MECHANISMS OF OXYGEN REDUCTION BY HYDROQUINONES

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Mechanisms of Oxygen Reduction by Hydroquinones

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ABSTRACT

Cytotoxic quinones such as 6-hydroxydopamine (6-OHDA), antitumour quinones, or phenolic metabolites of benzene exert toxic effects through cyclic reduction and reoxidation, producing active oxygen ($O_2^{\cdot-}$, H_2O_2, $^{\bullet}OH$). Mitochondrial respiration also produces active oxygen through autoxidation of reduced quinone intermediates. This thesis investigates the mechanisms of reaction of hydroquinones with oxygen. The thesis has three main parts: a) an evaluation of mitochondrial respiratory impairments on autoxidation of reduced quinone intermediates, b) investigations into roles of trace metals and radical propagators in the autoxidations of 6-hydroxydopamine and ascorbate, and c) a systematic comparison of the autoxidation mechanisms of hydroquinones differing in reduction potentials, aromaticity, and metal affinity.

Respiring submitochondrial particles did not measurably increase $O_2^{\cdot-}$ generation as reduced intermediates accumulate with a decline in oxygen tension to anoxia. Partial removal of cytochrome *c* however, as occurs in ischaemia-reperfusion injury, increased normoxic $O_2^{\cdot-}$ generation relative to oxygen consumption by 60-100%. A hypothesis is presented whereby, with cellular age, mitochondrial mutations which disrupt normal electron flow to oxygen, as with respiratory inhibitors, may increase oxidative stress through autoxidation of accumulating reduced intermediates.

The effects of metal chelators and superoxide dismutase on the reaction of 6-OHDA with oxygen revealed the importance of trace metals as initiators, and of $O_2^{\cdot-}$ as a reaction propagator. Superoxide dismutase inhibited autoxidation by up to 96%, but control experiments with albumin and denatured superoxide dismutase revealed synergistic coordination of trace metals by the apoprotein. Coordination of trace metals with desferrioxamine, histidine, or albumin inhibited autoxidation 30-40% and enhanced inhibition by catalytic amounts of superoxide dismutase from less than 25% to over 95%. Metal chelation thus precludes formation of a ternary 6-OHDA:metal:oxygen complex, and forces the autoxidation to occur by an outer sphere, $O_2^{\cdot-}$-propagated pathway. The

superoxide dismutase mimic, Mn-desferrioxamine (green complex), did not inhibit 6-OHDA autoxidation in the presence of excess desferrioxamine, instead catalysing autoxidation. With added transition metals, EDTA enhanced catalysis by Fe, but inhibited catalysis by Cu, Mn, or V. Desferrioxamine and histidine inhibited catalysis by each metal. A plot of catalytic activity versus reduction potential of metal:ligand complexes revealed a potential midway between reductant and oxygen as optimal for facile reduction and reoxidation. In ascorbate autoxidation, synergism between metal chelators and superoxide dismutase was not evident due to over 98% inhibition by chelators alone. Stronger inhibition by heat-denatured superoxide dismutase than native enzyme indicated a greater affinity of the unfolded protein for catalytic metals. The plasma antioxidant, uric acid, inhibited 6-OHDA autoxidation synergistically with ascorbate or superoxide dismutase, indicating metal-binding and cooperation with ascorbate and superoxide dismutase in its antioxidant activities.

The autoxidations of selected benzenediols, benzenetriols, and naphthalenediols differed in their responses to superoxide dismutase and metal chelators. In the autoxidation of benzenetriols (gallate; 2,3,4-trihydroxybenzoate; pyrogallol; 1,2,4-benzenetriol), 5 U/ml superoxide dismutase inhibited 17-60%, and metal chelators sensitized to further inhibition. With benzenediols (catechol and 1,4-hydroquinone), superoxide dismutase *accelerated* autoxidation (70% and 300%), and metal chelators did not produce inhibition. Superoxide dismutase evidently accelerates autoxidation when thermodynamic limitations to reduction of oxygen outweigh propagating actions of $O_2^{\cdot -}$. With the naphthalenediols (1,2- and 1,4-naphthalenediol), superoxide dismutase inhibited briefly but then accelerated autoxidation. More facile quinhydrone formation with naphthoquinones allows comproportionation to quickly supercede $O_2^{\cdot -}$ as a propagator . Whether an autoxidation proceeds by comproportionation or $O_2^{\cdot -}$-propagation, biologically important effects of superoxide dismutase are to limit formation of semiquinones and to facilitate redox pathways for excretion of cytotoxic quinones.

ACKNOWLEDGEMENTS

I much appreciate the contributions of the members of my graduate committee, Dr. Miriam Rosin, Dr. Glen Tibbits, and, in the early stages, Dr. Mike Gresser, to various aspects of my degree progression and thesis evolution. I also extend my gratitude to the Kinesiology technical staff, who have graciously helped me keep unruly instruments and computers operational, to the Kinesiology office staff, who have kindly and efficiently assisted in various ways in the face of crises and impending deadlines. I extend my warmest appreciation to the Kinesiology graduate students, faculty, and soccer teammates for making my time here enjoyable and rewarding, and to my coworkers in the Bioenergetics Lab. over the years, particularly Eunice Rousseau, Jym Moon, Anna Li, Luoping Zhang, Tony Kettle, Pauline Gee, Xuefeng Yin, Ann-Marie Gilbert, Linda Kowalski, Patrick Walter, James Weiler, Charles Tai, and Suiping Zhou for their help and friendship. I thank my family for their patience, love, and support. Special thanks to Jym Moon for his enthusiasm and moral support, and for many enlightening scientific discussions, many of which contributed to the development of this thesis. Most especially, I thank my Senior Supervisor, friend, and mentor, Professor Allan Davison, for sparking my interest in free radicals, for always being there for guidance, advice, support, and encouragement, for providing careful and insightful editing and fresh approaches and ideas, and for conveying a wonder and appreciation for science and nature to myself and other students of his laboratories.

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Chapter 1.
Redox Cycling in the Cytotoxicity of Quinones

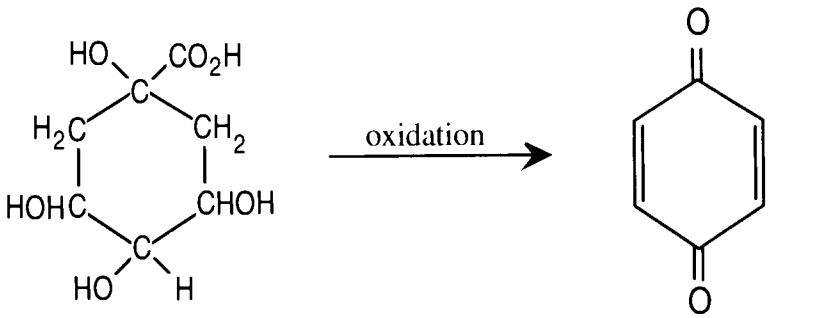
Abstract

The mechanisms by which hydroquinones react with oxygen require systematic characterization. We are exposed to potentially cytotoxic quinones from many sources. They are produced endogenously, prevalent in foods and the environment, and arise in the metabolism of xenobiotics, including aromatic pharmaceuticals or environmental pollutants. Pathologies involving or implicating quinone cytotoxicities include: Parkinson's disease and other neuropathies, catecholamine cardiotoxicity, antitumour quinone cardiotoxicity, benzene myelotoxicity and carcinogenicity, cigarette carcinogenicity, naphthalene ophthalmotoxicity, and cellular respiration-induced aging. Cytotoxicity of quinones arises mostly from two mechanisms: redox-cycling, with production of reactive oxygen species ($O_2^{\cdot-}$, H_2O_2, $^{\bullet}OH$, $^1O_2$), and electrophilic attack of quinones on cellular components such as proteins or DNA. Since quinones are reduced intracellularly to the corresponding hydroquinones, both mechanisms depend in part on the reaction of hydroquinones with oxygen. Due to a large activation free energy barrier, oxygen has a limited range of mechanisms available to react with hydroquinones. Catalytic metals and radical propagators are thus important participants. However, the relative contribution of different metals and radical propagators varies with different hydroquinones and needs systematic study. A major goal is to find generalizations relating hydroquinone structure to the mechanism of reaction with oxygen. In addition, we need to understand how different metal-binding and chain-breaking antioxidants interact to inhibit hydroquinone oxidations. Such interactions are fundamental to mechanisms of antioxidant action and to therapeutic strategies for protecting against quinone cytotoxicities.

Quinones as cytotoxic species: Current perspectives and future prospects

Quinones are among the most electronically active of all organic biomolecules. They are fundamental to life, abundant in nature, and highly active as natural and synthetic pharmaceuticals. The medicinal activity of a quinone preparation was first documented in Peru in 1638, when an extract from the bark of the chinchona tree, called "kina-kina" by the natives ("quina-quina" in Spanish), was used to cure a fever in the wife of the Spanish governor of Peru. Eventually the active ingredient was isolated and identified as a derivative from kinic acid, called "kinone" or "quinone" (figure 1). The major classes of quinones are shown in figure 2. In addition, several quinoid compounds such as quinonimines and phenazines (figure 3) show similar chemical properties. The unique chemistry of quinones results from their ability to undergo reversible reduction to the hydroquinone and reoxidation to the parent quinone (figure 4). The oxidation can occur either enzymatically (eg. by myeloperoxidase), or by non-enzymic reaction with oxygen (the subject of this thesis).

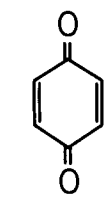
Quinones serve many functions biologically and industrially or clinically. Some relevant quinones or hydroquinones are shown in figure 5. Quinones provide a major function as electron transfer agents in cellular respiration (ubiquinone, flavoquinones) and photosynthesis (plastoquinones). Other biological functions of quinones include as hormones (catecholamines), as oxidation-reduction cofactors in protein carboxylation (vitamin K) and in monooxygenases or mixed-function oxidases (flavins, biopterins), as pigments (lawsone, tannins, melanins), as precursors to structural polymers (eg. lignin), and as natural pesticides or herbicides (eg. juglone in walnut hulls and leaves). The antioxidants vitamin C, vitamin E and bioflavonoids also have quinone or quinoid structures. Quinone or quinoid compounds used as pharmaceuticals include analgesics (acetaminophen), antibiotics, antihelmintics, and antitumour agents (eg. streptonigrin, mitomycin C, anthracyclines). Quinone containing extracts from plants such as rhubarb and aloe have been used as purgatives for over 4000 years, and are widely used in folk medicines. Industrially, quinones are used as stains (eg. alizarin) and dyes (eg. lawsone in henna and "natural" hair dyes).



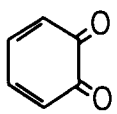
quinic acid

quinone
(chinon / kinone)

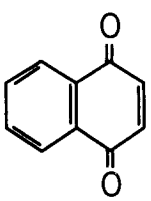
Figure 1. Oxidation of quinic acid to quinone.



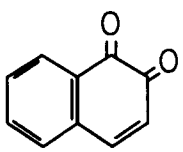
p-benzoquinone



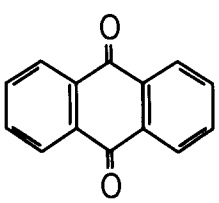
o-benzoquinone



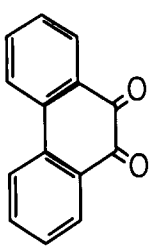
p-naphthoquinone



o-naphthoquinone



anthraquinone



phenanthrenequinone

Figure 2. Major classes of quinones

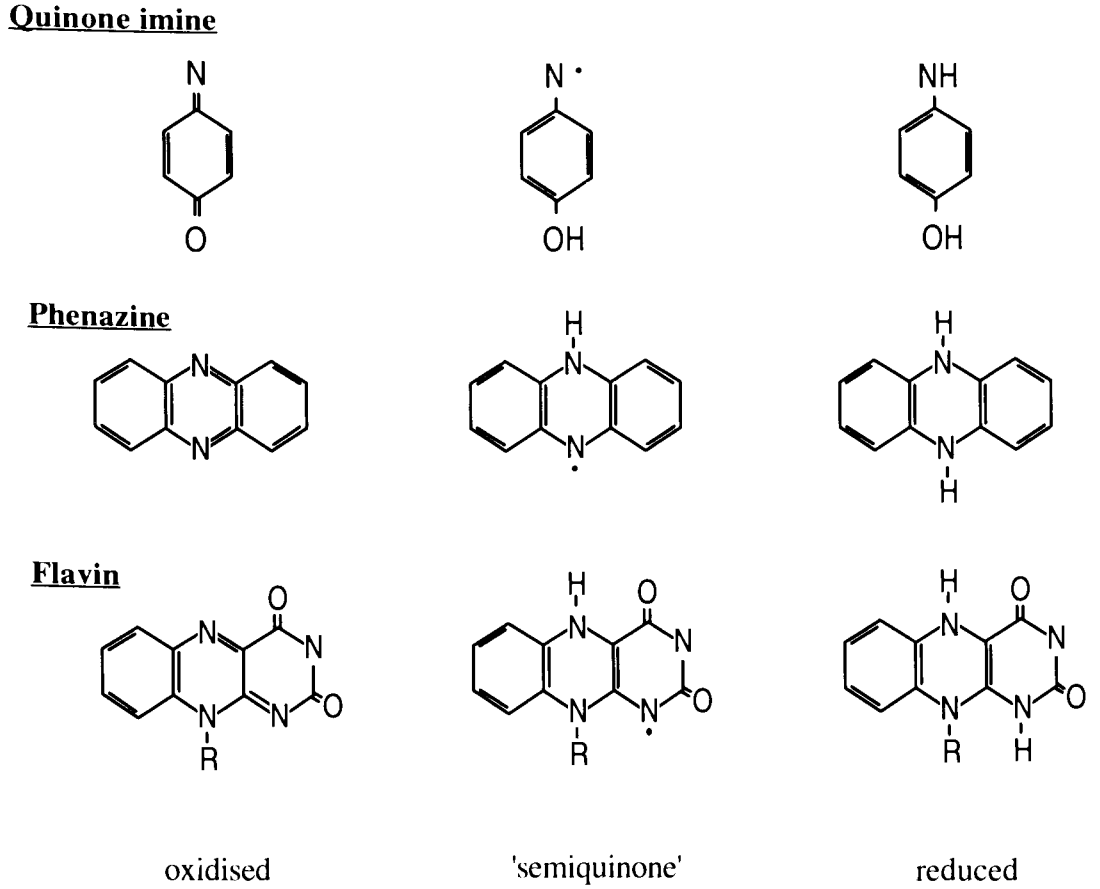


Figure 3. Structures of some quinoid compounds and their semiquinones

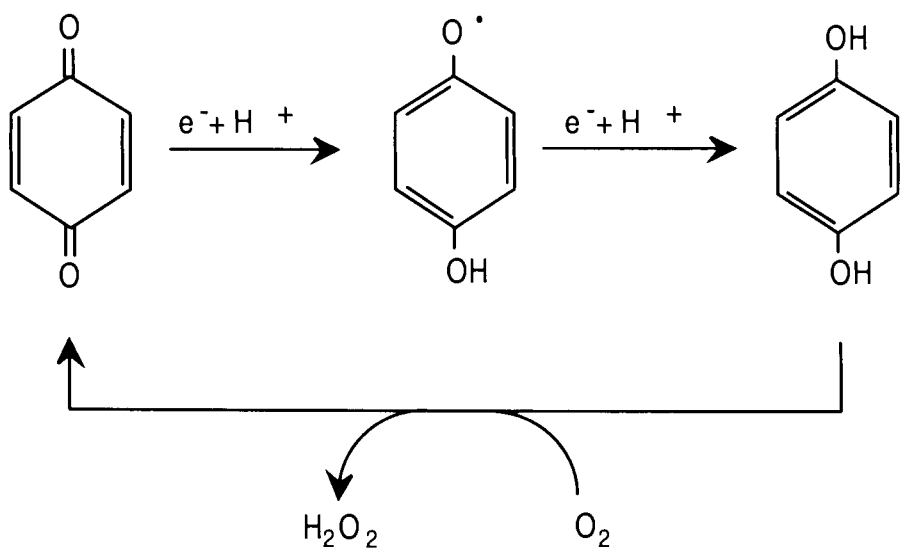
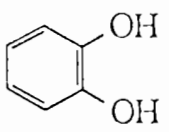
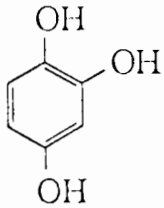


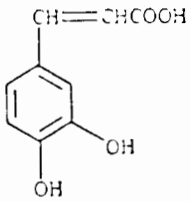
Figure 4. Reversible reduction/oxidation of *p*-benzoquinone



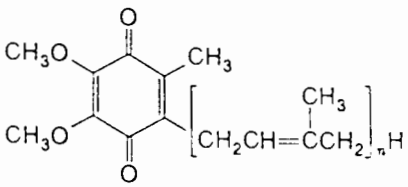
catechol



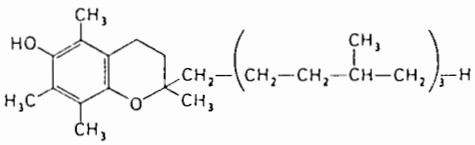
1,2,4-benzenetriol



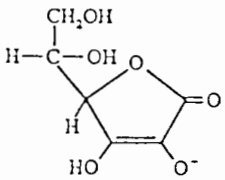
caffeic acid



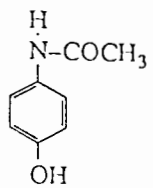
ubiquinone



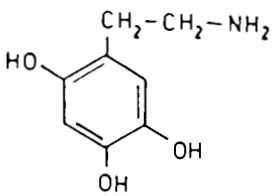
vitamin E



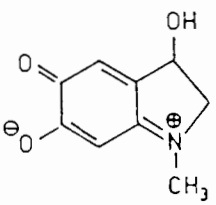
ascorbate



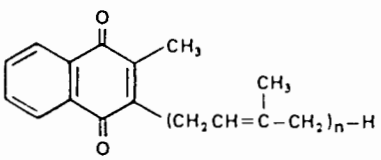
acetaminophen



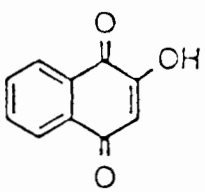
6-hydroxydopamine



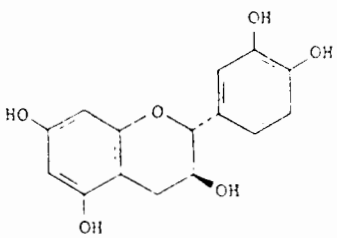
adrenochrome



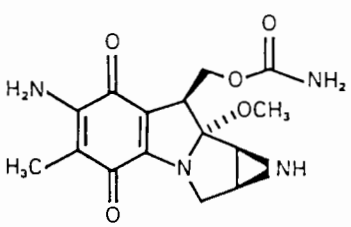
vitamin K_2



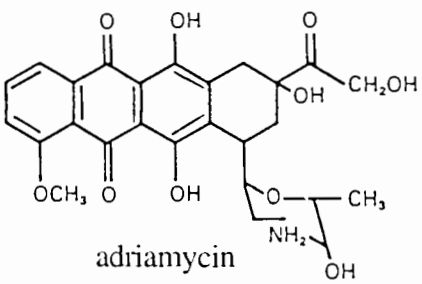
lawsone
("henna")



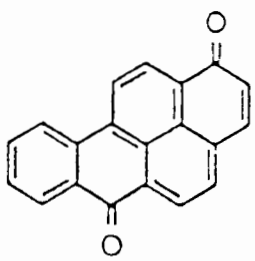
catechin
(bioflavonoid)



mitomycin C



adriamycin



benzo[a]pyrene quinone

Figure 5. Structures of some relevant quinones, hydroquinones and related compounds.

We are exposed to potentially beneficial and toxic quinones or hydroquinones from many sources, endogenous and xenobiotic. Teas and red wines for example contain a wide variety of polyphenolics, some of which are considered beneficial. Coffee contains catechol and caffeic acid. Potentially toxic quinones are present in cigarette smoke (eg. catechol), wood smoke (benzo[a]pyrene quinones), and atmospheric pollution (anthraquinone, phenanthrene quinone, benzo[a]pyrene quinones). Other toxic quinones arise through metabolism of aromatic compounds in pesticides (eg. chlorinated phenolics (Juhl et al 1991)) or of other environmental pollutants (eg. benzene, dioxins).

While quinones serve many therapeutic or useful purposes, both endogenous and xenobiotic quinones may be cytotoxic through cyclic reduction and reoxidation, producing reactive species of oxygen ($O_2^{\cdot-}$, H_2O_2, $^{\bullet}OH$, $^1O_2$) (figure 6). Autoxidative redox cycling can propagate cytotoxic damage to widespread and sensitive cellular targets through lipid peroxidation and metal site specific production of $^{\bullet}OH$ (as elaborated in a comprehensive reaction schema by Borg and Schaich 1984). Such redox cycling-induced oxidative stress contributes to the toxicities and mutagenicity of industrial chemicals, environmental pollutants, and quinone pharmaceuticals. Minimizing or protecting against such actions is of considerable interest. Quinone chemistry, biochemistry, and toxicology has received vast attention, and is the subject of several books (Thomson 1971, 1987; Patai 1974, 1988) and many excellent reviews (Brown 1980, Smith et al 1985, Nohl et al 1986, Powis 1987, 1989, Brunmark and Cadenas 1989, Willson 1990, Wardman 1990, Monks et al 1992). The reaction of hydroquinones with oxygen, and the contributions of active oxygen to quinone cytotoxicity are well documented. However, three aspects stand out as in need of attention: a) the roles of different metals and their ligands in catalysing the reactions of hydroquinones with oxygen, and b) systematic comparisons of the mechanisms by which different hydroquinones react with oxygen, and c) since antioxidants act together *in vivo*, interactions between different antioxidants (metal-binding and/or chain-breaking) in modulating quinone cytotoxicity.

