acid
0.7 mM TMPD
30 uM Cytochrome c
sample dosage

Assay Design

1. Set the electrical zero point on the recorder and then adjust the recorder to 100% with air saturated Phosphate/EDTA solution (25 C), allow 3-5 minutes of mixing for saturation.
2. Add Phosphate/EDTA solution, ascorbate, and TMPD, record the rate of consumption and determine the autoxidation rate for the latter two reagents.
3. Add the tissue sample using a syringe microburet attached to PE-50 tubing, and record oxygen consumption.
4. Inject the cytochrome c solution and record the rate of consumption.
5. Repeat steps 1-4, only this time reversing steps 3 & 4, so that the uptake rate due to the addition of cytochrome c alone can be determined. Total recorder range should equal 1 and total assay blanks should not exceed 0.02-0.03/min.
6. Repeat steps 1-5 so that duplicate measurements are obtained for each tissue sample.
7. A new chamber should be used after each determination. Traces of cytochrome c are removed by washing the chambers with 0.1 N HCl and rinsing thoroughly with distilled water.

Calculation of Activity

1. Determine the assay blank rates of consumption due to the ascorbate + TMPD and due to the cytochrome c, average them for the duplicate measurements, and subtract the total blank rate from the measured uptake rate to obtain the true consumption per unit time.
2. The catalytic activity of the sample is determined using the following equation:

$$O_2 \text{ consumption} = \frac{240 \times V}{v} \times \frac{X}{t} \text{ umoles oxygen consumed/L/second}$$

where:

240 = (equivalent to 240 umol O_2 dissolved/L at 25 C
X = oxygen consumption recorded (corrected for assay blank)
V = assay volume in ml
v = volume of sample added in ml
t = time for oxygen consumption in seconds

3. Since cytochrome oxidase catalyzes the transfer of one mole of electrons per 1/4 mole of oxygen, then 4 umoles of electrons are transfered per umole of oxygen. The katal is equivalent to the transfer of 1 mole of electrons per second. Therefore the rate of consumption can be converted to ukat/L by multiplying by 4.

Appendix E

Catalase Assay

Principle

The catalatic activity of catalase is assayed in crude tissue extracts using a rapid spectrophotometric variation of the technique for determining catalase activity by measuring H_2O_2 by titration with permanganate.

Reference

Cohen, G., D. Dembiec and J. Marcus., Anal. Biochem., 34:30-38, 1970.

Reagents

I.	Isotonic Buffered Solution		
	A. Stock Solution		
	1. NaCl	180.0	g
	2. Na_2HPO_4	27.3	g
	3. $NaH_2PO_4.H_2O$	4.86	g
	4. Distilled water	1.9	L
	5. Adjust pH to 7.40		
	6. Adjust volume with distilled water to make:	2.0	L
	B. Working Solution		
	1. Isotonic Buffer - Stock Solution	90.0	ml
	2. Distilled water to make:	1.0	L
II.	Ethanol		
	1. Ethanol (100%)	10.0	ml
III.	10% Triton X-100 (Sigma Chemical Co.)		
	1. Triton X-100	2.5	ml
	2. Add isotonic buffer to make:	25	ml

3. Dissolve in solution by sonifying.

IV. Monobasic Potassium Phosphate (1.0 M)

1.	KH_2PO_4	13.609 g
2.	Distilled water to make:	100 ml

V. Dibasic Potassium Phosphate (1.0 M)

1.	K_2HPO_4	17.418 g
2.	Distilled water to make:	100 ml

VI. Phosphate Buffer

A. Stock Solution (1.0 M)

1.	KH_2PO_4 (1.0 M)	9.0 ml
2.	K_2HPO_4 (1.0 M)	10.0 ml

B. Working Solution (0.01 M)

1.	Phosphate Buffer - Stock Solution	2.0 ml
2.	Distilled water to make:	200 ml
3.	Adjust pH to 7.0	

VII. Hydrogen Peroxide Solution (6 mM)

1.	30% H_2O_2	0.06 ml
2.	Phosphate Buffer to make:	100 ml

VIII. Sulfuric Acid (6 N)

1.	Concentrated H_2SO_4	168.0 ml
2.	Distilled water to make:	1.0 L

IX. Potassium permanganate solution (0.01 N)

1.	$KMnO_4$	0.316 g
2.	Distilled water to make:	1.0 L

X. Catalase solution

A. Stock Solution

1.	Catalase*	2.0 mg
2.	Distilled water to make:	10.0 ml

B. Working Solution

1.	Stock Catalase, at least 4 dilutions between	0.05-0.5 ml
2.	Distilled water to make:	10.0 ml

* Crystalline beef-liver catalase (Sigma Chemical Co., St. Louis, Mo., #C-40).