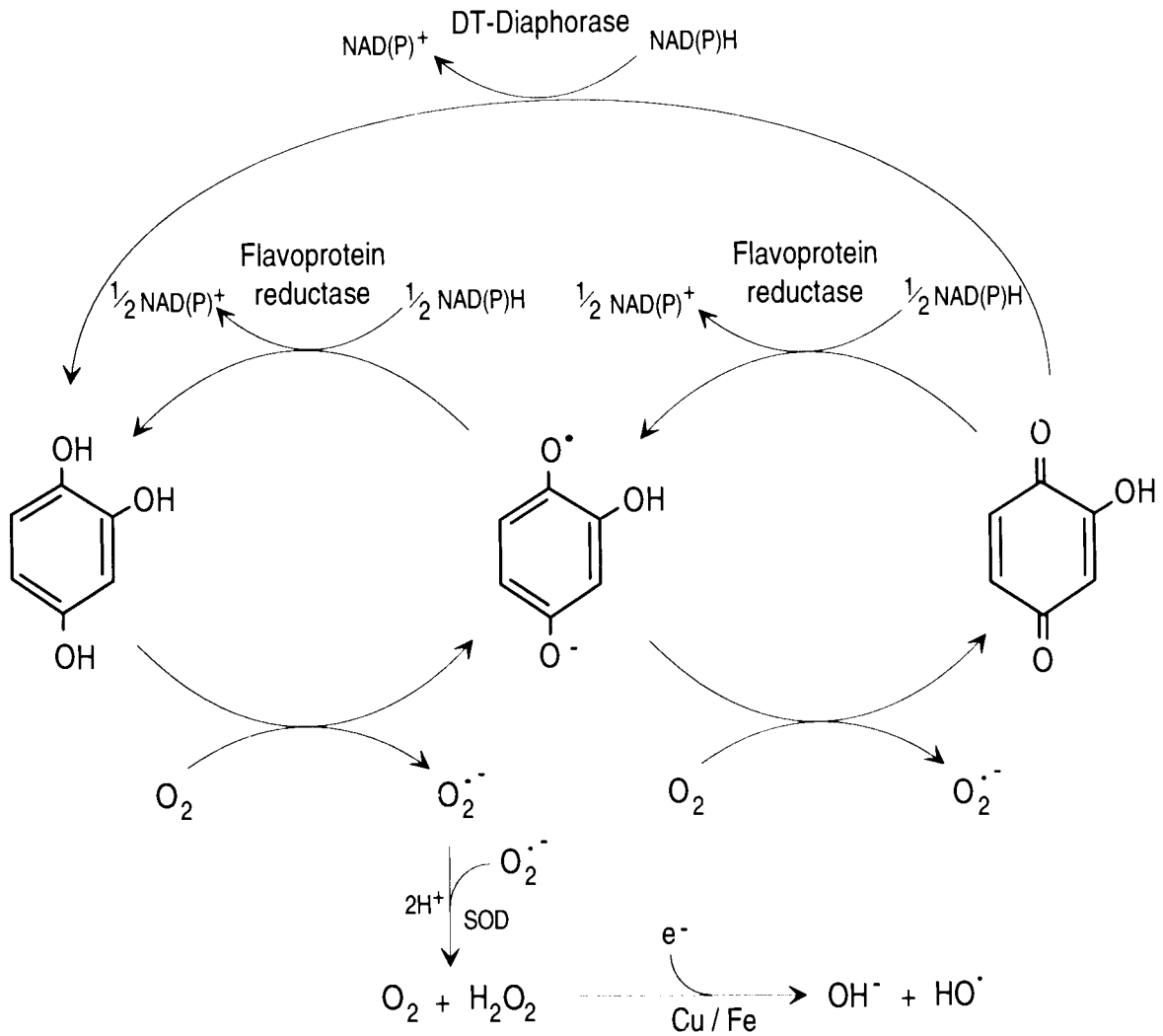


Figure 6. Redox cycling of 1,2,4-benzenetriol with production of active oxygen.

6-Hydroxydopamine and other catecholamines autoxidize to generate active oxygen.

Catecholamines are catabolized *in vivo* by monoamine oxidase and catechol-O-methyl transferase, but are also susceptible to non-enzymatic oxidation. The formation of pink oxidation products (*o*-quinones and aminochromes) can be readily observed in catecholamine solutions standing in air. The progressive accumulation of neuromelanin in catecholamine neurons implies that spontaneous oxidation also occurs *in vivo* (Graham 1978, 1979). The "autoxidation" of most natural catecholamines is slow at neutral pH, and would not on this basis be expected to be a significant source of cytotoxic oxygen species *in vivo* (Jewett et al 1989). However, metals such as copper, iron, and manganese greatly accelerate oxidation (Chaix et al 1950, Trautner and Bradley 1951, Gillette et al 1954, Heacock 1959, Graham 1984, Archibald and Tyree 1987). Oxygen mediated cytotoxicity thus becomes a danger in situations where catalytic metals become decompartmentalized in proximity to catecholamines.

Catalytic metals are available in brain to varying degrees. Certain regions of the brain (eg. *Substantia nigra*) concentrate transition metals (Poirier et al 1985), and cerebral spinal fluid contains significant catalytically active iron and copper complexes (Gutteridge et al 1982, Gutteridge 1984). Total free copper in sheep and mouse brains has been estimated at greater than 10^{-5} M (Lander and Austin 1976, Keen and Hurley 1976). Metals are generally sequestered by specific binding proteins, but the binding constants of certain cupro- and ferro-proteins implies a finite concentration of free metals to ensure catalytic activity. Metals are released in situations such as bleeding into brain or spinal chord following trauma, where iron becomes decompartmentalized from extravasated hemoglobin, or in ischaemia/reperfusion or inflammation, where superoxide induces release of iron from ferritin. In addition, metal storage sites can become overloaded on excessive ingestion or exposure to iron, copper, or manganese. For example, manganese poisoning in miners of manganese ore produces a neurological disorder (*locura manganura*) resembling Parkinsonism and an associated decrease in brain dopamine (Graham 1984, Segura-Aguilar and Lind 1989).

Reduction of oxygen by catecholamines is implicated in several neurologic and myocardial pathologies.

Dopamine. Loss of dopaminergic neurons originating in the *Substantia nigra* is notable in Parkinson's disease. This loss is likely due to reaction of oxygen with dopamine in these neurons, and the production of cytotoxic oxygen species (Graham et al 1978, Cohen 1985, Segura-Aguilar and Lind 1989). Administration of L-dopa boosts dopamine levels and helps alleviate symptoms of Parkinsonism, but may accelerate the underlying neuronal degeneration (Monks et al 1992). Ischaemic stroke, and amphetamine intoxication also produce release of dopamine in the striatum, which may contribute to neurotoxicity. An understanding of the factors contributing to dopamine oxidation, and the development of possible preventive or protective strategies is clearly of much interest.

Epinephrine. Elevated plasma levels of epinephrine (or isoproterenol) are cardiotoxic, producing functional and morphological changes, and ultimately myocardial necrosis (Singal et al 1982, Bindoli et al 1992). Moreover, ischaemia induces myocardial catecholamine release, with associated cardiac arrhythmia's (Reimmersma et al 1982). Also, β-adrenergic antagonists help protect against damage due to ischaemia-reperfusion (refs in Jewett et al 1989). Whether it is reactive oxygen associated with catecholamine autoxidation, or the cytotoxicity of adrenochrome oxidation products that produces the cardiomyopathy is a matter of debate (Jewett et al 1989, Bindoli et al 1992). Reduced adrenochrome autoxidizes much more rapidly than epinephrine, and adrenochrome redox cycles in mitochondria, microsomes, and sarcolemma with damaging consequences. However, much higher circulating concentrations of adrenochrome (10^{-4} M) than epinephrine ($>10^{-11}$ M) are needed to induce toxic effects. Catecholamine autoxidation in the absence of metal catalysts is arguably too slow to produce appreciable oxidative stress to myocardium in ischaemia-reperfusion (Jewett et al 1989). Instead, oxidation of epinephrine by microsomal enzymes or peroxidatic oxidation in the presence of ferritin or hemoproteins are suggested as possible sources of active oxygen (Jewett et al 1989). Alternatively, $O_2^{\cdot-}$ from xanthine oxidase may release catalytic iron from ferritin in ischaemia-reperfusion conditions.

6-Hydroxydopamine. The cytotoxic and neurotoxic properties of 6-hydroxydopamine (6-OHDA) have been documented extensively (Uretsky and Iversen 1969, 1970, Malmfors and Thoenen 1971, Heikkila et al 1973, Jonsson et al 1974, Heikkila and Cohen 1975, Jonsson and Sachs 1975, Davison et al 1984, Gee et al 1992), and 6-OHDA is often utilized as a model prooxidant in cell toxicity studies (eg. Giunta et al 1991). 6-Hydroxydopamine is selectively accumulated by the uptake systems of catecholaminergic neurons, where it generates reactive oxygen and destroys the neuron or nerve terminal. Injected systemically, 6-OHDA selectively destroys sympathetic adrenergic nerve terminals, producing chemical sypathectomy. Injected into brain (Ungerstedt 1968), 6-OHDA destroys dopaminergic neurons originating in the *Substantia nigra*, and produces experimental Parkinsonism. Intraventricular 6-OHDA depletes brain norepinephrine (Uretsky and Iversen 1969, 1970; Stein and Wise 1971), and produces behavioral deficits similar to schizophrenia (Stein and Wise 1971, Adams 1974). With neuroblastoma, a tumour of the sympathetic nervous system in children, 6-OHDA is used clinically to purge neuroblastoma cells (which take up catecholamines) from bone marrow prior to marrow transplantation (Angletti and Levi-Montalcini 1970, Graham et al 1978, Tiffany-Castiglioni et al 1982, Reynolds et al 1982, Bruchelt et al 1989). Ascorbate augments this chemotherapeutic action (Reynolds and Perez-Polo 1981) by redox cycling accumulated quinone, thereby increasing consumption of oxygen and production of cytotoxic species (Heikkila and Cohen 1972, Heikkila et al 1973).

6-Hydroxydopamine is a focus of interest in this thesis both as a prooxidant cytotoxin and as a model for autoxidation studies. 6-Hydroxydopamine differs from natural catecholamines in possessing an additional hydroxyl group across from the catechol residue, which accelerates its reaction with oxygen. This *p*-hydroxyl group makes 6-OHDA a stronger reductant by adding an additional electron donor group and by providing a more stable *p*-quinone oxidation product. Thus, 6-OHDA has properties both of catechols and of *p*-hydroquinones, and serves as a model reductant for studying mechanisms of oxygen reduction. Like other catecholamines, 6-OHDA has vicinal hydroxyl groups capable of coordinating metals. However, its oxidation product is primarily 2-hydroxy-*p*-quinone rather than the tautomeric *o*-quinone, and this quinone only slowly cyclizes to aminochrome. Previous studies on 6-OHDA autoxidation have shown that $O_2^{\cdot-}$ serves as a propagating intermediate, that H_2O_2 is the major oxygen-derived product (but can be reduced further to produce $^{\bullet}OH$), and that

metal catalysts are important to the reaction with oxygen (Heikkila and Cohen 1971, 1973, Cohen and Heikkila 1974, Liang et al 1976, Heikkila and Cabbat 1978, 1981, Borg et al 1978, Floyd and Wiseman 1979, Sullivan and Stern 1981, Gee and Davison 1984, 1985). This thesis focuses on the interdependencies of metal catalysts and radical propagators in the mechanisms of reaction of 6-OHDA with oxygen.

In particular, the influences of different metal-binding ligands on the rate of trace metal-catalysed oxidation and on the sensitivity to active oxygen scavengers (superoxide dismutase, catalase, ascorbate, urate) are assessed. Observations on the effects of ligands on catalysis by adventitious metals are extended to studies on the catalytic effectiveness of added metal-ligand combinations. The interactions of uric acid, a plasma antioxidant of current interest (Ames et al 1981, Becker 1993), ascorbate, and superoxide dismutase are explored.

Intermediary metabolism activates aromatic xenobiotics such as benzene and naphthalene to potentially cytotoxic hydroquinones.

In addition to being exposed to preformed quinones and polyphenolics in the environment, intermediary metabolism produces quinones during metabolic "detoxification" of other drugs or environmental toxins. Aromatic hydrocarbon hydroxylases and reductases of endoplasmic reticulum are essential for excretion of xenobiotics, but activate them to redox active metabolites. Aromatic hydrocarbons are generally lipophilic, and disrupt membranous structures and functions. Hydroxylation increases aqueous solubility, and allows for conjugation and excretion. Reductases such as cytochrome P_{450} reductase and DT-diaphorase (NAD(P)H:quinone oxidoreductase) keep quinones in a reduced state to prevent accumulation of electrophilic quinones and to allow sulphonation and glucuronidation, but leave them susceptible to reaction with oxygen. Redox cycling can thus produce substantial oxidative stress before the cytotoxin is excreted. Metal-binding and chain-breaking antioxidants help minimize the oxidative stress, but may become overwhelmed. Different hydroquinones vary in the rate and mechanisms by which they react

with oxygen. Understanding these mechanisms is crucial to designing optimal chemotherapeutic strategies (prooxidant or antioxidant).

Benzene. Chronic exposure to benzene is myelotoxic, producing aplastic anemia, leukopenia, and increasing risk of leukemia. Benzene is metabolized in the liver to phenol, catechol, 1,4-hydroquinone, and 1,2,4-benzenetriol. These metabolites accumulate in bone marrow, where they exert myelotoxic effects. The mechanisms by which benzene metabolites produce their toxic effects have not been fully resolved. The two main mechanistic possibilities are a) reduction of oxygen to cytotoxic species, and b) attack on cellular nucleophiles by electrophilic quinone products. Either case requires oxidation of the hydroquinone. The relative contribution of these pathways depends on the rates and mechanisms of oxidation, and the electrophilicity of the quinone products. Benzene metabolites bind to DNA and proteins *in vitro* and *in vivo*, indicating electrophilic attack by quinone oxidation products (refs in Brunmark and Cadenas 1989). Conversely, 1,2,4-benzenetriol reacts rapidly with oxygen (Greenlee et al 1981), is redox cycled by DT-diaphorase (Ollinger et al 1990), and produces cytotoxic and genotoxic effects through generation of active oxygen (Lewis et al 1988, Kawanishi et al 1989, Zhang et al 1993, 1994). 1,4-Hydroquinone reacts much less rapidly with oxygen than 1,2,4-benzenetriol, while catechol and phenol autoxidation was imperceptible *in vitro* (Greenlee and Sun 1981). Significant oxidation of these metabolites thus depends on other factors, such as metals and enzymes. Copper, and paradoxically superoxide dismutase stimulate the reaction of 1,4-hydroquinone with oxygen (Greenlee and Sun 1981, Li and Trush 1993). Bone marrow myeloperoxidase also provides enzymic oxidation of phenol, catechol, 1,4-hydroquinone, and perhaps 1,2,4-benzenetriol to electrophilic products. A difference in the balance between myeloperoxidase activity and DT-diaphorase activity may explain the differential sensitivity to benzene metabolites of bone marrow macrophages compared to fibroblast stromal cells (Monks et al 1992).

Naphthalene. Exposure to naphthalene, the major component of coal tar distillate, produces toxicity in the eye, resulting in lens opacification (eg. Russell et al 1991). In an analogous manner to benzene, naphthalene is metabolized in the liver to 1-naphthol, 1,2-naphthalenediol, 1,4-naphthalenediol, and 1,2,4-naphthalenetriol. (Brunmark and Cadenas

1989). 1-Naphthol is of some interest as a potential anticancer agent, as it, and its metabolites 1,2-naphthoquinone and 1,4-naphthoquinone, are selectively toxic to cultured colorectal tumour tissue. 1-Naphthol also has therapeutic potential in treating certain melanomas rich in tyrosinase activity, which activates 1-naphthol to 1,2-naphthoquinone with destructive consequences. As with metabolites of benzene, cytotoxicity can result through redox cycling and/or through electrophilic arylation (Brunmark and Cadenas 1989, Monks et al 1992). Evaluating the relative contribution of these pathways is a challenge that has received substantial attention (Monks et al 1992). In general, however, the naphthoquinones redox cycle more actively than benzoquinones (Oliinger et al 1990). While redox cycling of naphthoquinones by DT-diaphorase has received significant attention, the direct mechanisms of reaction of naphthohydroquinones with oxygen have received only preliminary characterization (Ishii and Fridovich 1990).

Respiratory flavoproteins produce redox-cycling semiquinone intermediates

DT-Diaphorase reduces quinones by a concerted two-electron mechanism, and thus redox cycling depends on the reactivity of the hydroquinone (rather than the semiquinone) with oxygen. With naphthoquinones, this redox cycling can be quite active (Buffington et al 1989, Ollinger et al 1990). In contrast to DT-diaphorase, mitochondrial or microsomal flavoenzymes reduce quinones by one-electron steps, producing significant steady state levels of semiquinones, which react even more readily with oxygen. Two much investigated examples of quinones which produce cytotoxicity through redox cycling in mitochondria and endoplasmic reticulum are menadione and the anthracycline antitumour quinones (eg. adriamycin). In addition, endogenous semiquinones (flavosemiquinones, ubisemiquinone) are susceptible to autoxidation under certain conditions.

Menadione. Menadione (2-methyl-1,4-naphthoquinone) is a synthetic naphthoquinone with vitamin K activity, which induces substantial oxidative stress in hepatocytes (Smith et al 1985), and has been studied extensively as a model redox-cycling prooxidant. Redox cycling of menadione by microsomal and mitochondrial flavoproteins produces large quantities of $O_2^{\cdot-}$ and H_2O_2. This oxidative stress causes rapid depletion of NAD(P)H and glutathione,

and release of calcium to cytosol from mitochondria and endoplasmic reticulum. The results of the oxidative stress are blebbing of the cell surface and eventual cell death.

Anthracyclines. Adriamycin and other anthracyclines produce antitumour activity largely through intercalation in and alkylation of replicating DNA (and perhaps by inducing oxidative damage to DNA). However, their effectiveness in cancer treatment is dose-limited by cardiotoxicity. The cardiotoxicity of anthracyclines results at least in part from the highly aerobic nature of heart tissue and the prevalence of respiratory flavoenzymes which redox cycle these quinones. Designing quinones which redox cycle less actively in myocardium but produce similar or greater antitumour activity, such as recently developed 5-iminodaunorubicin, would be of significant benefit. Knowledge of the fundamental mechanisms by which hydroquinones react with oxygen will aid in the rational design of such agents.

Endogenous semiquinones. Autoxidation of endogenous ubisemiquinone or flavosemiquinone intermediates produces significant active oxygen under some conditions. The respiratory inhibitors antimycin and cyanide impair conduction of electrons to cytochrome oxidase, blocking normal respiration. The resulting accumulation of reduced quinone intermediates leaves them susceptible to autoxidation, and produces substantial release of $O_2^{\cdot-}$ and H_2O_2 (Turrens and Boveris 1980, Cadenas and Boveris 1980, Ksenzenko et al 1983, Turrens et al 1985, Forman and Boveris 1982). In addition, respiratory inhibitors increase the reduction of exogenous quinones such as menadione to autoxidizable semiquinones and hydroquinones, accentuating the oxidative stress. *In vivo* situations for respiratory impairment include hypoxia, loss of cytochrome *c* from the surface of the mitochondrial inner membrane, or mutations to the terminal components of the respiratory chain. Mitochondrial generation of $O_2^{\cdot-}$ and H_2O_2 has been suggested to contribute to ischaemia-reperfusion injury (Fridovich 1979, Sjostrom and Crapo 1981, 1983, Flaherty and Weisfeldt 1988). Loss of cytochrome *c* (through ischaemia-induced mitochondrial swelling), or hypoxic accumulation of reduced intermediates, provide possible mechanisms. Mitochondrial mutations increase with age (Linnane et al 1989, Arnheim and Cortopassi 1992, Ozawa 1993), and mitochondria from older individuals release more $O_2^{\cdot-}$ and H_2O_2 (Nohl et al 1978, Sawada and Carlson 1987, Sohal 1993, Yen et al 1994). We have

suggested that mitochondrial mutations to terminal components of the respiratory chain provide a mechanism for this increased leakage of active oxygen (Bandy and Davison 1990).* The influences of mitochondrial impairments on the generation of active oxygen by respiratory quinones will be addressed in Part II of this thesis.

* This suggestion has currently come in focus due to a recent wealth of information and interest in mitochondrial genetic diseases (eg. reviews: DiMauro et al 1988, 1989, Shoffner and Wallace 1990, Kiester 1991, Wallace 1992a, 1992b, 1993, Byrne 1992, Bakker et al 1993, Schapira 1993, Schapira et al 1993, Schon 1993, Taylor et al 1993).

Hydroquinones can react with oxygen by a limited range of pathways

Transfer of an electron pair from hydroquinone to oxygen is spin restricted and thermodynamically unfavourable.

Consistent with molecular orbital theory (and Hund's rule of maximum multiplicity), oxygen behaves as a diradical. The molecular orbital diagram for ground state dioxygen is shown in figure 7. Oxygen has two unpaired electrons of the same spin in π^* antibonding orbitals. In contrast, the valence orbitals of non-radical organic molecules such as hydroquinones are occupied by paired electrons of opposite spin, thereby balancing spin magnetic moments. The valence orbitals of oxygen have an appropriate symmetry to overlap those of organic biomolecules in forming a transition state species. However, the occupancy is inappropriate for yielding two sets of spin-paired electrons. Thus, while oxygen is a relatively strong two-electron oxidant ($E^{o'}_{O_2/H_2O_2}$ = 0.33 V) (Table 1), the spin restriction creates an activation free energy barrier and prevents a direct two-electron reaction with organic biomolecules.

Oxygen can receive one electron from organic molecules such as hydroquinones, but thermodynamically the reaction is most often unfavourable. The reduction potentials for oxygen, ascorbate, and ubiquinone are shown in Table 1. Oxygen is a weak one-electron oxidant ($E^{o'}_{O_2/O_2^{\cdot-}}$ = -0.16 V), since formation of $O_2^{\cdot-}$ just fills one π^* antibonding orbital. Thus a reductant must have a reduction potential of less than -0.16 V to produce a favourable free energy change in reducing O_2 to $O_2^{\cdot-}$. Neither ascorbate ($E^{o'}_{A^{\cdot-}/AH^-}$ = 0.28 V) nor ubihydroquinone ($E^{o'}_{Q^{\cdot-}/QH_2}$ = 0.35 V) for example have favourable reduction potentials for reducing O_2 to $O_2^{\cdot-}$. In contrast, the semidehydroascorbate radical ($E^{o'}_{A/A^{\cdot-}}$ = -0.17 V) and ubisemiquinone radical ($E^{o'}_{Q/Q^{\cdot-}}$ = -0.23 V), once formed, may favourably reduce O_2 to $O_2^{\cdot-}$. Free energies, entropies, and enthalpies of activation have been calculated for at least one single-electron reduction of oxygen (Davison and Kaminsky 1971), with the inference that the main energy barrier results from the unfavourable entropy of solvation of superoxide.

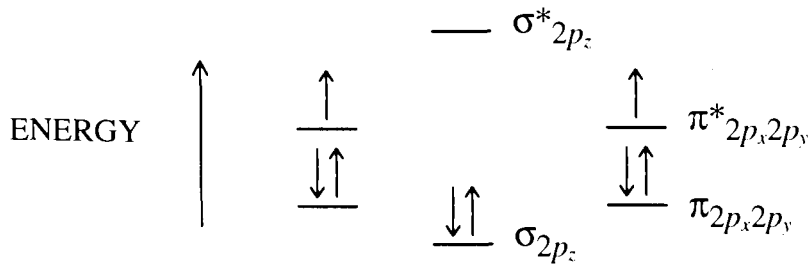


Figure 7. Molecular orbital diagram for valence electrons of ground state dioxygen.

Table 1. Standard reduction potentials for oxygen, ascorbate, and ubiquinone.

Reaction	**$E^{o'}$ (V)**
$O_2 + e^- \longrightarrow O_2^{\cdot -}$	-0.16
$O_2 + 2e^- \longrightarrow H_2O_2$	+0.33
$O_2^{\cdot -} + e^- \longrightarrow H_2O_2$	+0.94
$H_2O_2 + e^- + H^+ \longrightarrow H_2O + {}^{\bullet}OH$	+0.33
${}^{\bullet}OH + e^- + H^+ \longrightarrow H_2O$	+2.31
semidehydroascorbate + e^- + H^+ $\longrightarrow$ ascorbate	+0.28
dehydroascorbate + e^- $\longrightarrow$ semidehydroascorbate	-0.17
ubisemiquinone + e^- + H^+ $\longrightarrow$ ubiquinol	+0.35
ubiquinone + e^- + H^+ $\longrightarrow$ ubisemiquinone	-0.23

Catalysis by transition metal ions alleviates the spin restriction and decreases the free energy of activation that prevents the thermodynamically favoured reduction of O_2 to H_2O_2 (Miller et al 1990). The five partially filled d orbitals of transition metal ions permit formation of coordination complexes and facilitate transfer of electrons from one reactant to another. Thus Fe^{2+} can, for example, form a bidentate perferryl complex with O_2 (ie. $[Fe(IV):O_2]^{2+}$) where electrons of appropriate spin are available for transfer to oxygen. Moreover, metal ions allow formation of a ternary complex between reductant, metal, and oxygen. Oxidations of ascorbate (Khan and Martell 1967) and 6-hydroxydopamine (Gee and Davison 1984a, 1984b) reportedly occur by such inner sphere mechanisms. In this way the activation energy barrier for thermodynamically favourable reduction of O_2 to H_2O_2 is diminished. For example, the thermodynamically favourable oxidation of ascorbate to dehydroascorbate ($E^{o'}_{A/AH^-} = 0.054$ V) by oxygen ($E^{o'}_{O_2/H_2O_2} = 0.33$) becomes feasible.

In addition to reduction of oxygen within a ternary reductant:metal:oxygen complex, several other pathways are available to circumvent the spin restriction and thermodynamic barriers to reduction of oxygen by hydroquinones. For example, the thermodynamically unfavourable one-electron reduction of oxygen may be accelerated kinetically by removal of products ($O_2^{\cdot-}$ and $Q^{\cdot-}$) through disproportionation. Such a mechanism likely requires metal catalysts to decrease the activation energy and thus increase the rate at which equilibrium is established (Miller et al 1990).

$$QH_2 + O_2 \Leftrightarrow Q^{\cdot-} + O_2^{\cdot-} + 2H^+$$

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

$$2Q^{\cdot-} + 2H^+ \Rightarrow QH_2 + Q$$

Metal catalyzed oxidation can also proceed by an outer-sphere $O_2^{\cdot-}$-propagated pathway.

initiation:

$$QH_2 + M^{n+} \Leftrightarrow Q^{\cdot-} + M^{(n-1)+} + 2H^+$$

propagation:

$$Q^{\cdot-} + O_2 \Rightarrow Q + O_2^{\cdot-}$$

$$M^{(n-1)+} + O_2^{\cdot-} \Rightarrow M^{n+} + H_2O_2$$

$$QH_2 + O_2^{\cdot-} \Rightarrow Q^{\cdot-} + H_2O_2$$

H_2O_2 produced in inner sphere or outer sphere pathways may also propagate autoxidation.

$$QH_2 + H_2O_2 \Rightarrow Q^{\cdot-} + {}^{\bullet}OH + OH^-$$

Finally, once quinone product has accumulated, reaction with oxygen can be propagated by comproportionation.

$$QH_2 + Q \Rightarrow 2Q^{\cdot -} + 2H^+$$

$$Q^{\cdot -} + O_2 \Rightarrow Q + O_2^{\cdot -}$$

The contribution of each of these pathways depends on the hydroquinone and the metal catalysts present, as well as on the influences of metal-binding ligands and radical scavengers.

Metal binding ligands and radical scavengers influence the pathways of autoxidation.

Different hydroquinones coordinate catalytic metals with differing affinities. Catechols for example bind metal cations avidly, while 1,4-hydroquinones generally do not. Thus, the rate and mechanism of autoxidation depends not only on the reduction potentials involved, but also on the interactions with metal catalysts. Few, if any, direct comparisons of the mechanisms of (metal-catalyzed) oxidation of 1,2- and 1,4-hydroquinones are available. The results of such comparisons are presented in Part IV of this thesis.

The presence of metal-binding ligands influences both the reactivity of metal catalysts and the physical interactions of metal catalysts with hydroquinones. Ligands with a preferential affinity for the oxidized or reduced form of a metal ion decrease or increase its reduction potential, and thus influence its reactivity. EDTA for example binds Fe^{3+} more strongly than Fe^{2+}, and thus decreases the reduction potential of iron dramatically (from 0.77 to 0.12 V). By competing with hydroquinones and oxygen for coordination sites (d orbitals) on the metal, ligands sterically hinder access to the metal. Multidentate ligands such as desferrioxamine can occupy all coordination sites, and prevent inner-sphere electron transfers to oxygen. Other ligands such as histidine can form bi- or tri-molecular complexes (eg. histidine:Cu^{2+}:histidine) or mixed-ligand complexes (eg. catechol:Cu^{2+}:histidine). *In vivo*, metals are mostly sequestered by metal-binding proteins, such as ferritin. However, low molecular weight complexes are also found to a varying extent in tissues and biological fluids, particularly on release of metals from storage sites. Under such situations the available ligands are important in modulating unwanted catalytic activity. Potential ligands include ADP, citrate, histidine, albumin, and urate. Such ligands may allow redox activity or

may have metal-binding antioxidant activity. While coordination chemistry is an extensive field, and much is known of the principles of metal coordination, the influence of different ligands on redox activity in metal-catalysed oxidations has not been studied systematically. A systematic comparison of the influences of different ligands and ligand-metal complexes on the rates and mechanisms of oxygen reduction by 6-hydroxydopamine and ascorbate are presented in Part III of this thesis.

Scavengers of reactive intermediates can also influence the pathways of hydroquinone oxidation in several ways. Scavenging of $O_2^{\cdot-}$ by superoxide dismutase for example can prevent propagation of radical chains by $O_2^{\cdot-}$, and thus inhibit net reduction of oxygen.* Conversely, superoxide dismutase can accelerate reduction of oxygen by removing a product from equilibria which form $O_2^{\cdot-}$. Ascorbate can also scavenge $O_2^{\cdot-}$, but the resulting ascorbyl radical may either propagate further chains, or terminate them through disproportionation. Similarly, $^{\bullet}OH$ scavengers such as urate or formate may remove $^{\bullet}OH$, but the resulting (less reactive) radicals may more selectively propagate hydroquinone oxidation. *In vivo*, moreover, radical scavengers act in combination with other radical scavengers, and with metal-binding ligands. The influences of combinations of different scavengers and metal-binding ligands on hydroquinone autoxidations are explored in Parts III and IV of this thesis.

* By this means, superoxide dismutase can paradoxically diminish the yield of its product H_2O_2.

General Aims

To what extent does mitochondrial respiratory impairment influence the steady state level of autoxidizable (quinone) intermediates?

The influences of hypoxia and removal of cytochrome *c* on the generation of $O_2^{\cdot-}$ by submitochondrial particles are assessed. In addition, the possible influences of mitochondrial mutations on generation of active oxygen by mitochondria are evaluated on a theoretical basis.

To what extent do trace metals and radical intermediates participate as initiators and propagators of hydroquinone autoxidation?

Conversely, *in what ways do metal-binding ligands and radical scavengers interact to influence hydroquinone autoxidations?*

The effects of different combinations of ligands, added metals, and active oxygen scavengers on the autoxidations of 6-hydroxydopamine and ascorbate are assessed.

To what extent do thermodynamic (reduction potentials) and kinetic or steric (metal coordination) factors influence the rates and mechanisms of autoxidation?

The rates and kinetics of autoxidation, and the influences of metal chelators and superoxide dismutase are assessed for 1,2- and 1,4-, benzo- and naphtho- hydroquinones differing in metal affinities and reduction potentials.

REFERENCES

Adams, R.N. An overview of the 6-hydroxydopamine theory of schizophrenia. *Bull. Menninger Clin.* **38**:57-69; 1974.

Ames, B.N.; Cathcart, R.; Schwiers, E.; Hochstein, P. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: A hypothesis. *Proc. Natl. Acad. Sci. USA* **78**:6858-6862; 1981.

Angeletti, P.U.; Levi-Montalcini, R. Cytolytic effect of 6-hydroxydopamine on neuroblastoma cells. *Cancer Res.* **30**:2863-2869; 1970.

Archibald, F.S.; Tyree, C. Manganese poisoning and the attack of trivalent manganese upon catecholamines. *Arch. Biochem. Biophys.* **256**:638-650; 1987.

Arnheim, N.; Cortopassi, G. Deleterious mitochondrial DNA mutations accumulate in aging human tissues. *Mutation Res.* **275**:157-167, 1992.

Bakker, H.D.; Scholte, H.R.; Jeneson, J.A. Vitamin E in a mitochondrial myopathy with proliferating mitochondria. *Lancet* **342**:175-176; 1993.

Becker, B. Towards the physiological function of uric acid. *Free Rad. Biol. Med.* **14**:615-631; 1993.

Bindoli, A.; Rigobello, M.P.; Deeble, D.J. Biochemical and toxicological properties of the oxidation products of catecholamines. *Free Rad. Biol. Med.* **13**:391-405; 1992.

Borg, D.C.; Schaich, K.M.; Ellmore, J.J.Jr.; Bell, J.A. Cytotoxic reactions of free radical species of oxygen. *Photochem. Photobiol.* **28**:887-907; 1978.

Borg, D.C.; Schaich, K.M. Cytotoxicity from coupled redox cycling of autoxidizing xenobiotics and metals. A selective critical review and commentary on work in progress. *Israel J. Chem.* **24**:38-53; 1984.

Brown, J.P. A review of the genetic effects of naturally occurring flavonoids, anthraquinones and related compounds. *Mutation Research* **75**:243-277; 1980.

Brunmark, A.; Cadenas, E. Redox and addition chemistry of quinoid compounds and its biological implications. *Free Rad. Biol. Med.* **7**:435-477; 1989.

Buffington, G.D.; Ollinger, K.; Brunmark, A.; Cadenas, E. DT-diaphorase-catalysed reduction of 1,4-naphthoquinone derivatives and glutathionyl-quinone conjugates. *Biochem. J.* **257**:561-571; 1989.

Bruchelt, G.; Grygar, G.; Treuner, J.; Esterbauer, H.; Niethammer, D. Cytotoxic effects of 6-hydroxydopamine, merocyanine-540 and related compounds on human neuroblastoma- and hemopoietic stem cells. *Free Rad. Res. Comms.* **7**:205-212; 1989.

Byrne, E. New concepts in respiratory chain diseases. *Current Opinion in Rheumatology* **4**:784-793; 1992.

Cadenas, E.; Boveris, A. Enhancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria. *Biochem. J.* **188**:31-37; 1980.

Cadenas, E.; Mira, D.; Brunmark, A.; Lind, C.; Segura-Aguilar, J.; Ernster, L. Effect of superoxide dismutase on the autoxidation of various quinones - a possible role of superoxide dismutase as a superoxide:semiquinone oxidoreductase. *Free Rad. Biol. Med.* **5**:71-79; 1988.

Chaix, P.; Chauvet, J.; Jezequel, J.; etude cinéteque de l'oxydation de l'adrenaline en solution tampon phosphate. *Biochim. Biophys. Acta* **4**:471-483; 1950.

Chesis, P.L.; Levin, D.E.; Smith, M.T.; Ernster, L.; Ames, B.N. Mutagenicity of quinones: pathways of metabolic activation and detoxification. *Proc. Natl. Acad. Sci. USA* **81**:1696-1700; 1984.

Cohen, G.; Heikkila, R.E. The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents. *J. Biol. Chem.* **249**:2447-2452; 1974.

Cohen, G. Oxidative stress in the nervous system. In: Sies, H. ed. *Oxidative Stress* London: Academic Press; 1985.

Davison, A.J.; Kaminsky, L.S. Thermodynamic aspects of cytochrome *c* function. *South Africa Med. J.* **45**:144-147; 1971.

Davison, A.J.; Wilson, B.D.; Belton, P. Deterioration of axonal membranes induced by phenolic pro-oxidants. Roles of superoxide radicals and hydrogen peroxide. *Biochem. Pharmacol.* **33**:3887-3891; 1984.

Davison, A.J.; Tibbits, G.; Shi, Z.; Moon, J. Active oxygen in neuromuscular disorders. *Molec. Cell. Biochem.* **84**:199-216; 1988.

DiMauro, S.; Zeviani, M; Moraes, C.T.; Nakase, H.; Rizzuto, R.; Lombes, A.; Schanske, S.; Schon, E.A. Mitochondrial encephalomyopathies. *Progress in Clinical and Biological Research* **306**:117-128; 1989.

DiMauro, S.; Zeviani, M.; Servidei, S.; Prelle, A.; Miranda, A.F.; Bonilla, E.; Schon, E.A. Biochemical and molecular aspects of cytochrome C oxidase deficiency. *Adv. Neurol.* **48**:93-105; 1988.

Flaherty, J.T.; Weisfeldt, M.L. Reperfusion injury. *Free Rad. Biol. Med.* **5**:409-419; 1988.

Floyd, R.A.; Wiseman, B.B. Spin-trapping free radicals in the autooxidation of 6-hydroxydopamine. *Biochim. Biophys. Acta* **586**:196-207; 1979.

Forman, H. J.; Boveris, A. Superoxide radical and hydrogen peroxide in mitochondria. In: Prior W. A. ed. *Free Radicals in Biology.* Vol V. Orlando: Academic Press; 1982:65-90.

Fridovich, I. Hypoxia and oxygen toxicity. *Adv. Neurol.* **26**:255-259; 1979.

Gee, P.; Davison, A.J. "Autoxidation" of 6-hydroxydopamine involves a ternary reductant-metal-oxygen complex. *IRCS Med. Sci.* **12**:127; 1984.

Gee, P.; Davison, A.J. 6-Hydroxydopamine does not reduce molecular oxygen directly, but requires a co-reductant. *Arch. Bioch. Biophys.* **231**:164-168; 1984.

Gee, P.; Davison, A.J. Effects of scavengers of oxygen free radicals on the anaerobic oxidation of 6-hydroxydopamine by H_2O_2. *Biochim. Biophys. Acta* **838**:183-190: 1985.

Gee, P.; San, R.H.C.; Davison, A.J.; Stich, H.F. Clastogenic and mutagenic actions of active species generated in the 6-hydroxydopamine/oxygen reaction: effects of scavengers of active oxygen, iron, and metal chelating agents. *Free Rad. Res. Comms.* **16**:1-10; 1992

Gillette, J.R.; Watland, D.; Kalnitsky, G. The catalysis of the oxidation of some dihydroxybenzene derivatives by various metallic ions. *Biochim. Biophys. Acta* **15**:526-532; 1954.

Giunta, S.; Galeazzi, L.; Groppa, G. An in vitro bacterial model of cytotoxicity to living cells caused by dopamine and 6-hydroxydopamine oxidation at physiological pH. *Free Rad. Biol. Med.* **10**:297-303; 1991.

Graham, D.G.; Tiffany, S.M.; Bell, W.R.Jr.; Gutknecht, W.F. Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells in vitro. *Molec. Pharmacol.* **14**:644-653; 1978.

Graham, D.G. On the origin and significance of neuromelanin. *Arch. Pathol. Lab. Med.* **103**:359-362; 1979.

Graham, D.G. Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. *Molec. Pharmacol.* **14**:633-643; 1978.

Graham, D.G. Catecholamine toxicity: a proposal for the molecular pathogenesis of manganese neurotoxicity and Parkinson's disease. *Neurotoxicity* **5**:83-96; 1984.

Greenlee, W.F.; Sun, J.D.; Bus, J.S. A proposed mechainism of benzene toxicity: formation of reactive intermediates from polyphenol metabolites. *Toxicol. Appl. Pharmacol.* **59**:187-195; 1981.

Gutteridge, J.M.C.; Rowley, D.A.; Halliwell, B.; Westermarck, T Increased non-protein-bound iron and decreased protection against superoxide-radical damage in cerebrospinal fluid from patients with neuronal ceroid lipofuscinoses. *Lancet* **ii**:459-460; 1982.

Gutteridge, J.M.C. Copper-phenanthroline-induced site-specific oxygen-radical damage to DNA. Detection of loosely bound trace copper in biological fluids. *Biochem. J.* **218**:983-985; 1984.

Halliwell, B.; Gutteridge, J.M.C. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* **219**:1-14; 1984.

Halliwell, B.; Gutteridge, J.M.C. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.* **246**:501-514; 1986.

Halliwell, B.; Gutteridge, J.M.C. Oxygen radicals and the nervous system. *TINS* **Jan**:22-26; 1985.

Halliwell, B.; Gutteridge, J.M.C. The antioxidants of human extracellular fluids. *Arch. Biochem. Biophys.* **280**:1-8; 1990.

Heacock, R.A. The chemistry of adrenochrome and related compounds. *Chem. Rev.* **59**:181-237; 1959.

Heikkila, R.E.; Cohen, G. Inhibition of biogenic amine uptake by hydrogen peroxide: A mechanism for toxic effects of 6-hydroxydopamine. *Science* **172**:1257-1258; 1971.

Heikkila, R.E.; Cohen, G. Further studies on the generation of hydrogen peroxide by 6-hydroxydopamine. Potentiation by ascorbic acid. *Molec. Pharmacol.* **8**:241-248; 1972.

Heikkila, R.E.; Cohen, G. 6-Hydroxydopamine: evidence for superoxide radical as an oxidative intermediate. *Science* **181**:456-457; 1973.

Heikkila, R.E.; Mytilineou, C.; Cote, L.; Cohen, G. Evidence for degeneration of sympathetic nerve terminals caused by the *ortho*- and *para*-quinones of 6-hydroxydopamine. *J. Neurochem.* **20**:1345-1350; 1973.

Heikkila, R.E.; Cohen, G. Cytotoxic aspects of the interaction of ascorbic acid with alloxan and 6-hydroxydopamine. *Ann. N. Y. Acad. Sci.* **258**:221-230; 1975.

Heikkila, R.E.; Cohen, G. 6-Hydroxydopamine: evidence for superoxide radical as an oxidative intermediate. *Science* **181**:456-457; 1973.

Heikkila, R.E.; Cabbat, F.S. The stimulation of 6-hydroxydopamine autoxidation by bivalent copper: potential importance in the neurotoxic process. *Life Sci.* **23**:33-38; 1978.

Heikkila, R.E.; Cabbat, F.S. Inhibition of iron-stimulated catecholamine degradation by the iron-chelators DETAPAC and desferal. Potentially useful laboratory agents. *Biochem. Pharmacol.* **30**:2945-2947; 1981.

Ishii, T.; Fridovich, I. Dual effects of superoxide dismutase on the autoxidation of 1,4-naphthohydroquinone. *Free Rad. Biol. Chem.* **8**:21-24; 1990.

Jewett, S.L.; Eddy, L.J.; Hochstein, P. Is the autoxidation of catecholamines involved in ischemia-reperfusion injury? *Free Rad. Biol. Med.* **6**:185-188; 1989.

Jonsson, G.; Lohmander, S.; Sachs, C. 6-Hydroxydopamine induced degeneration of noradrenaline neurons in the scorbutic guinea-pig. *Biochem. Pharmacol.* **23**:2585-2593; 1974.

Jonsson, G.; Sachs, C. Actions of 6-hydroxydopamine quinones on catecholamine neurons. *J. Neurochem.* **25**:509-516; 1975.

Juhl, U.; Blum, J.K.; Butte, W.; Witte, I. The induction of DNA strand breaks and formation of semiquinone radicals by metabolites of 2,4,5-trichlorophenol. *Free Rad. Res. Comms.* **11**:295-305; 1991.

Kawanishi, S.; Inoue, S.; Kawanishi, M. Human DNA damage induced by 1,2,4-benzenetriol, a benzene metabolite. *Cancer Research* **49**:164-168; 1989.

Keen, C.L.; Hurley, L.S. Copper supplementation in quaking mutant mice: reduced tremors and increased brain copper. *Science* **193**:244-246; 1976.

Khan, M.M.T.; Martell, A.E. Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. II. Cupric and ferric chelate catalyzed oxidation. *J. Am. Chem. Soc.* **89**:7104-7111; 1967.

Kiester, E.Jr. A bug in the system. *Discover* **Feb**:70-76; 1991.

Ksenzenko, M.; Konstantinov, A. A.; Khomutov, G. B.; Tikhonov, A. N.; Ruuge, E. K. Effect of electron transfer inhibitors on superoxide generation in the cytochrome bc_1 site of the mitochondrial respiratory chain. *FEBS Lett.* **155**:19-24; 1983.

Lander, J.; Austin, L. Subcellular distribution of dopamine-beta-hydroxylase and inhibitors in the hippocampus and caudate nucleus in sheep brain. *J. Neurochem.* **26**:661-673; 1976.

Leibovitz, B.E.; Mueller, J.A. Bioflavonoids and polyphenols: Medical applications. *J. Optimal Nutr.* **2**:17-35; 1993.

Lewis, J.G.; Steward, W.; Adams, D.O. Role of oxygen radicals in induction of DNA damage by metabolites of benzene. *Cancer Res.* **48**:4762-4765; 1988.