Assay Procedure

The reagents are added sequentially at fixed intervals and allowed to react for exactly 3 minutes. The remaining H_2O_2 is then reacted with a standard excess of $KMnO_4$ and the absorbance of the residual $KMnO_4$ is measured at 480 nm. All reactions are carried out in an ice-water bath (0-2 C).

1. Place duplicate 0.5 ml aliquots of the cold catalase samples (either standards or diluted tissue samples) into cold 20-40 ml test tubes.
2. Include duplicate blanks consisting of 0.5 ml distilled water.
3. For tissues exhibiting weak catalase activity (e.g., brain) it is necessary to include a different blank (i.e., by simply reversing the order of addition of the acid and the peroxide) to correct for substances that can reduce H_2O_2 or $KMnO_4$.
4. Initiate the enzymic reactions sequentially at fixed intervals by adding 5.0 ml of cold 6 mM H_2O_2 and vortex thoroughly.
5. After exactly 3 minutes, stop the reactions sequentially at the same fixed intervals by rapidly adding 1.0 ml 6N H_2SO_4 and vortexing.

Spectrophotometry

The H_2O_2 is measured by reacting it with a standard excess of $KMnO_4$ and then measuring the residual $KMnO_4$ spectrophotometrically. The temperature of the cell within the spectrophotometer is maintained at 2-4 C.

1. Prepare duplicate spectrophotometric standards by adding 7.0 ml of 0.01 N $KMnO_4$ to a mixture of 5.5 ml of 0.01 M phosphate buffer and 1.0 ml 6 N H_2SO_4.
2. Combine the duplicate spectrophotometric standards and read the absorbance at 480 nm using a single beam spectrophotometer.
3. For convience in performing later calculations, adjust the digital readout to read 1.000 absorbance unit for the standard.
4. The remaining enzyme-reaction samples and the blanks are taken one at a time. To each, rapidly add 7.0 ml $KMnO_4$ reagent, mix

thoroughly, and read at 480 nm within 30-60 seconds.

5. To avoid precipitation of MnO_2 in the cell, rinse with distilled water between samples.

Calculations

As described, the decomposition of H_2O_2 by catalase follows first order kinetics (See Equation 1).

$$k = \log (S_o/S_3) \times 2.3/t \qquad (1)$$

where:

k = first order reaction rate constant,

t = time interval over which reaction is allowed to occur (3 minutes),

S_o = substrate concentration at time zero,

and S_3 = substrate concentration at 3 minutes.

The values S_o and S_3 are expressed in absorbance units.

1. To obtain S_o, subtract the average absorbance of the reaction system blanks from the absorbance of the spectrophotometric standard.
2. To obtain S_3, subtract the absorbance of the reaction samples from the spectrophotometric standard.
3. Results can now be expressed in terms of the first order reaction rate constant (k).
4. Alternatively, a value k may be expressed in terms of units of activity as described by one of the following three methods. (Luck, 1965).

   a) A catalase unit as defined by Bergmeyer is the amount of enzyme which liberates half the peroxide oxygen from a H_2O_2 solution in 100 seconds at 25 C. Since the subtrate concentration is reduced by one half for a given half life then:

$$\text{tau} = \frac{\ln N_1/N_2}{k} = \frac{\ln 2}{k} = \frac{0.693}{k} \ [\text{sec}^{-1}] \qquad (2)$$