Liang, Y.O.; Wightman, R.M.; Adams, R.N. Competitive oxidation of 6-hydroxydopamine by oxygen and hydrogen peroxide. *Eur. J. Pharmacol.* **36**:455-458; 1976.

Linnane, A.W.; Marzuki, S.; Ozawa, T.; Tanaka, M. Mitochondrial DNA mutations as an important contribution to aging and degenerative diseases. *Lancet* **i**:642-645, 1989.

Malmfors, T.; Thoenen, H. eds. *6-Hydroxydopamine and Catecholamine Neurones.* Amsterdam: North-Holland; 1971.

Miller, D.M.; Buettner, G.R.; Aust, S.D. Transition metals as catalysts of "autoxidation" reactions. *Free Rad. Biol. Med.* **8**:95-108; 1990.

Monks, T.J.; Hanzlik, R.P.; Cohen, G.M.; Ross, D.; Graham, D.G. Quinone chemistry and toxicity. *Toxicol. Appl. Pharmacol.* **112**:2-16; 1992.

Nohl, H.; Breuninger, V.; Hegner, D. Influence of mitochondrial radical formation on energy-linked respiration. *Eur. J. Biochem.* **90**:385-390, 1978.

Nohl, H.; Jordan, W.; Youngman, R.J. Quinones in biology: functions in electron transfer and oxygen activation. *Adv. Free Rad. Biol. Med.* **2**:211-279; 1986.

Ollinger, K.; Buffington, G.D.; Ernster, L.; Cadenas, E. Effect of superoxide dismutase on the autoxidation of substituted hydro- and semi-naphthoquinones. *Chem.-Biol. Interact.* **73**:53-76; 1990.

Ozawa, T. Mitochondrial DNA mutations associated with age. *Aging* **5**:155-158, 1993.

Patai, S. ed. *The Chemistry of the Quinoid Compounds* New York: John Wiley & Sons; 1974.

Patai, S.; Rappoport, Z. eds. *The Chemistry of Quinonoid Compounds. Vol II* New York: John Wiley & Sons; 1988.

Poirier, J.; Donaldson, J.; Barbeau, A. The specific vulnerability of the substantia nigra to MPTP is related to the presence of transition metals. *Biochem. Biophys. Res. Commun.* **128**:25-33; 1985.

Powis, G. Free radical formation by antitumor quinones. *Free Rad. Biol. Med.* **6**:63-101; 1989.

Powis, G. Metabolism and reactions of quinoid anticancer agents. *Pharmac. Ther.* **35**:57-162; 1987.

Reimmersma, R.A. Myocardial catecholamine release in acute myocardial ischaemia: Relationship to cardiac arrhythmias. In: Parrat, J.R., ed. *Early arrhythmias resulting from myocardial ischaemia.* London: Macmillan; 1982:125-138.

Reynolds, C.P.; Perez-Polo, J.R. Ascorbic acid enhances the cytotoxic effect of 6-hydroxydopamine for human neuroblastoma cell lines. *Neurosci. Lett.* **26**:151-155; 1981.

Reynolds, C.P.; Reynolds, D.A.; Frenkel, E.P.; Smith, R.G. Selective toxicity of 6-hydroxydopamine and ascorbate for human meuroblastoma in vitro: a model for clearing marrow prior to autologous transplant. *Cancer Res.* **42**:1331-1336; 1982.

Russell, P.; Yamada, T.; Xu, G.-T.; Garland, D.; Zigler, J.S.Jr. Effects of naphthalene metabolites on cultured cells from eye lens. *Free Rad. Biol. Med.* **10**:255-261; 1991.

Sawada, M.; Carlson, J.C. Changes in superoxide radical and lipid peroxide formation in the brain, heart, and liver during the lifetime of the rat. *Mech. Aging Dev.* **41**:125-137, 1987.

Schapira, A.H. Mitochondrial cytopathies. *Current Opinion in Neurobiology* **3**:760-767; 1993.

Schapira, A.H.; Hartley, A.; Cleeter, M.W.; Cooper, J.M. Free radicals and mitochondrial dysfunction in Parkinson's disease. *Biochem. Soc. Trans.* **21**:367-370; 1993.

Schon, E.A. Mitochondrial disorders in muscle. *Current Opinion in Neurology and Neurosurgery* **6**:19-26; 1993.

Segura-Aguilar, J.; Lind, C. On the mechanism of the Mn^{3+} induced neurotoxicity of dopamine: Prevention of quinone-derived oxygen toxicity by DT-diaphorase and superoxide dismutase. *Chem.-Biol. Interact.* **72**:309-324; 1989.

Shoffner, J.M.; Wallace, D.C. Oxidative phosphorylation diseases. Disorders of two genomes. *Advances in Human Genetics* **19**:267-330; 1990.

Singal, P.K.; Napur, N.; Dhillon, K.S.; Beamish, R.E.; Dhalla, N.S. Role of free radicals in catecholamine-induced cardiomyopathy. *Can. J. Physiol. Pharmacol.* **60**:1390-1397; 1982.

Sjostrom, K.; Crapo, J.D. Adaptation to oxygen by preexposure to hypoxia: Enhanced activity of manganic superoxide dismutase. B*ull. Europ. Physiopath. Resp.* **17(suppl.)**:111-116; 1981.

Sjostrom, K.; Crapo, J.D. Structural and biochemical adaptive changes in rat lungs after exposure to hypoxia. *Lab. Invest.* **48**:68-79; 1983.

Smith, M.; Evans, C.G.; Thor, H.; Orrenius, S. Quinone-induced oxidative injury to cells and tissues. In: Sies, H., ed. *Oxidative Stress.* London: Academic Press; 1985:91-113.

Sohal, R.S. Aging, cytochrome oxidase activity, and hydrogen peroxide release by mitochondria. *Free Rad. Biol. Med.* **14**:583-588, 1993.

Stein, L.; Wise, C.D. Possible etiology of schizophrenia: progressive damage to the noradrenergic reward system by 6-hydroxydopamine. *Science* **171**:1032-1036; 1971.

Sullivan, S.G.; Stern, A. Effects of superoxide dismutase and catalase on catalysis of 6-hydroxydopamine and 6-aminodopamine autoxidation by iron and ascorbate. *Biochem. Pharmacol.* **30**:2279-2285; 1981.

Taylor, R.W.; Birch-Machin, M.A.; Lowerson, S.; Sherratt, H.S.; West, I.C.; Bartlett, K.; Turnbull, D.M. Defects of oxidative phosphorylation in man. *Biochem. Soc. Trans.* **21(pt 3)**:804-807; 1993.

Thomson, R.H. *Naturally Occuring Quinones.* London and New York; Academic Press; 1971.

Thomson, R.H. *Naturally Occurring Quinones. III. Recent Advances.* London: Chapman & Hall; 1987.

Tiffany-Castiglioni, E.; Saneto, R.P.; Proctor, P.H.; Perez-Polo, J.R. Participation of active oxygen species in 6-hydroxydopamine toxicity to a human neuroblastoma cell line. *Biochem. Pharmacol.* **31**:181-188; 1982.

Tikkanen, L.; Matsushima, T.; Natori, S.; Yoshihira, K. Mutagenicity of natural naphthoquinones and benzoquinones in the Salmonella/microsome test. *Mutation Res.* **124**:25-34; 1983.

Trautner, E.M.; Bradley, T.R. The early stages of the oxidation of adrenaline in dilute solution. *Aust. J. Sci. Res.* **B4**:303-343; 1951.

Turrens, J. F.; Alexandre, A.; Lehninger, A. L. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch. Biochem. Biophys.* **237**:408-414; 1985.

Turrens, J. F.; Boveris, A. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem. J.* **191**:421-427; 1980.

Ungerstedt, U. 6-Hydroxy-dopamine induced degeneration of central monoamine neurons. *Eur. J. Pharmacol.* **5**:107-110; 1968.

Uretsky, N.J.; Iversen, L.L. Effects of 6-hydroxydopamine on noradrenaline-containing neurones in the rat brain. *Nature* **221**:557-559; 1969.

Uretsky, N.J.; Iversen, L.L. Effects of 6-hydroxydopamine on catecholamine containing neurons in the rat brain. *J. Neurochem.* **17**:269-278; 1970.

Wallace, D.C. Diseases of the mitochondrial DNA. *Ann. Rev. Biochem.* **61**:1175-1212; 1992.

Wallace, D.C. Mitochondrial genetics: a paradigm for aging and degenerative diseases. *Science* **256**:628-632; 1992.

Wallace, D.C. Mitochondrial diseases: genotype versus phenotype. *Trends in Genetics* **9**:128-133; 1993.

Wardman, P. Reduction potentials of one-electron couples involving free radicals in aqueous solution. *J. Phys. Chem. Ref. Data* **18**:1637-1755; 1989

Wardman, P Bioreductive activation of quinones: redox properties and thiol reactivity. *Free Rad. Res. Comms.* **8**:219-229; 1990.

Willson, R.L. Quinones, semiquinone free radicals and one-electron transfer reactions: a walk in the literature from Peru to S.O.D. *Free Rad. Res. Comms.* **8**:201-217; 1990.

Yen, T-C.; King, K-L.; Lee, H-C.; Yeh, S-H.; Wei, Y-H. Age-dependent increase of mitochondrial DNA deletions together with lipid peroxides and superoxide dismutase in human liver mitochondria. *Free Rad. Biol. Med.* **16**:207-214, 1994.

Zhang, L.; Robertson, M.; Kolachana, P.; Davison, A.J.; Smith, M.T. Benzene metabolite, 1,2,4-benzenetriol induces micronuclei and oxidative DNA damage in human lymphocytes and HL60 cells. *Env. Mol. Mutagenesis* **21**:339-348; 1993.

Zhang, L.; Venkatesh, P.; Robertson-Creek, M.L.; Smith, M.T. Detection of 1,2,4-benzenetriol induced aneuploidy and microtubule disruption by fluorescence in situ hybridization and immunocytochemistry. *Mutation Res.* **320**:315-327; 1994.

Chapter 2.

Effects of Respiratory Impairment on the Autoxidation of Reduced Quinone Intermediates in Submitochondrial Particles

by

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ABSTRACT

Hypoxia, and loss of cytochrome *c*, impair mitochondrial respiration in ischaemic tissues, and result in the accumulation of autoxidizable respiratory intermediates. We therefore assessed the effects of oxygen depletion and of cytochrome *c* removal on the release of $O_2^{\cdot-}$ by beef heart submitochondrial particles. Rapidly respiring submitochondrial particles (SMPs) consuming NADH in air-saturated buffer produced 0.40 nmol $O_2^{\cdot-}$/min/mg protein (or 0.82 nmol $O_2^{\cdot-}$/μmol O_2 consumed), as detected by superoxide dismutase-sensitive reduction of acetylated cytochrome *c*. Respiration on succinate produced only 0.1-0.2 nmol $O_2^{\cdot-}$/min/mg protein (0.66 nmol $O_2^{\cdot-}$/μmol O_2 consumed). Increasing the level of reduced intermediates by adding the respiratory inhibitor antimycin A increased $O_2^{\cdot-}$ generation 2-fold. In contrast, increasing the steady state level of reduced quinone intermediates by allowing respiration to deplete oxygen to hypoxic levels, did not measurably increase $O_2^{\cdot-}$ generation. Presumably the low Km of cytochrome oxidase for oxygen minimizes autoxidation of reduced intermediates even at very low oxygen tensions. Partial removal of cytochrome *c* to a level which decreased oxygen consumption 50%, did not change the absolute rate of $O_2^{\cdot-}$ generation, but increased the generation of $O_2^{\cdot-}$ per μmole O_2 consumed by 80-90%. Thus, while hypoxia may not increase $O_2^{\cdot-}$ release by mitochondria, loss of cytochrome *c* can, on reoxygenation, result in an increase in oxidative stress relative to the capacity for oxidative phosphorylation.

INTRODUCTION

Mitochondrial flavoproteins and quinone oxidoreductases produce significant $O_2^{\cdot-}$ and H_2O_2 in state IV, or inhibited respiration.

Mitochondria from mammalian sources generate 0.2-1.0 nmol H_2O_2/min/mg protein during respiration in state IV, where the absence of ADP gives slow respiration and largely reduced respiratory intermediates (reviewed in Forman and Boveris 1982). This H_2O_2 generation accounts for 1-2% of state IV oxygen consumption (Boveris et al 1972, Loschen et al 1974). Under more physiological conditions (state III: presence of ADP and rapid respiration) H_2O_2 generation decreases to less than 25% that of state IV (Boveris et al 1972, Boveris and Chance 1973, Forman and Boveris 1982). Submitochondrial particles (free of endogenous superoxide dismutase) produce 0.8-5.2 nmol $O_2^{\cdot-}$ /min/mg protein in the presence of respiratory inhibitors antimycin or cyanide (Forman and Boveris 1982). The rate of $O_2^{\cdot-}$ generation in the absence of respiratory inhibitors is very low (due to rapid uncoupled respiration in SMP's) and is not often measured or reported.

The autoxidizable components of the mitochondrial respiratory chain responsible for the "leak" of $O_2^{\cdot-}$ and H_2O_2 during respiration include reduced ubiquinone and flavoquinone intermediates (Forman and Boveris 1982). Ubiquinol reacts only slowly with oxygen ($k = 1.5\ M^{-1}\ sec^{-1}$) (Sugioka et al 1988, Cadenas et al 1977), and is likely not a major source of $O_2^{\cdot-}$ and H_2O_2. The effects of specific respiratory inhibitors and of ubiquinone extraction on $O_2^{\cdot-}$ or H_2O_2 generation, and the effects of superoxide dismutase and oxygen on ESR signals, suggest enzymically produced ubisemiquinone as the component of complex III (ubiquinol:cytochrome *c* oxidoreductase) which reacts with O_2 (Boveris et al 1976, Cadenas et al 1977, Trumpower and Simmons 1979, Sugioka et al 1980, Ksenzenko et al 1983, Turrens et al 1985, Sugioka et al 1988). While the bulk of evidence supports autoxidation of ubisemiquinone as the major source of $O_2^{\cdot-}$ and H_2O_2 from this complex, other evidence suggests the Rieske iron-sulphur protein (DegliEsposti et al 1990) or cytochrome b_{566} (Nohl and Jordan 1986, Nohl et al 1990) as autoxidizable components. In NADH dehydrogenase

(complex I), flavosemiquinone or iron-sulphur clusters are the likely sources of $O_2^{\cdot-}$ (Cadenas et al 1977, Turrens and Boveris 1980, Kang et al 1983). Succinate dehydrogenase (complex II) reportedly does not produce $O_2^{\cdot-}$ and H_2O_2 (Boveris et al 1976, Forman and Boveris 1982), except by supplying substrate (ubiquinol) for complex III.

In vivo, hypoxia or ischaemia/reperfusion injury may increase mitochondrial generation of $O_2^{\cdot-}$ and H_2O_2

Hypoxia, and damage to or dissociation of respiratory chain components, produce situations where reduced intermediates may accumulate to react with O_2. The state III Km of cytochrome oxidase for oxygen is 0.5 - 1.0 μM (Chance 1957, Wilson et al 1979, Ludwig et al 1983). At oxygen concentrations below about 3 μM (in isolated cells or mitochondria) mitochondrial respiration drops off and the cytochrome components become more reduced (Longmuir 1957; Sugano et al 1974; Wilson et al 1977, 1979; Ludwig et al 1983). Above 3 μM respiration is almost independent of oxygen tension (Longmuir 1957, Wilson et al 1979) and the cytochromes are largely oxidized (Chance 1957, Sugano et al 1974, Wilson et al 1979, Ludwig et al 1983). In hyperoxia, $O_2^{\cdot-}$ and H_2O_2 generation during state IV or antimycin inhibited respiration increases directly with oxygen tension (Boveris and Chance 1974; Freeman and Crapo 1981; Turrens et al 1982a, 1982b).

Conversely, it has been suggested (Fridovich 1979, 1980; Sjostrom and Crapo 1981, 1983) that hypoxia in actively respiring (ie. state III) mitochondria may provide an increased opportunity for spontaneous oxidation which at some pO_2 may increase generation of $O_2^{\cdot-}$ and H_2O_2. The generation of $O_2^{\cdot-}$ by ferredoxin:NADPH oxidoreductase, for example, peaks at low oxygen tensions (Misra and Fridovich 1972a). ESR measurements show an increase in the radical signal from FeS proteins and from ubisemiquinone during myocardial ischaemia (Suzuki 1975, Ledenev et al 1986, Baker et al 1988, Baker and Kalyanaraman 1989). Because of this evidence, and the possibility that mitochondrial generation of $O_2^{\cdot-}$

and H_2O_2, either during ischaemia, or on reoxygenation may contribute to cellular injury, we chose to measure the effect of pO_2 on mitochondrial generation of superoxide.

Disruption of the electron transport chain creates another situation where mitochondria may accumulate reduced intermediates and in normoxia, or on reoxygenation after ischaemia, increase their release of $O_2^{\cdot-}$ and H_2O_2. Mitochondria are among the first sites of injury in ischaemia or on reoxygenation (reviewed in Steen et al 1983). Mitochondria in ischaemic or reperfused tissues appear swollen, have disrupted cristae, and accumulate hydroxyapatite crystals due to uptake of excessive cytoplasmic calcium. Mitochondria isolated from ischaemic tissues display decreased respiratory capacity (state III respiration) and increased (ie. uncoupled) state IV respiration, resulting in loss of respiratory control.

Measurements of $O_2^{\cdot-}$ and H_2O_2 generation by mitochondria from ischaemic tissues has yielded mixed results. Succinate-supported H_2O_2 generation (in absence of respiratory inhibitors) was lower in mitochondria from ischaemic rat brain or hypoxic rat heart, perhaps due to decreased reverse electron flow through NADH dehydrogenase (Cino and DelMaestro 1989, Paraidathathu et al 1992). With NADH as substrate however, mitochondria from ischaemic or reperfused myocardium generate more $O_2^{\cdot-}$ and H_2O_2 than control mitochondria, either in the presence of respiratory inhibitors (Guarnieri et al 1985, Ledenev and Ruuge 1991) or in their absence (Turrens et al 1991). This increased generation of $O_2^{\cdot-}$ and H_2O_2 was most evident at the level of NADH dehydrogenase, and was associated with inhibited electron transfer from NADH dehydrogenase to complex III, and uncoupled respiration (Guarnieri et al 1985, Ledenev and Ruuge 1986, Turrens et al 1991). The findings could be mimicked by exposure of control mitochondria to high Ca^{2+} or unsaturated fatty acids. These results suggested a mechanism whereby Ca^{2+} activates phopholipase A_2 to release free fatty acids, causing uncoupling, swelling, and impaired interaction between complexes I and III (Turrens et al 1991). The observation that ubisemiquinone only autoxidizes on exposure to protic media (Nohl 1990), gives a rationale for increased release of $O_2^{\cdot-}$ and H_2O_2 on disruption of the aprotic membrane environment.

Ischaemia also results in losses of cytochrome *c* (loosened by mitochondrial swelling) and an associated decrease in state III respiration in isolated mitochondria (Toleikis 1983, Steen et al 1983). Loss of cytochrome *c* may result in prolonged accumulation of reduced intermediates on reintroduction of O_2, leaving them susceptible to autoxidation. In vitro removal of cytochrome *c* from isolated mitochondria increased the proportion of H_2O_2 formed per oxygen consumed about 3-fold (comparison of figures 2,4 and 5 in Turrens et al 1985), but it slightly inhibited succinate-supported state IV generation of H_2O_2 per mg mitochondrial protein (and inhibited antimycin-stimulated H_2O_2 formation 50-60%, as might be expected) (Loschen et al 1974, Turrens et al 1985). However, the influence of cytochrome *c* removal on $O_2^{\cdot-}$ or H_2O_2 generation in active state III (or uncoupled) respiration, which would predominate following hypoxia, has not been tested.

The goals of the studies reported here were to measure low level $O_2^{\cdot-}$ generation by SMPs in the absence of respiratory inhibitors, and to assess $O_2^{\cdot-}$ generation under conditions where reduced intermediates accumulate: i) as oxygen is depleted to hypoxic levels and ii) in normoxia or hyperoxia after extraction of cytochrome *c*.

MATERIALS AND METHODS

Reagents

All reagents were from Sigma Chemical Co. Epinephrine was dissolved to 10 mM in H_2O at pH 2.0 to prevent autoxidation (Misra and Fridovich 1972b). Acetylated cytochrome *c* was prepared as previously described (Azzi et al 1975, Boveris 1984) by reacting cytochrome *c* (Sigma type VI) with acetic anhydride (resulting in approximately 60% acetylation of lysine residues) and dialyzing against distilled water and 50 mM phosphate buffer. Concentrations of cytochrome *c* and acetylated cytochrome *c* solutions were determined from the dithionite-reduced minus ferricyanide-oxidized absorbance spectra (E_{550} = 21100 M^{-1} cm^{-1}).

Preparation of mitochondria and submitochondrial particles

Mitochondria were prepared in a cold-room from a fresh beef heart transported on ice in sucrose/Tris/EDTA buffer from a local slaughter house. The heart was homogenized and mitochondria isolated by differential centrifugation according to established procedures (Smith 1967). Aliquots of the mitochondrial preparation were frozen in liquid nitrogen and stored at -80°C. Cytochrome *c* was extracted from samples of these mitochondria by osmotic swelling and subsequent washing, as described (Lenaz and MacLennan 1967), and refrozen. Submitochondrial particles were prepared from mitochondria and cytochrome *c* - extracted mitochondria by sonication and ultracentrifugation as previously described (Beyer 1967, Lee 1979). Protein concentrations of mitochondrial and submitochondrial particle preparations were determined by the method of Lowry et al (1951).

$O_2^{\cdot-}$ generation and oxygen consumption measurements

Cytochrome *c*, acetylated cytochrome *c*, and epinephrine worked equally well as superoxide dismutase-sensitive measures of $O_2^{\cdot-}$ generated by xanthine oxidase. Reduction of cytochrome *c* by an aged solution of xanthine oxidase however was partially (40%) insensitive to inhibition by superoxide dismutase (as, I later found, had been described in a footnote of a previous article (McCord and Fridovich 1969). Reduction of acetylated cytochrome *c* by this aged enzyme, on the other hand, was 100% inhibitable by superoxide

dismutase, suggesting hindered access of the acetylated protein to a loosened active site. Acetylation of cytochrome *c* inhibits enzymic reduction and reoxidation by the mitochondrial respiratory chain, making it more suitable than native cytochrome *c* as a probe for $O_2^{\cdot -}$ generated by SMP's (Azzi et al 1975, Boveris 1984).

Assays for $O_2^{\cdot -}$ generation by SMP's were conducted initially in a split beam Beckman DB-GT at 550 nm for reduction of acetylated cytochrome *c* (E_{550} = 0.0211 μM^{-1} cm^{-1}) or 480 nm for formation of adrenochrome (E_{480} = 0.00402 μM^{-1} cm^{-1}). Control of light scattering effects due to swelling and shrinking of SMP's required addition of SMP's and substrate (succinate or NADH) to both sample and reference cuvettes, causing increased noise at high sensitivity settings. Buffers isotonic with the SMP sonication medium (sucrose-Tris, sucrose-HEPES, KCl-phosphate) did not prevent light scattering effects. Epinephrine assays were complicated by spontaneous autoxidation, particularly in Tris buffers, but could be largely controlled by addition of EDTA or DTPA (but not desferrioxamine). 50 mM Potassium phosphate/0.1 mM EDTA (pH 7.4) was therefore established as the buffer system for these experiments. Later experiments were conducted with a dual wavelength photometer to better control effects of light scattering (see below).

Hypoxia: Measures of $O_2^{\cdot -}$ generation and oxygen consumption were performed simultaneously in a specially designed and constructed spectrophotometer cuvette equipped with a Clarke oxygen electrode and a water turbine-powered magnetic stirrer (figure 1). Because stirring rate and water temperature through the turbine influenced measurements in the cuvette, water pressure was controlled with a regulator and water temperature adjusted to 25°C with the aid of a thermistor inserted in the outflow water line. Differences in light scattering between sample (closed cuvette) and reference cuvettes on exhaustion and reintroduction of oxygen complicated initial attempts to follow adrenochrome formation as SMP's consumed oxygen to anoxia. Subsequent experiments therefore were conducted with a dual wavelength spectrophotometer (TCS model 3000) using 550 nm minus 530 nm measurements for reduction of acetylated cytochrome *c* and 490 nm minus 550 nm for adrenochrome formation.

Normoxia and Hyperoxia: $O_2^{\cdot -}$ generation by SMP's and cytochrome *c* - extracted SMP's (-cSMP's) was monitored by superoxide dismutase-sensitive reduction of acetylated cytochrome *c* at 550 minus 530 nm (TCS dual wavelength photometer) in a standard open cuvette with continuous stirring. Experiments were conducted in air-saturated or oxygen-saturated 50 mM phosphate/0.1 mM EDTA buffer (pH 7.4) at 25°C. Oxygen consumption by the SMP preparations was measured separately in the sample chamber of a YSI model 53 oxygen monitor and stirrer assembly. Oxygen concentrations were calculated using a value of 246 μM for air-saturated buffer at the altitude of our laboratory. Rates of $O_2^{\cdot -}$ generation were calculated from the rates of acetylated cytochrome *c* reduction in the presence and absence of excess superoxide dismutase.

RESULTS

SMP's respiring on succinate produced 0.1-0.2 nmol $O_2^{\cdot-}$ /min/mg protein, depending on the succinate oxidase activity of the SMP preparation (figure 2). Antimycin increased $O_2^{\cdot-}$ generation 2-fold, and blocked significant consumption of oxygen (figure 3). Respiration on NADH produced 0.40 nmol $O_2^{\cdot-}$ /min/mg protein and consumed oxygen more rapidly, but it also produced greater superoxide dismutase-insensitive reduction of acetylated cytochrome *c* (figure 4), as noted previously in lung SMP's (Turrens et al 1982).

$O_2^{\cdot-}$ generation by SMP's during oxygen consumption to hypoxia

Oxygen consumption on succinate accelerated to a rate of 0.16 μmol/min/mg protein (figure 5a), displaying a latent period for full activity of succinate dehydrogenase (as observed previously (Kearney 1957, Lee 1979)). The rate of acetylated cytochrome *c* reduction decreased as oxygen tension declined (figure 5a). At the point of oxygen exhaustion the 550 - 530 nm absorbance showed a sudden small increase. However, this sudden increase also occurred in the absence of acetylated cytochrome *c* (figure 5b) or in the presence of superoxide dismutase, so was likely due to sudden reduction of endogenous cytochrome *c*.

Epinephrine was also useful for detecting $O_2^{\cdot-}$, but was hampered somewhat by spontaneous oxidation and by light scattering effects on addition of substrate or exhaustion of O_2 (figure 6). Although the measurements were influenced by a change in light scattering, oxidation of epinephrine also did not reveal any change in $O_2^{\cdot-}$ generation at low oxygen tensions (figure 6). In this measurement, the cuvette was left open to allow slow diffusion of oxygen into the stirred suspension. Only very slow oxidation of epinephrine continued under these conditions. These studies were not conclusive, but they showed no evidence of an increase in $O_2^{\cdot-}$ generation at low oxygen tensions.

$O_2^{\cdot-}$ generation by cytochrome c deficient SMP's

Extraction of cytochrome *c* by the procedures used does not give complete removal from heart mitochondria (Jacobs and Sanadi 1960, Lenaz and Maclennan 1967, Turrens et al 1985), but produced SMP's with a 50-60% decrease in the rate of oxygen consumption (figure 7). Addition of native cytochrome *c*, but not acetylated cytochrome *c*, increased oxygen consumption by these SMP's 70-80% (figure 7). Addition of cytochrome *c* also stimulated respiration of control SMP's 10-15%, indicating some loss of cytochrome *c* during preparation.

A first preparation of -cSMP's respiring on succinate seemed to generate slightly more $O_2^{\cdot-}$ /min/mg protein (figure 8, table I). However, the succinate oxidase activity of these preparations declined with time, leading to low rates of oxygen consumption and acetylated cytochrome *c* reduction (figure 9). The pH of the sonicating medium was thus decreased from 8.2 to 7.8 at 4°C in subsequent preparations (as recommended by Lee 1979). Succinate is sometimes added to the sonication medium to stabilize succinate dehydrogenase (Kearney 1957, Lee 1979), but was withheld in these SMP preparations since it is not present in the buffer for extraction of cytochrome *c* from mitochondria (to assist mitochondrial swelling and loosening of cytochrome *c*).

With fresh preparations of cytochrome *c* - extracted mitochondria, -cSMP's, and SMP's, we used oxygen-saturated buffer and NADH to maximize $O_2^{\cdot-}$ generation from both complex I and complex III. Succinate-supported reduction of acetylated cytochrome *c* was non-linear and partially insensitive to superoxide dismutase (figure 10). Respiration on succinate was also low, suggesting partial inactivation of succinate dehydrogenase. NADH produced a consistent linear reduction of acetylated cytochrome *c* (figure 11), which was partially insensitive to superoxide dismutase. The rate of acetylated cytochrome *c* reduction and its sensitivity to superoxide dismutase depended on the concentration of acetylated cytochrome *c* (as found previously for native cytochrome *c* (Boveris and Cadenas 1975)), 5 μM proving suitable for distinguishing $O_2^{\cdot-}$ under the conditions used here (figure 12). The superoxide

dismutase-sensitive reduction of acetylated cytochrome *c* was distinguishable in air-saturated buffer, but was more evident in oxygen-saturated buffer (figure 13). The cytochrome *c* deficient SMP's produced $O_2^{\cdot -}$ at approximately the same rate per milligram protein as the control SMP's (table II). The amount of $O_2^{\cdot -}$ produced per oxygen consumed however was 80-90% higher in -cSMP's.

DISCUSSION

Optimal conditions for measuring $O_2^{\cdot-}$ generation by SMP's.[*]

Generation of $O_2^{\cdot-}$ was measurable using either epinephrine oxidation or acetylated cytochrome *c* reduction. Each method had advantages and disadvantages, depending on the situation. Spontaneous oxidation and sensitivity to light scattering effects (due to the broad wavelength difference measured) complicated the epinephrine assay. Superoxide dismutase-insensitive reduction of acetylated cytochrome *c* complicated the acetylated cytochrome *c* assay with NADH as the respiratory substrate. Acetylation of cytochrome *c* apparently does not inhibit enzymic reduction by NADH:cytochrome *c* reductase, as it does for succinate:cytochrome *c* reductase (Minakami et al 1958, Azzi et al 1975, Boveris 1983). Optimal detection of $O_2^{\cdot-}$ by this assay required a suitable balance between the concentrations of acetylated cytochrome c, SMP's, and superoxide dismutase. For the range 0.05 - 0.1 mg SMP protein/ml used in these studies, an acetylated cytochrome *c* concentration of 5 μM proved suitable for maximal inhibition by 40 U/ml superoxide dismutase.

Succinate oxidase activity appeared unstable in the current preparations, declining in activity on storage, and displaying a latent period for full activation. For use of succinate as substrate, care should be taken to preserve succinate oxidase activity. Addition of substrate (succinate or malonate) helps stabilize succinate dehydrogenase (Kearney 1957, Lee 1979) and may also help prevent loss of cytochrome *c* from SMP preparations.

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[*] Measuring low level $O_2^{\cdot-}$ generation by SMP's proved feasable, but measures of H_2O_2 generation by whole mitochondria would likely present less technical difficulties for future studies, and would allow comparisons under more physiological, coupled conditions.

Hypoxia did not measurably increase $O_2^{\cdot-}$ generation

Within the limitations of the preparations and assays used here, no increase in $O_2^{\cdot-}$ was detectable at oxygen tensions where the respiratory intermediates become more reduced. Presumably the very low Km of cytochrome oxidase for oxygen keeps the respiratory intermediates in a mostly oxidized steady state until the oxygen concentration is too low to give significant autoxidation of accumulating reduced intermediates. Slow diffusion of oxygen into the system did not produce measurable $O_2^{\cdot-}$, likely because oxygen tension was kept very low by respiratory activity. Generation of $O_2^{\cdot-}$ would not expectedly increase on introduction of oxygen unless damage to the respiratory chain prevented enzymic reoxidation of accumulated reduced intermediates.

Respiratory deficiency due to loss of cytochrome c increased $O_2^{\cdot-}$ generation relative to oxygen consumption.

Removal of sufficient cytochrome *c* to decrease respiratory activity by 50-60% did not increase $O_2^{\cdot-}$ generation by SMP's in these assays. Complete respiratory inhibition by antimycin A or cyanide increases the free radical signal from SMP's (Commoner and Hollocher 1960, Suzuki 1975, Sugioka et al 1988) and stimulates $O_2^{\cdot-}$ formation (Cadenas and Boveris 1980, Turrens and Boveris 1980, Sugioka et al 1988). The remaining respiratory activity in cytochrome *c* - extracted SMP's was apparently sufficient to prevent significant accumulation of autoxidizable intermediates.* However, the $O_2^{\cdot-}$ generated by these preparations was 3-fold higher than controls when expressed as a function of oxygen consumed. In ischaemia-reperfusion, where cytochrome *c* may be partially lost due to mitochondrial swelling, the capacity of the mitochondria to produce $O_2^{\cdot-}$ would remain,

* Extraction of cytochrome *c* from heart mitochondria is inevitably incomplete. Thus, cytochrome *c* removal does not allow us to assess the influence of abolition of cytochrome oxidase activity, as might occur with a mitochondrial mutation (Bandy and Davison 1990). Cytochrome *c* is more readily extracted from liver mitochondria however, leaving respiratory rates of 0-5% (Jacobs and Sanadi 1960).

while the ability to generate ATP would decrease. Moreover, the decreased respiration would increase the steady-state membrane oxygen tension (on reperfusion), adding to reperfusion hyperoxia from the Bohr effect (Wolbarsht and Fridovich 1989), and increasing the possibility for autoxidation of intermediates.

CONCLUSIONS

Hypoxia alone does not measurably increase generation of $O_2^{\cdot -}$ by submitochondrial particles. In the absence of respiratory impairment, the low Km of cytochrome oxidase for oxygen apparently limits autoxidation of reduced intermediates. Respiratory impairment through loss of cytochrome *c* increases the rate of $O_2^{\cdot -}$ release by SMP's relative to oxygen consumption. Thus, in ischaemia-reperfusion, loss of cytochrome *c* from mitochondria may increase $O_2^{\cdot -}$ and H_2O_2 production relative to ATP production. Reperfusion hyperoxia would amplify such oxidative stress.

REFERENCES

Azzi, A.; Montecucco, C.; Richter, C. (1975) The use of acetylated ferricytochrome *c* for the detection of superoxide produced in biological membranes. *Biochem. Biophys. Res. Comm.* **65**:597-603.

Baker, J.E.; Felix, C.C.; Olinger, G.N.; Kalyanaraman, B. (1988) Myocardial ischemia and reperfusion: Direct evidence for free radical generation by electron spin resonance spectroscopy. *Proc. Natl. Acad. Sci. USA* **85**:2786-2789.

Baker, J.E.; Kalyanaraman, B. (1989) Ischemia induced changes in myocardial paramagnetic metabolites: Implications for intracellular oxy-radical generation. *FEBS Lett.* **244**:311-314.

Bandy, B.; Davison, A.J. (1990) Mitochondrial mutations may increase oxidative stress: Implications for carcinogenesis and aging? *Free Rad. Biol. Med.* **8**:523-539.

Beyer, R.E.. (1967) Preparation, properties and conditions for assay of phosphorylating electron transport particles (ETPH) and its variations. *Methods Enzymol.* **10**:186-194.

Boveris, A.; Oshino, N.; Chance, B. (1972) The cellular production of hydrogen peroxide. *Biochem. J.* **128**:617-630.

Boveris, A.; Chance, B. (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem. J.* **134**:707-716.

Boveris, A.; Chance, B. (1974) Optimal rates of hydrogen peroxide production in hyperbaric oxygen. In: *Alcohol and Aldehyde Metabolizing Systems* (R.G. Thurman, T. Yonetani, J.R. Williamson, and B. Chance, eds.) pp207-214, Academic press, New York and London.

Boveris, A.; Cadenas, E. (1975) Mitochondrial production of superoxide anions and its relationship to the antimycin insensitive respiration. *FEBS Lett.* **54**:311-314.

Boveris, A.; Cadenas, E.; Stoppani, A.O.M. (1976) Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. *Biochem. J.* **156**:435-444.

Boveris, A. (1984) Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. *Methods Enzymol.* **105**:429-435.

Cadenas, E.; Boveris, A. (1980) Enhancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria. *Biochem. J.* **188**:31-37.

Cadenas, E.; Boveris, A.; Ragan, C.I.; Stoppani, A.O.M. (1977) Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome *c* reductase from beef-heart mitochondria. *Arch. Biochem. Biophys.* **180**:248-257.

Chance, B. (1957) Cellular oxygen requirements. *Fed. Proc.* **16**:671-680.

Cino, M.; Del Maestro, R (1989) Generation of hydrogen peroxide by brain mitochondria: The effect of reoxygenation following postdecapitative ischemia. *Arch. Biochem. Biophys.* **269**:623-638.

Commoner, B.; Hollocher, T.C.Jr. (1960) Free radicals in heart muscle mitochondrial particles: General characteristics and localization in the electron transport system. *Proc. Natl. Acad. Sci. USA* **46**:405-416.

DegliEsposti, M.; Ballester, F.; Timoneda, J.; Crimi, M.; Lenaz, G. (1990) The oxidation of ubiquinol by the isolated Rieske iron-sulfur protein in solution. *Arch. Biochem. Biophys.* **283**:258-265.

Flohe, L.; Loschen, G.; Azzi, A.; Richter, C. (1977) Superoxide radicals in mitochondria. In: *Superoxide and Superoxide Dismutases* (A.M. Michelson; J.M. McCord; I. Fridovich eds) pp323-334.

Forman, H.J.; Boveris, A. (1982) Superoxide radical and hydrogen peroxide in mitochondria. In: *Free Radicals in Biology* (W.A. Pryor, ed.) **vol V**:65-90, Academic Press.

Fridovich, I. (1979) Hypoxia and oxygen toxicity. *Adv. Neurol.* **26**:255-259.

Freeman, B.A.; Crapo, J.D. (1981) Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *J. Biol. Chem.* **256**:10986-10992.

Guarnieri, C.; Muscari, C.; Ventura, C.; Mavelli, I. (1985) Effect of ischemia on heart submitochondrial superoxide production. *Free Rad. Res. Comms.* **1**:123-128.

Jacobs, E.E.; Sanadi, D.R. (1960) The reversible removal of cytochrome *c* from mitochondria. *J. Biol. Chem.* **235**:531-534.

Kang, D.; Narabayashi, H.; Sata, T.; Takeshige, K. (1983) Kinetics of superoxide formation by respiratory chain NADH-dehydrogenase of bovine heart mitochondria. *J. Biochem.* **94**:1301-1306.

Kearney, E.B. (1957) Studies on succinate dehydrogenase. IV. Activation of the beef heart enzyme. *J. Biol. Chem.* **229**:363-375.

Ksenzenko, M.; Konstantinov, A.A.; Khomutov, G.B.; Tikhonov, A.N.; Ruuge, E.K. (1983) Effect of electron transfer inhibitors on superoxide generation in the cytochrome bc_1 site of the mitochondrial respiratory chain. *FEBS Lett.* **155**:19-24.

Ledenev, A.N.; Ruuge, E.K. (1986) Generation of superoxide radicals by mitochondria of the ischemic heart. *Exp. Cardiol.* **9**:1204-1206.

Lee, C.P. (1979) Tightly coupled beef heart submitochondrial particles. *Methods Enzymol.* **55**:105-112.

Lee, C.P. (1981) Submitochondrial particles and energy transduction. In: *Mitochondria and Microsomes* (C.P. Lee, G. Schatz, G. Dallner, eds.) pp121-123, Addison-Wesley Publishing Company, Reading.

Lenaz, G.; MacLennan, D.H. (1967) Extraction and estimation of cytochrome *c* from mitochondria and submitochondrial particles. *Methods Enzymol.* **10**:499-504.

Longmuir, I.S. (1957) Respiration rate of rat-liver cells at low oxygen concentrations. *Biochem. J.* **65**:378-382.

Loschen, G.; Azzi, A.; Flohe, L. (1974) Mitochondrial hydrogen peroxide formation. In: *Alcohol and Aldehyde Metabolising Systems* (R.G. Thurman, T. Yonetani, J.R. Williamson, and B. Chance, eds.) pp215-229, Academic press, New York and London.

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.

Ludwig, G.D.; Kuby, S.A.; Edelman, G.M.; Chance, B. (1983) A kinetic study of the oxidation by molecular oxygen of the cytochrome chain of intact yeast cells, *acetobacter suboxydans* cells, and of particulate suspensions of heart cells. *Enzyme* **29**:73-85.

McCord, J.M.; Fridovich, I. (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244**:6049-6055.

Minakami, S.; Titani, K.; Ishikura, H. (1958) The structure of cytochrome *c*. II. Properties of acetylated cytochrome *c*. *J. Biochem.* **45**:341-348.

Misra, H.P.; Fridovich, I. (1972a) The univalent reduction of oxygen by reduced flavins and quinones. *J. Biol. Chem.* **247**:188-192.

Misra, H.P.; Fridovich, I. (1972b) The role of superoxide ion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* **247**:3170-3175.

Nohl, H.; Jordan, W. (1986) The mitochondrial site of superoxide formation. *Biochem. Biophys. Res. Comm.* **138**:533-539.

Nohl, H. (1990) Is redox cycling ubiquinone involved in mitochondrial oxygen activation. *Free Rad. Res. Comms.* **8**:307-315.

Packer, L.; Tappel, A.L. (1960) Light scattering changes linked to oxidative phosphorylation in mitochondrial membrane fragments. *J. Biol. Chem.* **235**:525-530.

Paraidathathu, T.; de Groot, H.; Kehrer, J.P. (1992) Production of reactive oxygen by mitochondria from normoxic and hypoxic rat heart tissue. *Free Rad. Biol. Med.* **13**:289-297.

Sjostrom, K.; Crapo, J.D. (1981) Adaptation to oxygen by preexposure to hypoxia: Enhanced activity of manganic superoxide dismutase. Bull. euop. *Physiopath. resp.* **17(suppl.)**:111-116.

Sugano, T.; Oshino, N.; Chance, B. (1974) Mitochondrial functions under hypoxic conditions. The steady states of cytochrome *c* reduction and of energy metabolism. *Biochim. Biophys. Acta* **347**:340-358.

Sjostrom, K.; Crapo, J.D. (1983) Structural and biochemical adaptive changes in rat lungs after exposure to hypoxia. *Lab. Invest.* **48**:68-79.

Smith, A.L. (1967) Preparation, properties, and conditions for assay of mitochondria: Slaughterhouse material, small scale. *Methods Enzymol.* **X**:81-86.

Sugioka, K.; Nakano, M.; Totsune-Nakano, H.; Minakami, H.; Tero-Kubota, S; Ikegami, Y. (1988) Mechanism of $O_2^{\cdot-}$ generation in reduction and oxidation cycles of ubiquinones in a model of mitochondrial electron transport systems. *Biochim Biophys Acta* **936**:377-385.

Suzuki, Y. (1975) Studies on the free radicals in myocardial mitochondria by electron spin resonance (ESR) spectrometry (Studies on experimental infarction dogs). *Jap. Circulation J.* **39**:683-691.

Steen, R.G.; Newman, W.H.; Lindenmayer, G.E. (1983) Modification of ischemia-induced myocardial damage. In: *Pathobiology of Cell Membranes* **vol III**:63-85, Academic Press.

Toleikis, A. (1983) Cytochrome oxidase activity of mitochondria from ischemic and reperfused myocardium. *Adv. Myocard.* **4**:409-418.

Turrens, J.F.; Boveris, A. (1980) Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem. J.* **191**:421-427.

Turrens, J.; Freeman, B.A.; Levitt, J.G.; Crapo, J.D. (1982) The effect of hyperoxia on superoxide production by lung submitochondrial particles. *Arch. Biochem. Biophys.* **217**:401-410.

Turrens, J.; Freeman, B.A.; Crapo, J.D. (1982) Hyperoxia increases H_2O_2 release by lung mitochondria and microsomes. *Arch. Biochem. Biophys.* **217**:411-421.

Turrens, J.F.; Alexandre, A.; Lehninger, A.L. (1985) Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch. Biochem. Biophys.* **237**:408-414.

Turrens, J.F.; Beconi, M.; Barilla, J.; Chavez, U.B.; McCord, J.M. (1991) Mitochondrial generation of oxygen radicals during reoxygenation of ischemic tissues. *Free Rad. Res. Comms.* **12/13**:681-689.