   A unit of enzyme activity is therefore related to the half-life time (tau) of a first order reaction:

$$1 \text{ unit} = \frac{100}{\text{tau observed}} = \frac{100}{0.693/k} = \frac{k \text{ observed}}{0.00693} \ [\text{sec}] \qquad (3)$$

b) An international unit of catalase is defined as the umoles of substrate converted per mg protein per min at 25 C. To convert to I.U. the H_2O_2 concentration must be defined. Since, according to Bergmeyer, 1 unit is defined for t = 100 seconds and therefore for a first order reaction k = 0.00693 sec^{-1}, it follows that by substituting this value for k and S_o = 30 umoles H_2O_2/5 ml in equation 1, that approximately 10.2 umoles H_2O_2 are decomposed in 60 seconds. (The value 30 is from an initial H_2O_2 concentration of 10 mM or 30 umoles H_2O_2/5 ml). The value 10.2 is obtained from the difference between 30 and the value obtained from equation 1 with the above substitutions. Therefore 10.2 International Units are equal to 1 unit according to Bergmeyer or I.U. = 10.2 x units according to Bergmeyer.

c) Activity can also be expressed in terms of Kat.f. units (Katalasefahigkeit) in order to facilitate comparisons of crude tissue homogenate activities:

$$\text{Kat.f.} = 1/2.3 \times k/W \qquad (4)$$

where k is the first order reaction rate constant [min^{-1}], the value 2.3 is for logarithmic conversion and W is the amount of protein in a reaction volume of 50.0 ml. The value of W is equal to (50.0/5.5) x the amount of protein in the 0.5 ml homogenate volume for the method described here.

d) Protein concentrations are determined using the method of Lowry (Oyama and Eagle, 1956), so that activity can be expressed on a per mg protein basis.

Appendix F

Glutathione Peroxidase Assay

Principle

Glutathione peroxidase catalyzes the oxidation of glutathione by H_2O_2 or an organic hydroperoxide. The oxidized form of glutathione (GSSG) can be converted back to the reduced form (GSH) through the addition of exogenous glutathione reductase and NADPH, thereby maintaining substrate concentration for the enzyme. Glutathione peroxidase activity can therefore be measured indirectly by following the decrease in absorbance of the reaction mixture at 340 nm as NADPH is converted to NADP.

Reference

Paglia, D.E. and W.N. Valentine., J. Lab. Clin. Med., 70:158-169, 1969.

Reagents

A. Potassium Phosphate buffer/EDTA (0.05 M phosphate; 5 mM EDTA)

1.	K_2HPO_4	0.531 g
2.	KH_2PO_4	0.265 g
3.	EDTA-Na_2	0.186 g
4.	Distilled water	90 ml
5.	Adjust pH to 7.0	
6.	Adjust volume with distilled water to make:	100 ml

B. Reduced Nicotinamide Adenine Dinucleotide Phosphate (8.4 mM)

1.	NADPH	0.016 g
2.	Distilled water to make:	2 ml

C. Glutathione Reductase (Type VII; 75 units/mg solid; Lot #22F-8080; Sigma Chemical Co., St. Louis, MO)

1.	Glutathione reductase	1.3 mg

2. Distilled water to make: 1.0 ml

IV. Sodium Azide (1.125 M)

1. NaN_3 1.829 g
2. Distilled water to make: 25 ml

V. Glutathione (0.15 M)

1. GSH 1.153 g
2. Distilled water to make: 25 ml

VI. Hydrogen Peroxide (0.0022 M)

1. 30% H_2O_2 (w/v) 25 ul
2. Distilled water to make: 100 ml

VII. Sucrose (0.25 M)

1. Sucrose 8.558 g
2. Distilled water to make: 100 ml

Assay Procedure

1. The following reagents are mixed in a 3 ml cuvette or precombined in large fresh pools and kept on ice, so as to expedite sample measurements.
   a. Phosphate buffer/EDTA 2.58 ml
   b. B-NADPH 0.10 ml
   c. Glutathione reductase 0.01 ml
   d. Sodium azide 0.01 ml
   e. Reduced glutathione 0.10 ml
2. The above mixture is pipetted into the reference and sample cuvettes, which are then placed into the thermostatically controlled spectrophotometer and allowed to equlibrate to 25 C for 2-3 minutes.
3. The tissue sample (100 ul) is then added to the sample cuvette, while an equivalent volume of distilled water is added to the reference cuvette and mixed. Sample size is reduced to 50 ul to more accurately measure tissues which exhibit a greater degree of activity (i.e. liver). The difference in volume is made up with phosphate buffer.
4. The reaction is initiated with the addition of 0.1 ml of the H_2O_2 solution to both the reference and sample cuvettes.