Trumpower, B.L.; Simmons, Z. (1979) Diminished inhibition of mitochondrial electron transfer from succinate to cytochrome *c* by thenoyltrifluoroacetone induced by antimycin. *J. Biol. Chem.* **254**:4608-4616.

Videla, L. (1991) Respective roles of free radicals and energy supply in hypoxic rat liver injury after reoxygenation. *Free Rad. Res. Comms.* **14**:209-215.

Wilson, D.F.; Erecinska, M.; Drown, C.; Silver, I.A. (1977) Effect of oxygen tension on cellular energetics. *Am. J. Physiol.* **233**:C135-C140.

Wilson, D.F.; Erecinska, M.; Drown, C.; Silver, I.A. (1979) The oxygen dependence of cellular energy metabolism. *Arch. Biochem. Biophys.* **195**:485-493.

Wolbarsht, M.L.; Fridovich, I. (1989) Hyperoxia during reperfusion is a factor in reperfusion injury. *Free. Rad. Biol. Med.* **6**:61-62.

Table I. Comparison of succinate-supported respiration and $O_2^{\cdot-}$ generation in SMP's and -cSMP's (preparation 1).

	VO_2 (μmol/min/mg)	$O_2^{\cdot-}$ (nmol/min/mg)	$O_2^{\cdot-}/O_2$ (nmol/μmol)
SMP	0.152 ± 0.02 (n=4)	0.10 (n=1)	0.66
-cSMP	0.054 ± 0.01 (n=4)	0.17 (n=1)	3.15

Table II. Comparison of NADH-supported respiration and $O_2^{\cdot-}$ generation in SMP's and -cSMP's (preparation 2).

	VO_2 (μmol/min/mg)	$O_2^{\cdot-}$ (nmol/min/mg)	$O_2^{\cdot-}/O_2$ (nmol/μmol)
air-saturated			
SMP	0.584 ± 0.086 (n=6)	0.402 (n=2)	0.825
-cSMP	0.282 ± 0.028 (n=5)	0.355 (n=2)	1.51
O_2-saturated			
SMP	-	0.588 ± 0.115 (n=5)	1.21 ± 0.20
-cSMP	-	0.498 ± 0.088 (n=5)	2.12 ± 0.11

Legends to Figures

Figure 1. *Apparatus for simultaneous measurement of oxygen consumption and $O_2^{\cdot-}$ generation.* Air- or oxygen- saturated buffer, SMP's, and reagents were added to the cuvette to a final volume of 7.4 ml. The oxygen electrode was inserted into the suspension through a snug sleeve in an epoxy stopper (secured to the cuvette with an elastic), and head-space gas forced out through a side arm. The apparatus was inserted into the cuvette chamber of a dual wavelength photometer equipped with a water-powered magnetic stirrer. Reactions were initiated by addition of substrate through the side arm with a syringe.

Figure 2. *Measurement of succinate-supported generation of $O_2^{\cdot-}$ by SMP's.* The reaction medium consisted of 50 mM phosphate buffer (pH 7.4), 0.1 mM EDTA, 3 μM acetylated-cytochrome *c*, and 0.042 mg/ml SMP's. Respiration was initiated by addition of 5 mM succinate and reduction of acetylated-cytochrome *c* followed at 550-530 nm. Blips in the trace represent surges in water pressure through the magnetic stirrer before adjustment of the pressure regulator.

Figure 3. a) *Effects of antimycin on succinate-supported generation of $O_2^{\cdot-}$.* Reaction conditions as in figure 2, but acetylated-cytochrome *c* reduction was followed in a standard open cuvette without stirring. **b)** *Effect of antimycin on succinate-supported respiration.* Antimycin (2 μM) was added to 0.042 mg/ml SMP's respiring on succinate and oxygen consumption monitored polarographically.

Figure 4. *Measurement of NADH-supported generation of $O_2^{\cdot-}$.* Reaction conditions as shown. Acetylated-cytochrome *c* reduction was followed in an open cuvette with stirring.

Figure 5. *Acetylated-cytochrome c reduction during oxygen consumption to anoxia.* Reactions were conducted using the closed-system cuvette described in figure 1. **a)** *Simultaneous oxygen consumption and acetylated-cytochrome c reduction by SMP's respiring on succinate.* Reaction conditions consisted of 7.4 ml 50 mM phosphate/EDTA

(pH 7.4), 3 μM acetylated-cytochrome *c*, 0.06 mg/ml SMP's. Respiration was initiated by addition of 3 mM succinate. **b)** *Absorbance at 550-530 nm in the* ***absence*** *of acetylated-cytochrome c during consumption of oxygen by SMP's.*

Figure 6. *$O_2^{\cdot-}$ generation by SMP's as detected by epinephrine oxidation.* Epinephrine oxidation was monitored at 490-550 nm in a stirred open cuvette (traces **a** and **b**). Consumption of oxygen to hypoxia in the time span shown in trace **b** was confirmed in separate oxygen consumption measurements under similar conditions.

Figure 7. *Oxygen consumption by SMP's and cytochrome c-extracted SMP's (-cSMP's) respiring on NADH.* Oxygen consumption by 0.05 mg/ml SMP's or -cSMP's was followed on addition of 0.5 mM NADH. Cytochrome *c* or acetylated-cytochrome *c* (10 μM) were added as indicated.

Figure 8. *Initial comparison of $O_2^{\cdot-}$ generation by SMP's and -cSMP's.* SMP's and -cSMP's were prepared simultaneously, under identical conditions, from previous preparations of mitochondria and cytochrome *c*-extracted mitochondria and stored for 3 days at -80°C prior to these assays. Acetylated-cytochrome *c* reduction by SMP's and -cSMP's during respiration on succinate was followed at 550-530 nm in a stirred, open cuvette.

Figure 9. a) *Oxygen consumption by SMP's and -cSMP's respiring on succinate.* Oxygen consumption by 0.05 mg/ml SMP's or -cSMP's was followed on addition of 1 mM succinate. Cytochrome *c* (10 μM) was added as indicated. Oxygen consumption on the day of the assays shown in figure 8 is compared with oxygen consumption by SMP's and -cSMP's after 3 days further storage. **b)** *Succinate-supported generation of $O_2^{\cdot-}$ by SMP's and -cSMP's after storage.* Assays of acetylated-cytochrome *c* reduction on subsequent days from those shown in figure 8.

Figure 10. *Succinate-supported generation of $O_2^{\cdot-}$ by preparation-2 SMP's and -cSMP's.* SMP's and -cSMP's were prepared from mitochondria and a fresh preparation of cytochrome *c*-extracted mitochondria and stored for 3 days prior to these assays.

Figure 11. *NADH-supported generation of $O_2^{\cdot-}$ by preparation-2 SMP's and -cSMP's.* Acetylated-cytochrome *c* reduction was followed at 550-530 nm during respiration on 0.5 mM NADH in stirred, air-saturated suspensions.

Figure 12. *Dependence of superoxide dismutase-sensitive reduction of acetylated-cytochrome c on concentration of acetylated cytochrome c.* Reduction of differing concentrations of acetylated-cytochrome *c* was followed during respiration on NADH in air-saturated suspensions.

Figure 13. *NADH-supported generation of $O_2^{\cdot-}$ in oxygen-saturated suspensions of SMP's and -cSMP's.* Reduction of acetylated-cytochrome *c* by preparation 2 SMP's and -c SMP's was followed during respiration on NADH in oxygen saturated suspensions.

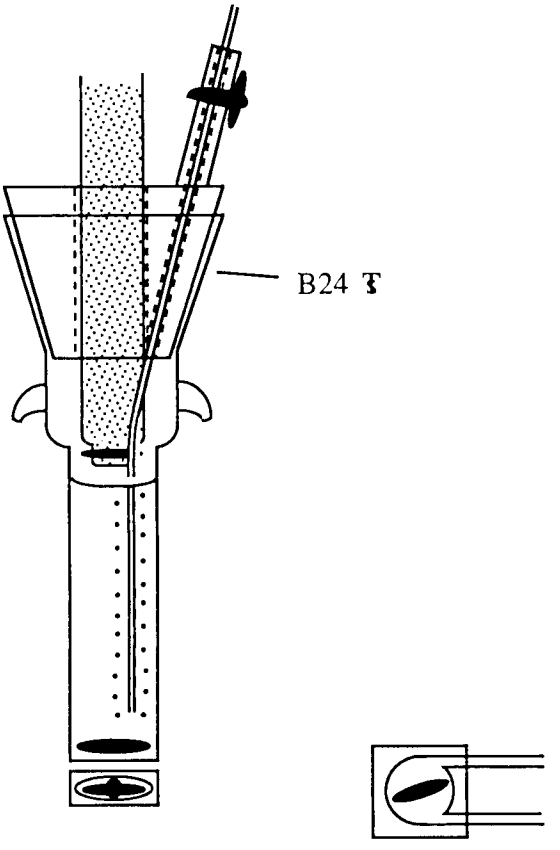


Figure 1.

Figure 2.

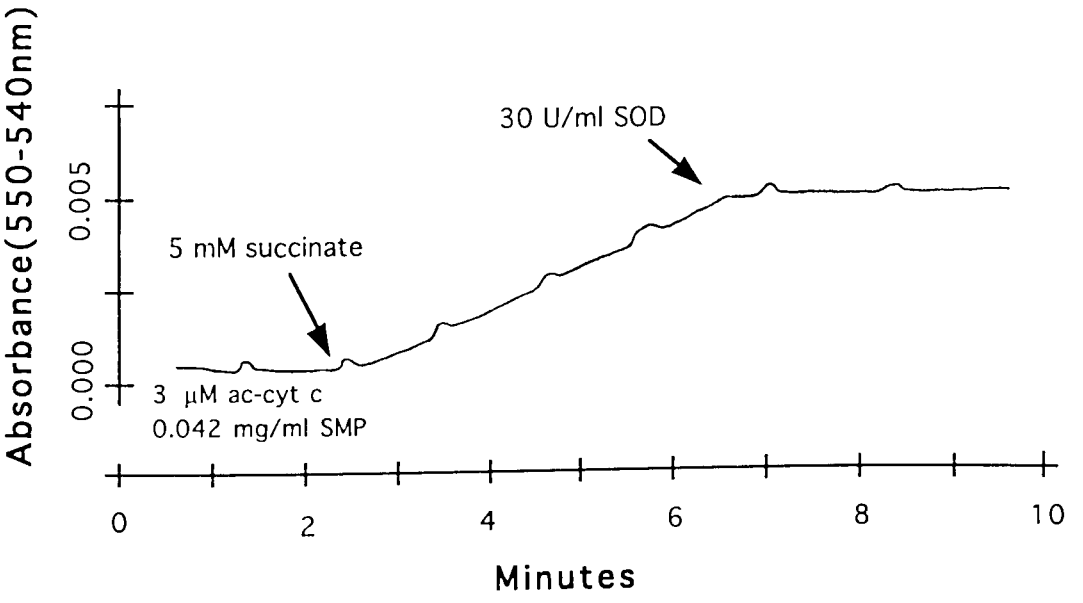
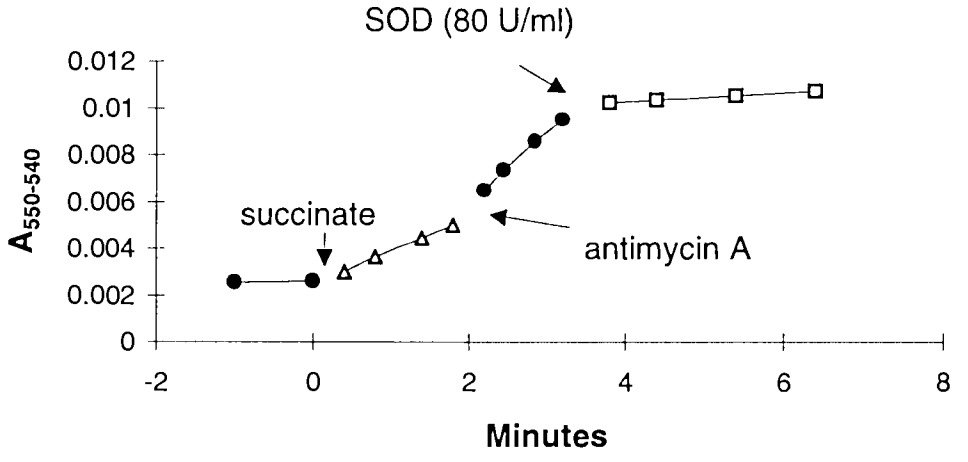

Absorbance(550-540nm)
0.005
0.000
30 U/ml SOD
5 mM succinate
3 μM ac-cyt c
0.042 mg/ml SMP
0
2
4
6
8
10
Minutes

Figure 3.

a) **Effect of antimycin on succinate-supported $O_2^{\cdot-}$ generation**



b) **Effect of antimycin on succinate-supported respiration.**

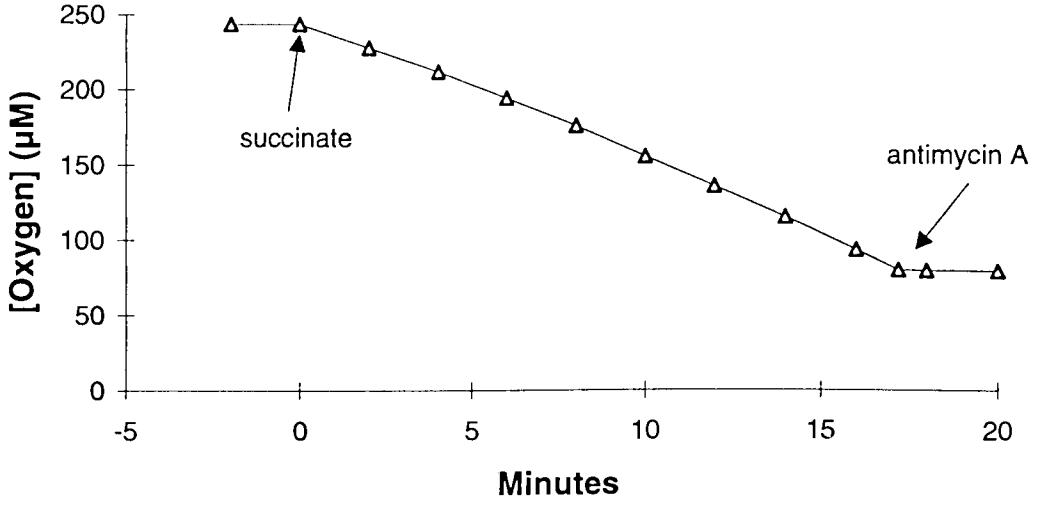


Figure 4.

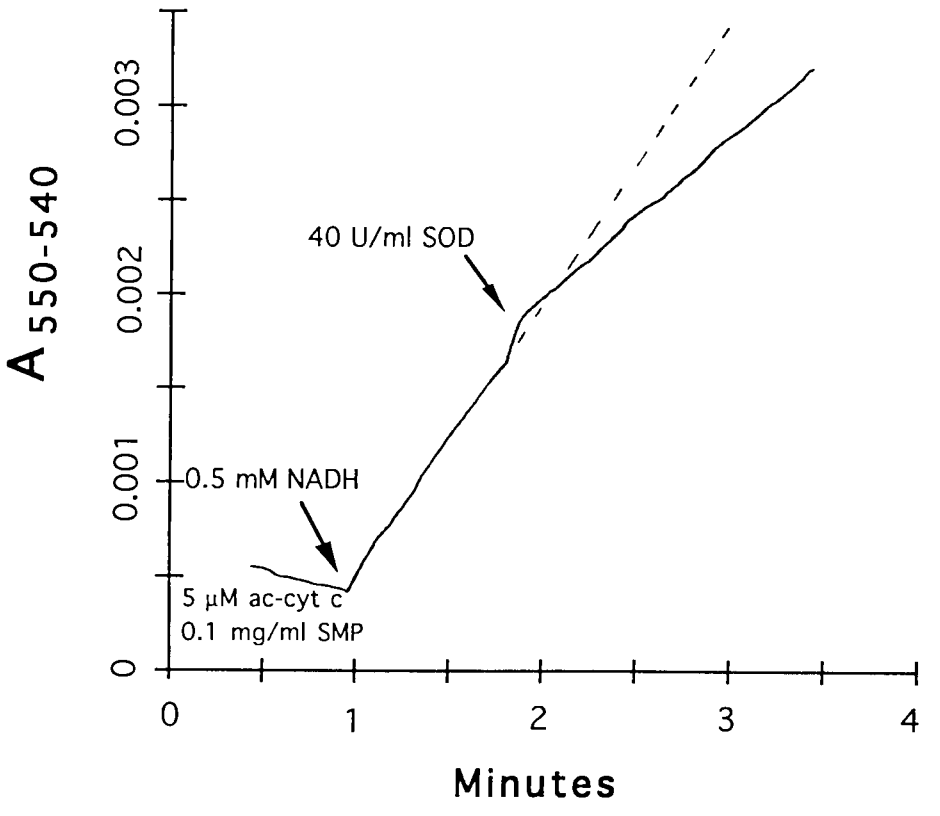

A 550-540
0.003
0.002
0.001
0
40 U/ml SOD
0.5 mM NADH
5 µM ac-cyt c
0.1 mg/ml SMP
0
1
2
3
4
Minutes

Figure 5a.

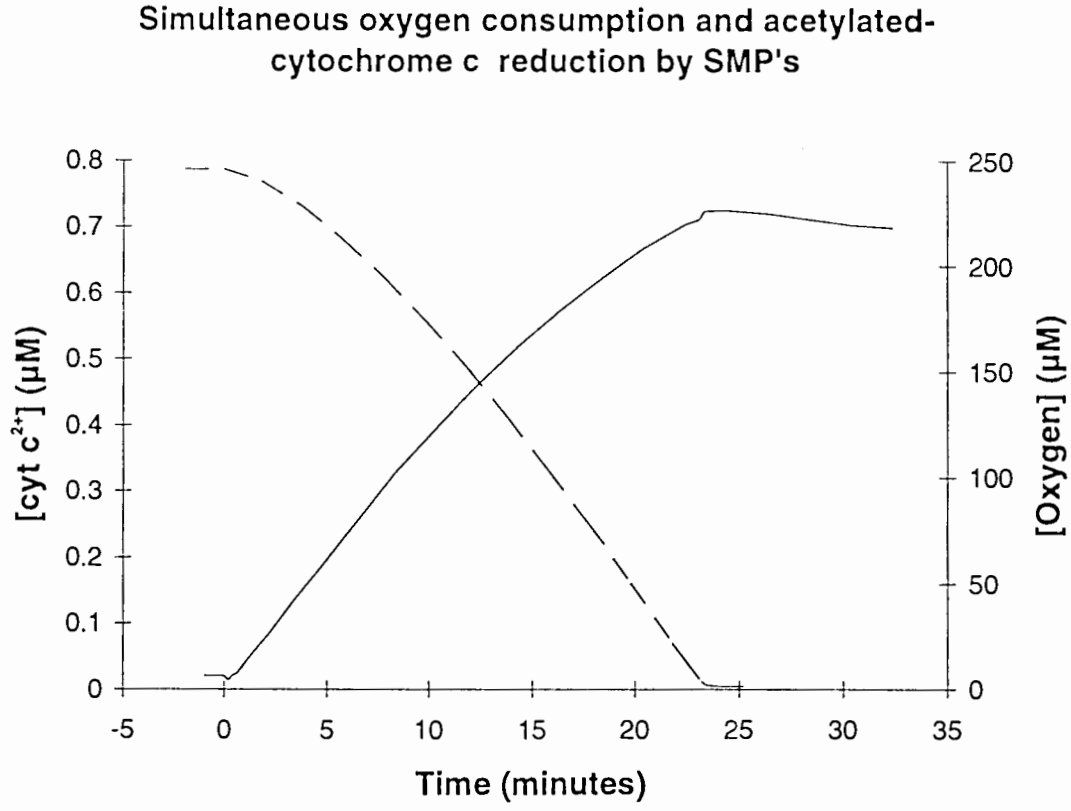


Figure 5b.

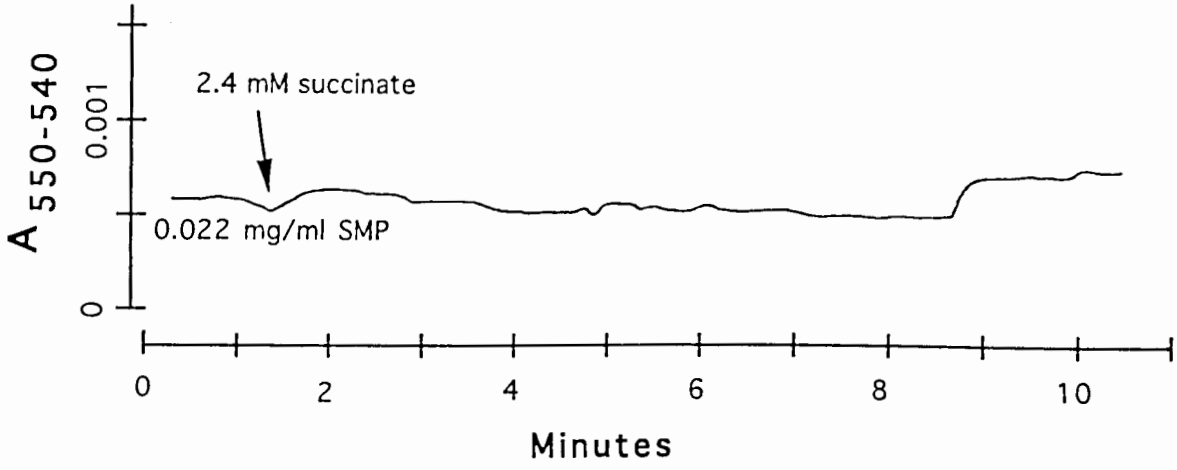


Figure 6.

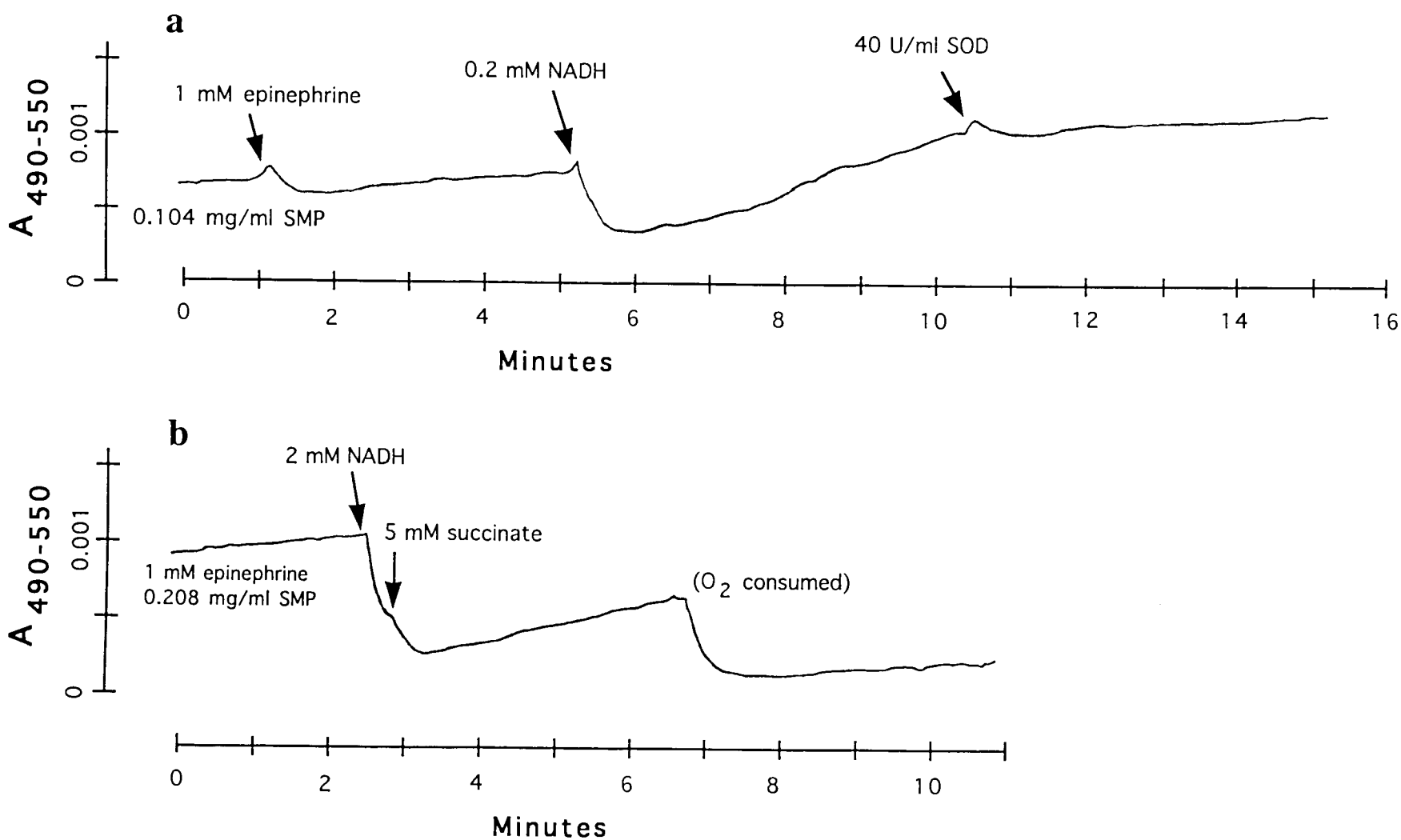

a
A 490-550
0.001
0
1 mM epinephrine
0.2 mM NADH
40 U/ml SOD
0.104 mg/ml SMP
0
2
4
6
8
10
12
14
16
Minutes
b
A 490-550
0.001
0
2 mM NADH
5 mM succinate
1 mM epinephrine
0.208 mg/ml SMP
(O2 consumed)
0
2
4
6
8
10
Minutes

Figure 7.

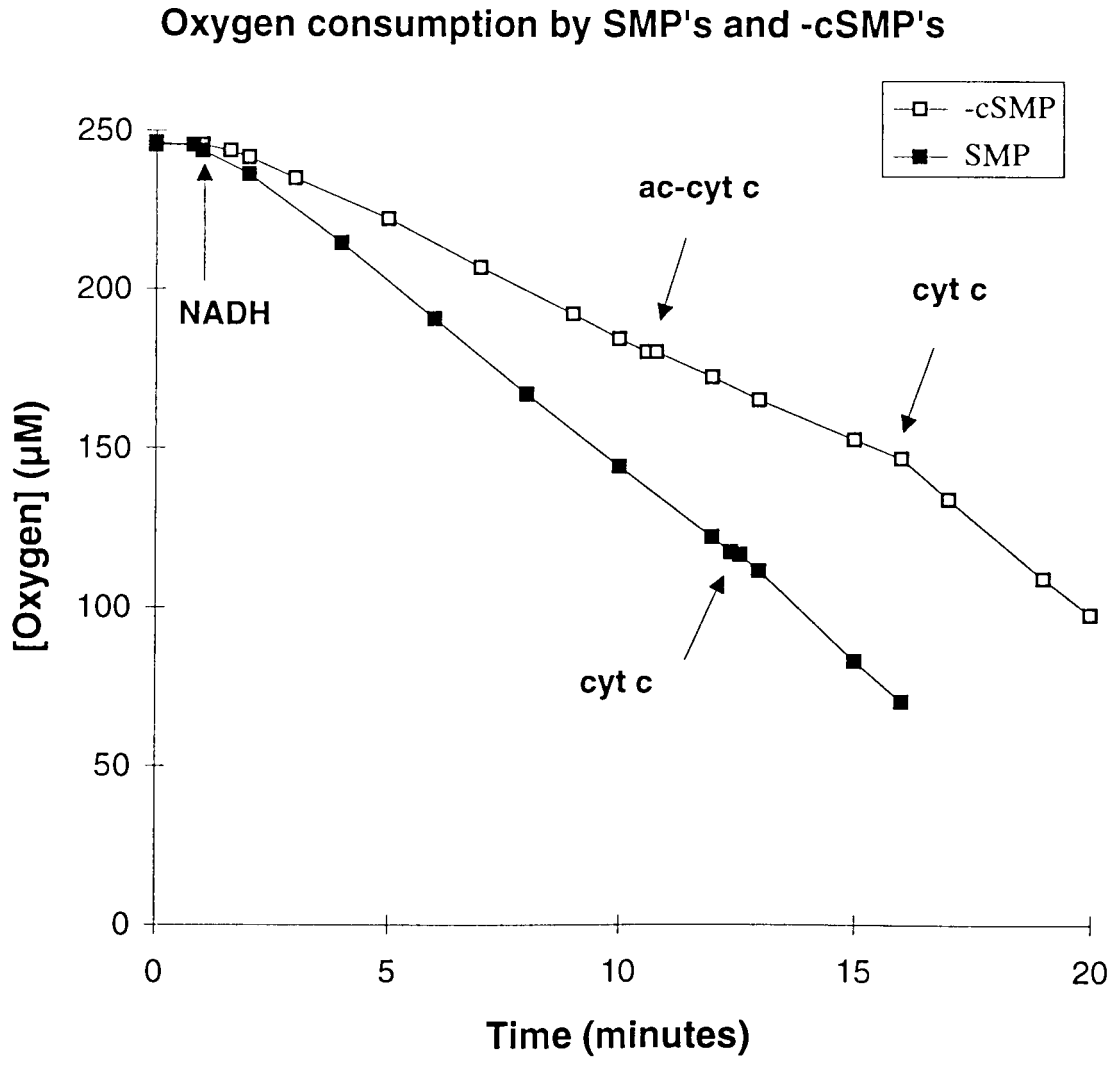

Oxygen consumption by SMP's and -cSMP's
-cSMP
SMP
ac-cyt c
cyt c
NADH
cyt c
[Oxygen] (µM)
250
200
150
100
50
0
0
5
10
15
20
Time (minutes)

Figure 8.

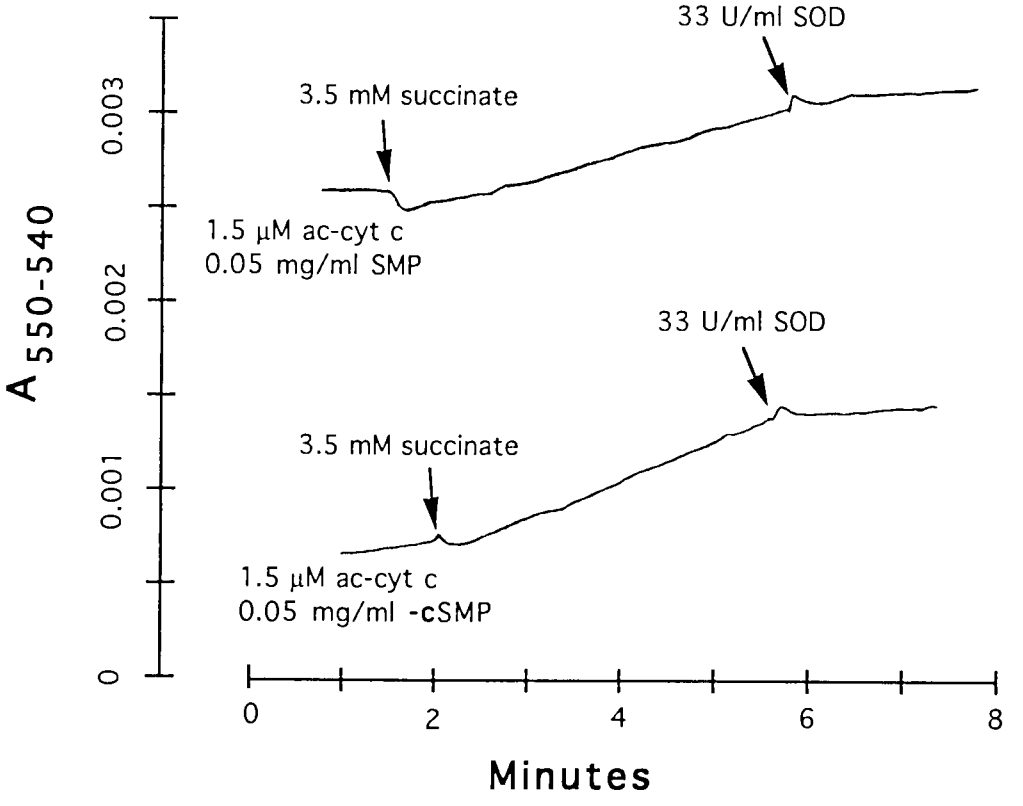

33 U/ml SOD
3.5 mM succinate
1.5 μM ac-cyt c
0.05 mg/ml SMP
33 U/ml SOD
3.5 mM succinate
1.5 μM ac-cyt c
0.05 mg/ml -cSMP
A 550-540
0
0.001
0.002
0.003
0
2
4
6
8
Minutes

Figure 9a.

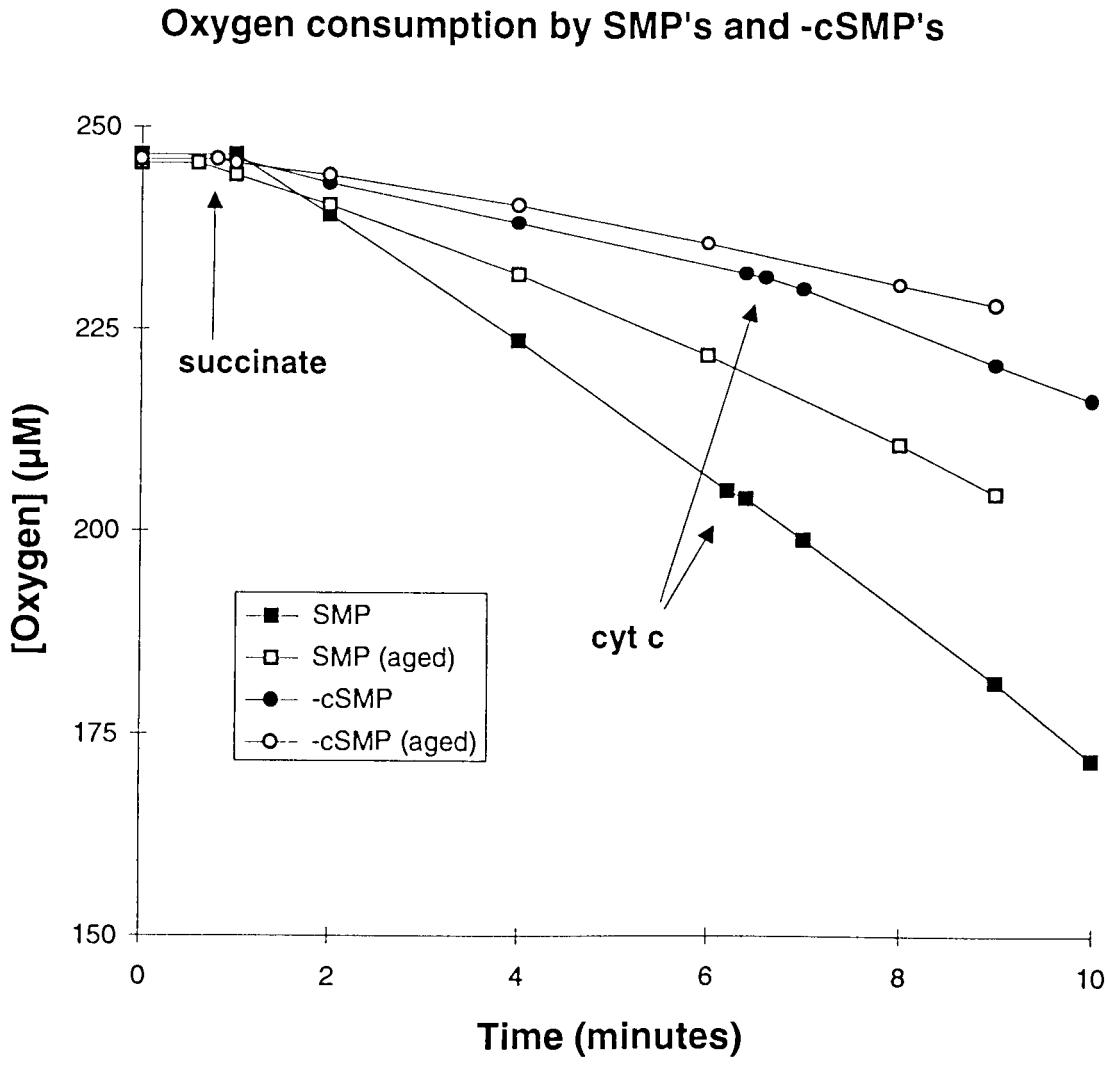

Oxygen consumption by SMP's and -cSMP's
250
225
200
175
150
[Oxygen] (µM)
0
2
4
6
8
10
Time (minutes)
succinate
cyt c
SMP
SMP (aged)
-cSMP
-cSMP (aged)

Figure 9b.

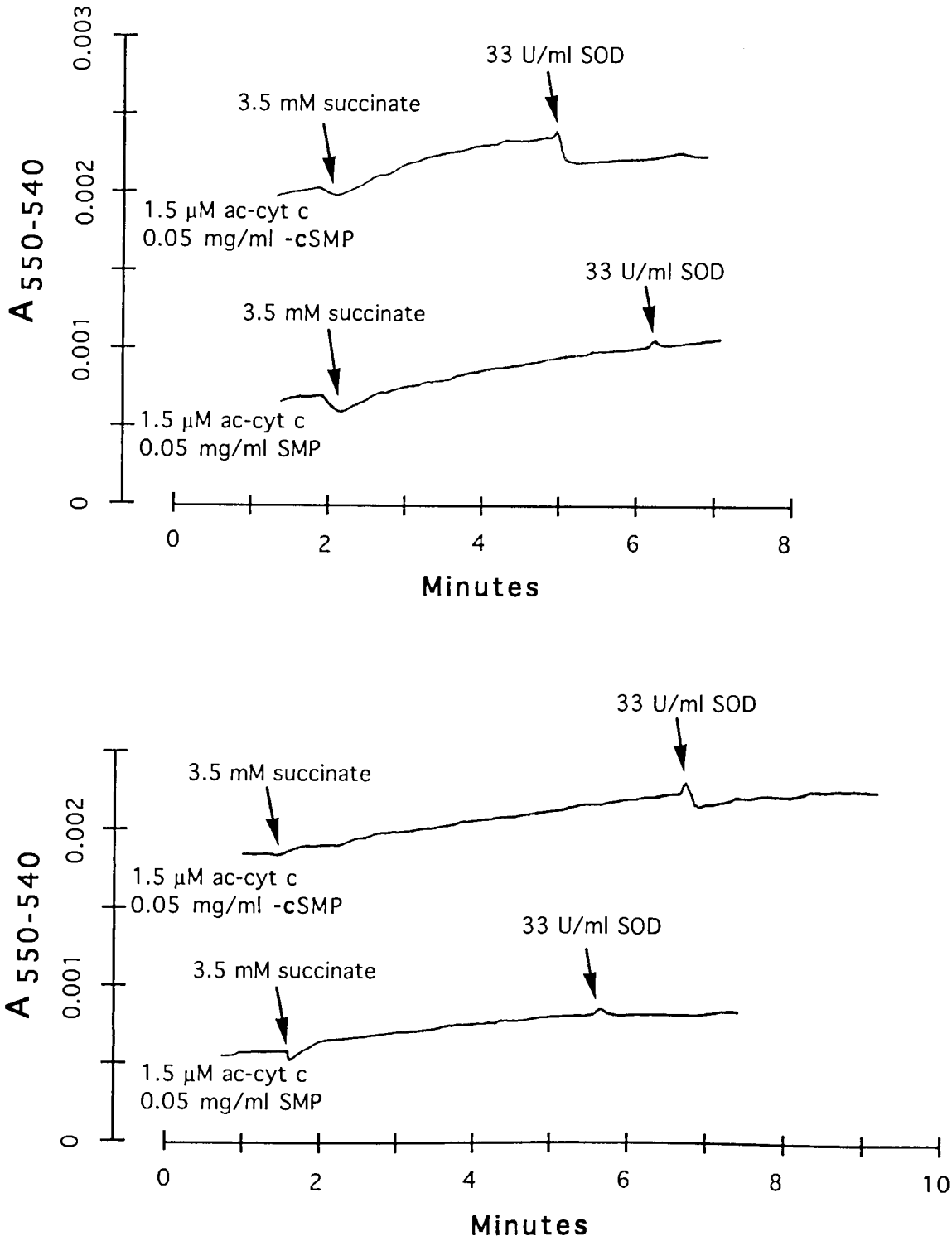

A 550-540
0.003
0.002
0.001
0
33 U/ml SOD
3.5 mM succinate
1.5 µM ac-cyt c
0.05 mg/ml -cSMP
33 U/ml SOD
3.5 mM succinate
1.5 µM ac-cyt c
0.05 mg/ml SMP
0
2
4
6
8
Minutes
A 550-540
0.002
0.001
0
33 U/ml SOD
3.5 mM succinate
1.5 µM ac-cyt c
0.05 mg/ml -cSMP
33 U/ml SOD
3.5 mM succinate
1.5 µM ac-cyt c
0.05 mg/ml SMP
0
2
4
6
8
10
Minutes

Figure 10.

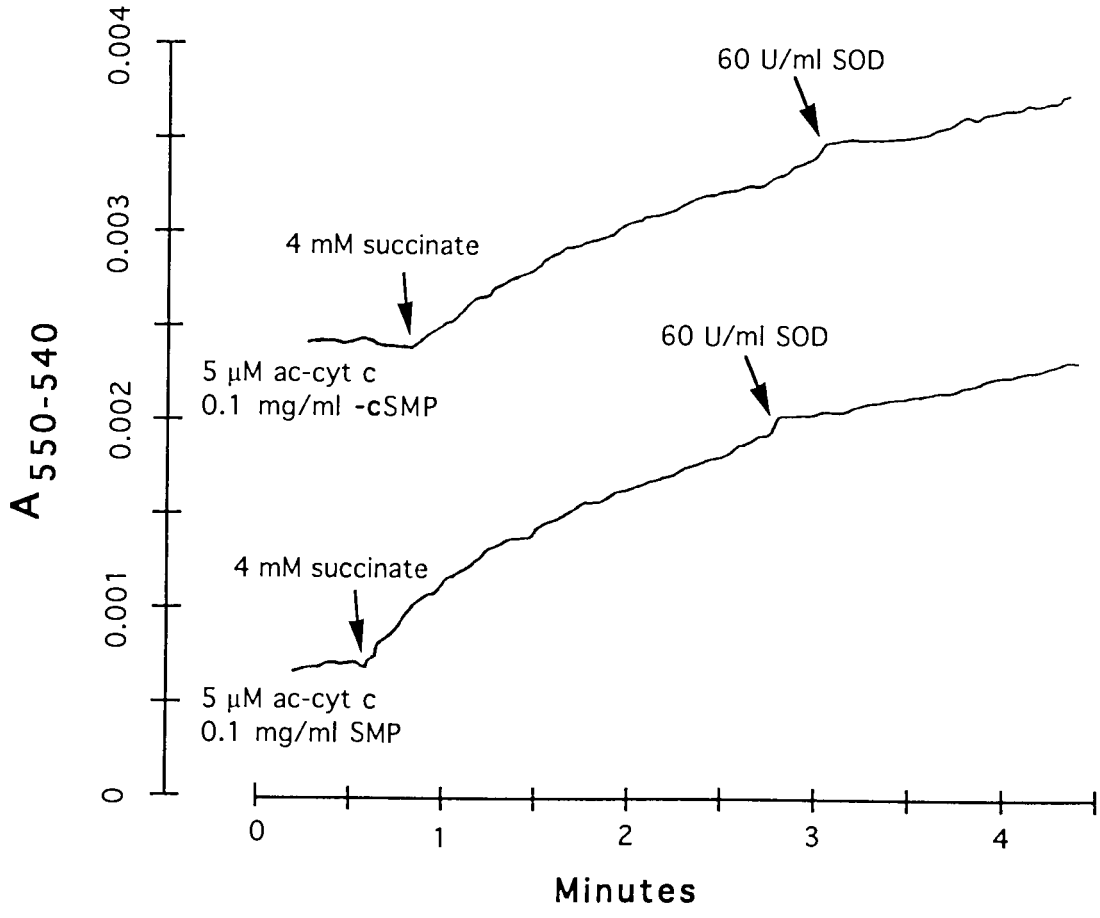

60 U/ml SOD
4 mM succinate
5 µM ac-cyt c
0.1 mg/ml -cSMP
60 U/ml SOD
4 mM succinate
5 µM ac-cyt c
0.1 mg/ml SMP
A550-540
0.004
0.003
0.002
0.001
0
0
1
2
3
4
Minutes

Figure 11.