Spectrophotometry

Enzyme activity is measured indirectly by following the decrease in absorbance of the reaction mixture at 340 nm as NADPH is converted to NADP at 25 C. A Beckman DB-G Spectrophotometer equipped with a linear recorder is used to obtain a continuous recording of the absorbance changes. True enzymatic activity is measured through the use of a dual beam spectrophotometer which subtracts out the nonenzymatic oxidation of GSH which occurs simultaneously in the reference cuvette.

Calculations

Glutathione peroxidase activity was determined from the molar extinction coefficient for NADPH at 340 nm, which is 6220 $l.mol^{-1}.cm^{-1}$ (Mattenheimer, 1971). Protein determinations were made using the method of Bradford (1976) so that activity could be expressed as mUnits/mg protein or $nmoles.min^{-1}.mg\ protein^{-1}$. The general formula for the calculation of the international enzyme unit is:

$$\frac{A}{E \times d} \times 10^6 \times \frac{1}{t} \times \frac{TV}{SV} = nmoles.ml^{-1}.min^{-1} \text{ or } mU.ml^{-1}$$

where: A = change in absorbance

E = molar extinction coefficient

d = diameter of the cuvette in cm

TV = total assay volume

SV = sample volume

t = reaction time in minutes

mU/ml = milliunits/ml or nmoles/ml/min

and 10^6 converts moles/liter to nmoles/ml

Appendix G

Assay for Estimation of Sulfhydryl Content

Principle

This procedure is a simple spectrophotometric method for the routine determination of the sulfhydryl content in the protein-bound (PB-SH) and nonprotein-bound (NP-SH) fractions of various tissues. The method is based on the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) by SH groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH. The nitromercaptobenzoic acid anion has an intense yellow color which absorbs at 412 nm and can be used to measure SH groups.

Reference

Sedlak, J. and R.H. Lindsay. Anal. Biochem., 25:192-205, 1968.

Reagents

A.	5,5'-dithiobis-(2-nitrobenzoic acid) solution (0.01 M)	
	1. DTNB	0.099 g
	2. Methanol (absolute)	25 ml
B.	EDTA-Na_2 solution (0.2 M)	
	1. EDTA-Na_2	37.224 g
	2. Distilled water to make:	500 ml
C.	Tris buffer (0.2 M) pH 8.2	
	1. Trizma base	13.369 g
	2. Trizma-HCl	14.157 g
	3. EDTA-Na_2 (0.2 M EDTA)	100 ml
	4. Distilled water	850 ml
	5. Adjust pH with 1N HCl to 8.2	
	6. Adjust volume with distilled water to make:	1.0 L
D.	Tris buffer (0.4 M; pH 8.9)	

1. Trizma base 10.633 g
2. Trizma-HCl 1.928 g
3. EDTA-Na_2 (0.2 M) 25 ml
4. Distilled water 200 ml
5. Adjust pH with 1 N HCl to 8.9
6. Adjust volume with distilled water to make: 250 ml

V. EDTA-Na_2 solution (0.02 M)
   1. EDTA-Na_2 7.445 g
   2. Distilled water to make: 1.0 L

VI. Methanol-Absolute
   1. 100% Methanol 100 ml

VII. 50% Trichloroacetic acid
   1. Trichloroacetic acid (100%) 25 ml
   2. Distilled water to make: 50 ml

Assay Procedure

I. Total sulfhydryl content is determined by reacting 0.5 ml of the tissue homogenates buffered in 0.2M Tris at pH 8.2 with Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) in the presence of absolute methanol.

   A. All buffers and solutions should be gassed 2-3 minutes with a vigorous stream of nitrogen prior to their use in the assay.

   B. Make up the following reaction mixtures in an ice water bath (0-2C).

      1. Tissue homogenate (Run in duplicate)
         a. Place 0.5 ml of tissue homogenate in 15.0 ml test tubes.
         b. Add 1.5 ml of 0.2 M Tris buffer (pH 8.2).
         c. Add 0.1 ml of 0.01 M DTNB solution and mix.
         d. Bring up to 10.0 ml with 7.9 ml of methanol.
      2. Sample blank
         a. Place 0.5 ml of tissue homogenate in 15.0 ml test tube.
         b. Add 1.5 ml of 0.2 M Tris buffer (pH 8.2).
         c. Bring up to 10.0 ml with 8.0 ml of methanol.
      3. Reagent blank (Run in duplicate)

a. Place 2.0 ml of 0.2 M Tris buffer (pH 8.2) in 15.0 ml test tubes.

b. Add 0.1 ml of 0.01 M DTNB solution and mix.

c. Bring up to 10.0 ml with 8.0 ml of methanol.

C. Stopper the tubes and let stand 30 minutes with occassional shaking.

D. Filter twice through the same folded filter paper such that a clear filtrate is produced.

E. An alternative method would be to allow color to develop over 15 minutes then centrifuge at 3000g at room temperature for 15 min.

F. The duplicate reagent blanks are mixed and used to set the spectrophotometer to full scale (100% Transmittance).

G. The absorbance of the filtrates is read at 412 nm in a Beckman DB-G dual beam spectrophotometer using 1 cm quartz cuvettes with the respective sample blanks in the reference cuvette.

II. Non-protein bound sulfhydryl content is determined using 2.5 ml of the tissue homogenate and reacting it with 50% trichloroacetic acid in order to precipitate out the proteins. Color is developed within 10-15 minutes and the mixture is filtered in order to obtain a clear filtrate.

A. All solutions should be gassed with 100% N_2 for 2-3 minutes.

B. Make up the following reaction mixture in an ice water bath (0-2C).

1. Tissue homogenates (Run in duplicate).

a. Place 2.5 ml of tissue homogenates in 15.0 ml test tubes.

b. Add 2.0 ml of distilled water.

c. Add 0.5 ml of 50% trichloroacetic acid.

d. Shake tubes 10-15 minutes.

e. Filter twice through the same folded filter paper such that a clear filtrate is produced.

C. A 2 ml aliquot of the filtrate buffered with 0.4 M Tris at pH 8.9 is then reacted with Ellman's reagent and the absorbance measured against a reagent blank at 412 nm within 5 minutes. Make up the following reaction mixtures for spectroscopy.

1. Tissue homogenates
   a. Place 2.0 ml of the clear filtrate (from part B above) into 15.0 ml test tubes.
   b. Add 4.0 ml of 0.4 M Tris buffer (pH 8.9) and mix.
   c. Add 0.1 ml of 0.01 M DTNB, mix and read absorbance within 5 minutes.
2. Reagent blank (Run in duplicate).
   a. Place 6.0 ml of 0.4 M Tris buffer (pH 8.9) into 15.0 ml test tubes.
   b. Add 0.1 ml of 0.01 M DTNB, mix and read absorbance within 5 minutes.

D. The duplicate reagent blanks are mixed and used to set the spectrophotometer to full scale (100% Transmittance).

E. The absorbance of the tissue homogenates is read at 412 nm in a Beckman DB-G dual beam spectrophotometer using 1 cm quartz cuvettes with the reagent blank in the reference cuvette.

III. Determination of Protein-Bound Sulfhydryls.

A. Protein-bound sulfhydryl content is determined as the difference between the total and nonprotein-bound sulfhydryl groups.

Calculation of Sulfhydryl Content

1. The total sulfhydryl and non-protein sulfhydryl content of the tissues is calculated from the experimentally determined molar extinction coefficient of 13,100 $l.mol^{-1}.cm^{-1}$ at 412 nm. The general formula for the calculation is:

$$\frac{A}{E \times d} \times 10^6 \times \frac{TV}{SV} = \text{umoles.ml}^{-1}$$

where:

A = absorbance

E = molar extinction coefficient

d = diameter of the cuvette in cm

TV = total assay volume

SV = sample volume

and 10^6 converts moles/liter to umoles/ml

2. The protein bound sulfhydryl content is calculated by subtracting out the non-protein bound sulfhydryls from the total sulfhydryl content of the tissues.
3. The Lowry method (Oyama and Eagle, 1956), was used to determine the protein concentration so that activity could be expressed on a per mg protein basis.

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