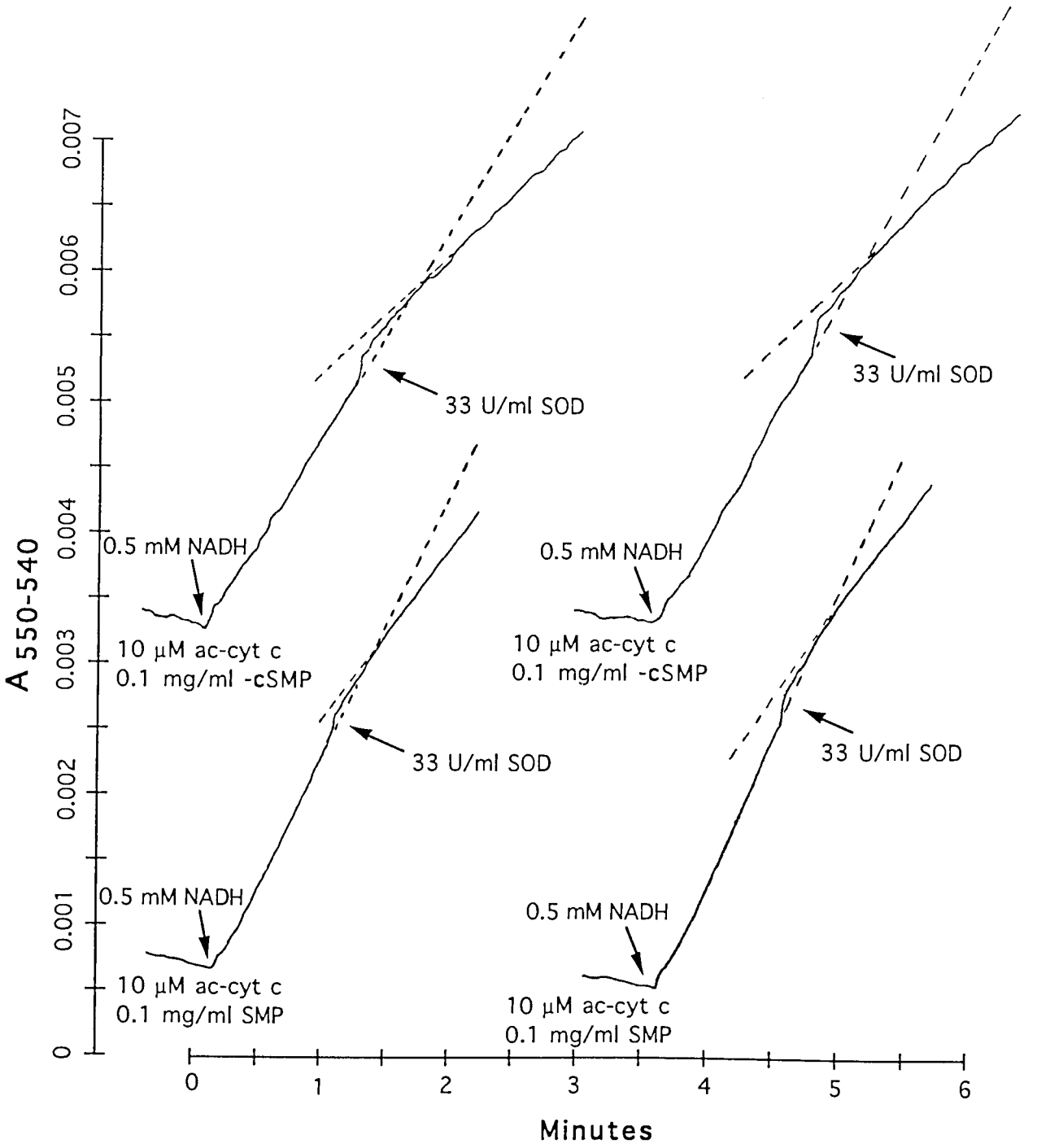

A 550-540
0
0.001
0.002
0.003
0.004
0.005
0.006
0.007
0.5 mM NADH
10 µM ac-cyt c
0.1 mg/ml -cSMP
33 U/ml SOD
0.5 mM NADH
10 µM ac-cyt c
0.1 mg/ml -cSMP
33 U/ml SOD
0.5 mM NADH
10 µM ac-cyt c
0.1 mg/ml SMP
33 U/ml SOD
0.5 mM NADH
10 µM ac-cyt c
0.1 mg/ml SMP
33 U/ml SOD
0
1
2
3
4
5
6
Minutes

Figure 12.

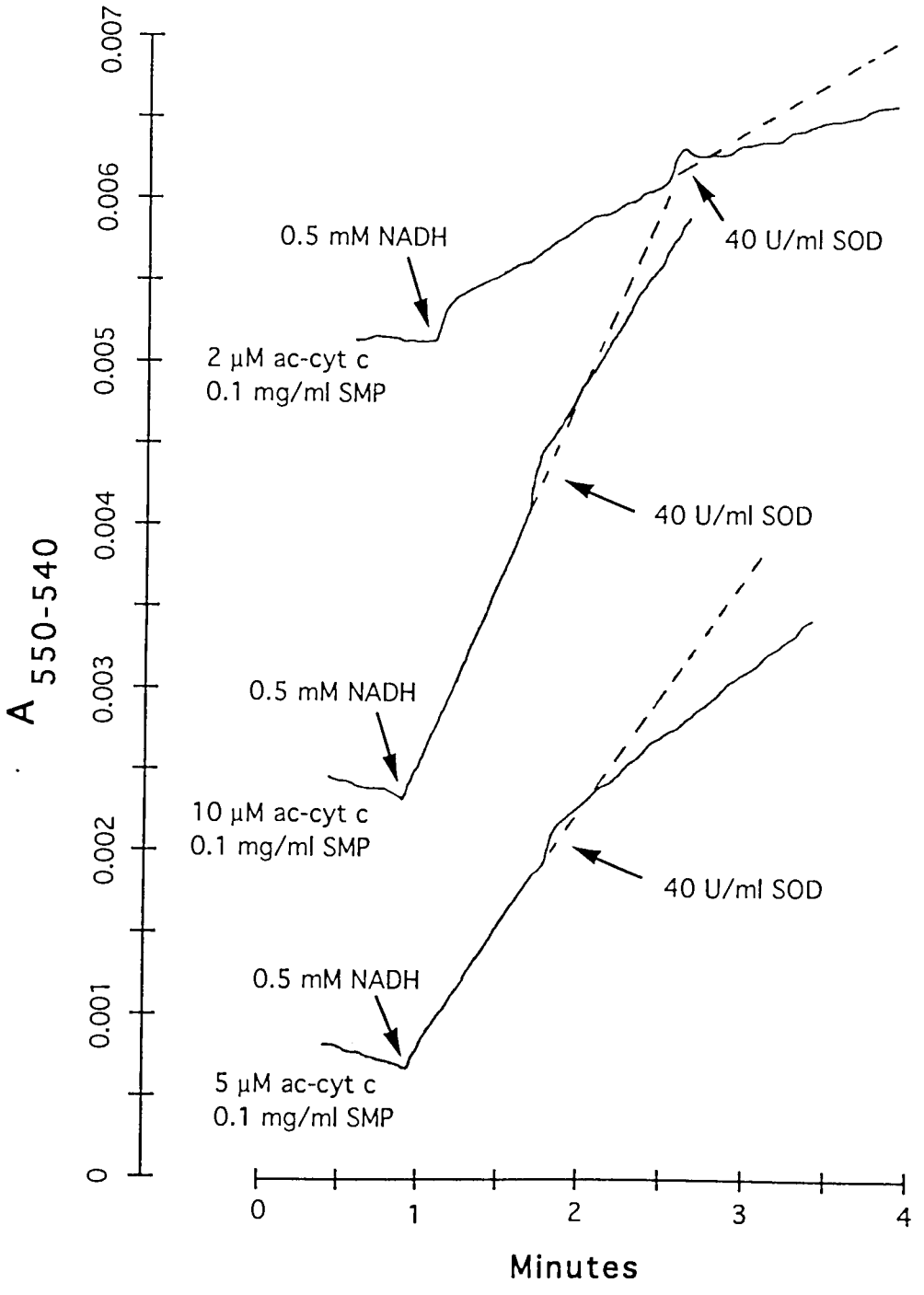
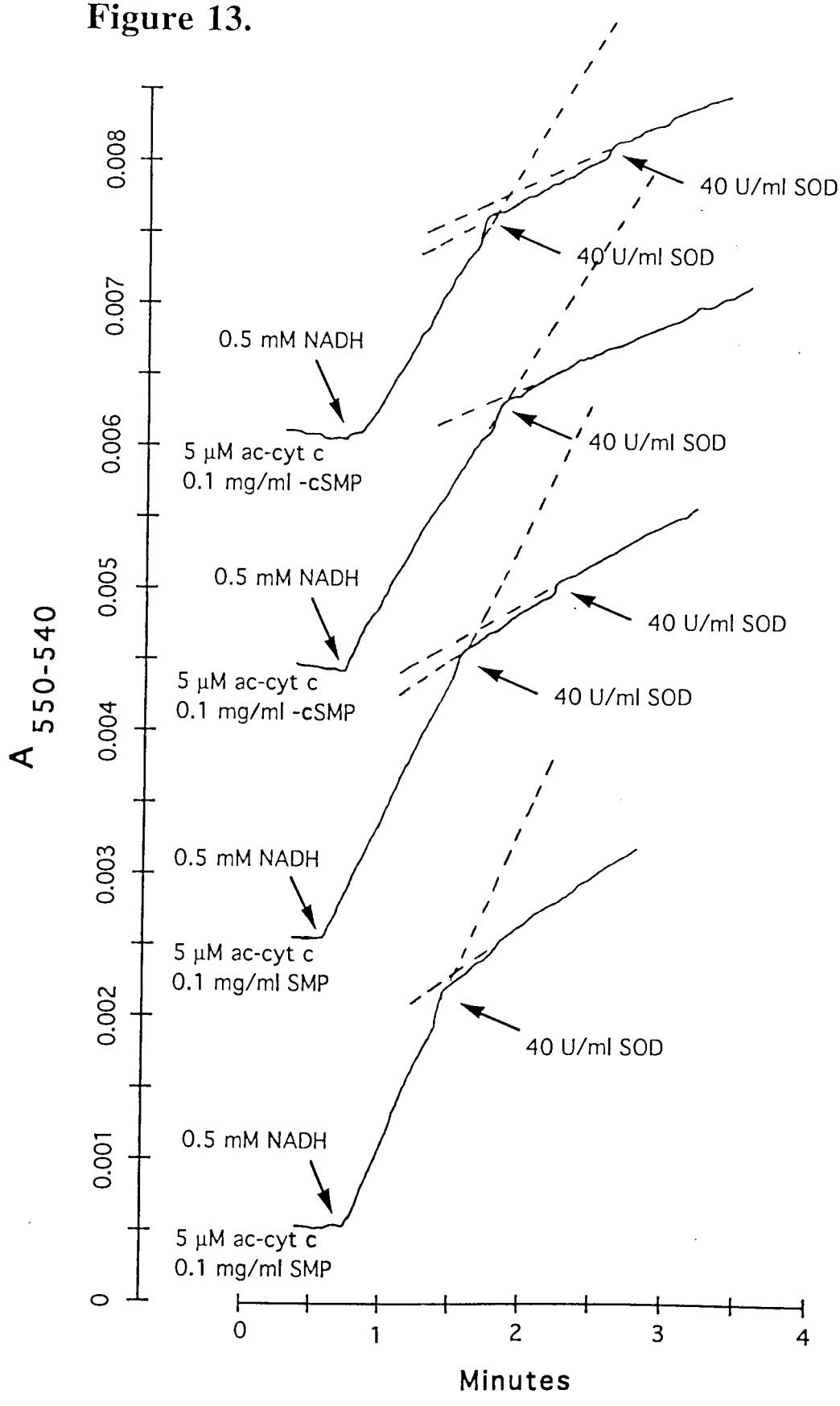

0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
A 550-540
0.5 mM NADH
40 U/ml SOD
2 µM ac-cyt c
0.1 mg/ml SMP
40 U/ml SOD
0.5 mM NADH
10 µM ac-cyt c
0.1 mg/ml SMP
40 U/ml SOD
0.5 mM NADH
5 µM ac-cyt c
0.1 mg/ml SMP
0
1
2
3
4
Minutes

Figure 13.


A 550-540
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
0.5 mM NADH
5 µM ac-cyt c
0.1 mg/ml -cSMP
40 U/ml SOD
40 U/ml SOD
0.5 mM NADH
5 µM ac-cyt c
0.1 mg/ml -cSMP
40 U/ml SOD
40 U/ml SOD
0.5 mM NADH
5 µM ac-cyt c
0.1 mg/ml SMP
40 U/ml SOD
0.5 mM NADH
5 µM ac-cyt c
0.1 mg/ml SMP
0
1
2
3
4
Minutes

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Chapter 3.

Hypothesis Paper

MITOCHONDRIAL MUTATIONS MAY INCREASE OXIDATIVE STRESS: IMPLICATIONS FOR CARCINOGENESIS AND AGING?

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Abstract—The sensitivity of mitochondrial DNA to damage by mutagens predisposes mitochondria to injury on exposure of cells to genotoxins or oxidative stress. Damage to the mitochondrial genome causing mutations or loss of mitochondrial gene products, or to some nuclear genes encoding mitochondrial membrane proteins, may accelerate release of reactive species of oxygen. Such aberrant mitochondria may contribute to cellular aging and promotion of cancer.

Keywords—Mitochondrial mutations, Oxidative stress, Carcinogenesis, Cellular aging, Mitochondrial respiration, Superoxide, Lipid peroxidation, Intracellular calcium, Free radicals

INTRODUCTION

Not all mitochondrial abnormalities in cancer cells result from ischemia

Mitochondrial abnormalities in cancer cells are many. These abnormalities include[1,2]:

1. changes in ultrastucture;
2. deficiencies in energy-linked functions (such as ADP-stimulated state III respiration);
3. increased state IV (ADP-limited) respiration;
4. loss of ability to undergo swelling and ATP-induced contraction cycles;
5. disturbed Ca^{2+} or K^+ transport;
6. impaired protein synthesis, and
7. loss of electron transport components.

Warburg wrote[3]: "Cancer arises because lack of oxygen, or respiratory enzymes, produces fermentation in the body cells and leads to a destruction of the differentiation of these cells." For many years cancer research focused on the loss of respiratory metabolism common to tumor cells. Tumor cells are not always grossly impaired in respiration however, and the connections between mitochondrial function or dysfunction and neoplasia (e.g., activation of nuclear oncogenes) are not obvious. Mitochondrial aberrations may be attributed to the poor perfusion and anoxic state of many tumors. Mitochondrial aberrations in cancer cells which are not ischemic however, such as the leukemias,[4] or ascites, or cultured tumor cells,[cf. 1] require other explanations. Recent reports that mitochondrial DNA is highly sensitive to mutagens and that cytoplasm transfer supresses tumorigenicity, has prompted suggestions of a more direct role of mitochondria in transformation.[2,5–11] Impaired calcium regulation,[2,8] or incorporation of mitochondrial DNA fragments into the nuclear genome[9,11] are two suggested mechanisms by which mitochondria might contribute to neoplastic transformation.

Release of active oxygen by mitochondria may promote transformation

The possibility that mitochondria contribute to tumorigenesis as an intracellular source of excited oxygen deserves consideration. Reactive species of oxygen can participate in the initiation or promotion of cancer through their ability to cause point mutations, DNA cross-links, and DNA strand breaks in nuclear chromosomes. Such injuries may activate an oncogene or inactivate a tumor-suppressor gene. Mitochondria consume over 90% of the oxygen used by most cells and are a significant source of active oxygen under normal conditions. Any condition which increases this generation, or decreases protective systems, will likely

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have pathological consequences; both to the mitochondria and the cell. The toxicity and carcinogenicity of the dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, for example, may be related to generation of $O_2^{\cdot -}$ and H_2O_2 in mitochondria.[12]

Oxidative stress and intracellular redox balance can also influence cellular differentiation and proliferation through effects on intracellular messengers.[13-16] The ability of respiratory stress to influence expression of the nuclear genome is evident in the appearance of chromosome "puffs" in certain regions (e.g., heat shock genes) on exposure to respiratory poisons (e.g., antimycin, rotenone, menadione, dintrophenol), hyperoxia, or intermittent anoxia.[17-21]

Release of active oxygen by mitochondria can contribute to aging

The free radical theory of aging receives wide support, and the contributions of mitochondria to this process are widely accepted.[22-32] Maximum life span potential relates inversely to the rate of oxygen metabolism and positively to antioxidant capacity in different mammalian species or insects.[30-33] Oxidized lipid pigments (e.g., lipofuscin) accumulate in aged cells and at least partly represent peroxidized mitochondria.[34,35] Other common observations in aged cells include fewer mitochondria, increased mitochondrial structural abnormalities, losses in mitochondrial enzyme activities, and decreased respiratory control.[23,36] Moreover, mitochondria from older rats show higher levels of lipid peroxides, losses of polyunsaturated fatty acids, and correspondingly increased oxygen radical generation.[23,37,38] In houseflies, rates of mitochondrial generation of superoxide are inversely associated with life expectancy, and increase with age.[39]

Aged cells also show losses or injury of mitochondrial DNA.[24,40] For example, deletions and insertions in mitochondrial DNA of mouse liver accumulate with age.[41] Thus, in addition to sustaining damage to proteins and lipids from oxygen radicals generated during respiration, mitochondria lose ability to regenerate functional mitochondria. Decreased ability of mitochondria to replicate in terminally differentiated cells leads to age-related declines in functionally intact mitochondria and ATP production. Cellular aging is conceivably caused by intrinsic injury to the mitochondrial genome from oxygen radicals generated during normal mitochondrial respiration, with consequent loss of respiratory capacity.[24,28,40] This loss depends on imbalance between mitochondrial turnover and oxygen radical generation in terminally differentiated cells (which have the highest respiratory rates and slowest mitochondrial turnover). We elaborate that injury to the mitochondrial genome may *increase* generation of oxygen radicals, which then more readily overcome protective systems than the O_2 radicals generated in normal respiration.

Hypothesis

We suggest that some mutations to the mitochondrial genome (or to certain nuclear genes) produce aberrant mitochondria which reduce oxygen to reactive intermediates. Such mutations give a mechanism for mitochondria to contribute to cancer and aging. Substantiation of this hypothesis requires: 1) evidence that mitochondrial mutations influence release of active oxygen, and 2) evidence that mitochondrial generation of active oxygen can contribute to neoplastic transformation and aging. In this manuscript we: (i) review evidence implicating mitochondrial mutations in carcinogenesis, (ii) outline mechanisms by which mitochondrial mutations predispose to release of active oxygen, and (iii) review evidence implicating mitochondrial release of active oxygen in cancer and aging.

Mitochondria are vulnerable to mutagens

Mitochondrial DNA is susceptible to damage by genotoxins for a variety of reasons.[2-10] Reasons for this vulnerability include the lack of protective histones or nonhistone proteins ("naked" DNA), limited DNA repair mechanisms, low replication fidelity, the presence of respiratory enzymes which can activate chemical carcinogens, and the sensitive supercoiled structure of the DNA. The high lipid content and lipid-to-DNA ratio make mitochondria particularly susceptible to lipophilic chemicals. Attachment of mitochondrial DNA to the inner membrane makes it sensitive to membrane disturbances and a prime target for electrophiles generated within the membrane (e.g., peroxides, epoxides, nitroxides, semiquinones).

Several studies on exposure of cells or animals to carcinogens report accumulation of the carcinogen in mitochondria and selective modification of mitochondrial DNA. For example, carcinogenic polycyclic aromatic hydrocarbons,[42-44] azo dyes,[45] and nitrosamines[46] accumulate preferentially in the mitochondria of animal cells. In addition, polycyclic aromatic compounds of differing carcinogenicity bind to the mitochondrial DNA of cultured mouse embryo cells 50 to more than 500 times more readily than to nuclear DNA.[44] The lipophilic inner and outer mitochondrial membranes contain the enzymes to metabolize these compounds to their active forms. Similarly a carcinogenic dihydrodiol-epoxide derivative of benzo(a)pyrene covalently modifies mitochondrial DNA 40

to 90 times more extensively than nuclear DNA in mammalian cell cultures.[47] Also, treatment of rats and hamsters with *N*-(^{14}C)methyl-*N*-nitrosourea[43] or (^{14}C)dimethylnitrosamine[48] methylates mitochondrial DNA up to five times more heavily than nuclear DNA. Cyclophosphamide alkylates mitochondrial DNA in rat liver over 100 times more readily than nuclear DNA.[et al. 7] Moreover, dimethylnitrosamine decreases mitochondrial DNA which can be isolated in the closed circular form and correspondingly increases open circular or linear forms which result from single-strand breaks in the normally supercoiled circular DNA.[49] *N'*-nitro-*N*-nitrosoguanidine and 4-nitroquinoline 1-oxide also cause strand scission of mitochondrial DNA in animal cells, with formation of open circular forms.[50] This damage was not repaired during 20 h of posttreatment incubation. Aflotoxin B_1 administered to experimental animals binds covalently to liver mitochondrial DNA at concentrations three to four times higher than to nuclear DNA.[51] The adducts remained unrepaired even after 24 h, inhibiting mitochondrial transcription and translation. Chromium is reduced by the mitochondrial respiratory chain and preferentially forms adducts in mitochondrial DNA.[52,53] Carbon tetrachloride is activated by rat liver mitochondria,[54] and binds covalently to mitochondrial DNA 20–50 times more extensively than to nuclear DNA in livers of poisoned rats.[55] In addition, lipid peroxidation of mitochondrial membranes or increased oxidative stress damages mitochondrial DNA.[56,57]

The mitochondrial genome is thus particularly susceptible to mutations. The spontaneous frequency of mitochondrial petite mutations in yeasts is more than one thousand times the nuclear mutation rate.[58] In mammalian cells, mitochondrial DNA fixes mutations 5 to 10 times faster than nuclear DNA, consistent with the faster evolution of mitochondrial DNA.[59,60] Comparing mutations to specific genes, mitochondrial genes sustained mutations at 17 times the rate of a nuclear encoded subunit of ATP synthase.[60] Similarly, oxidative damage to mitochondrial DNA occurs at 16 times the level in nuclear DNA.[57]

Consistent with their supposed procaryotic origins, mitochondria are also sensitive to viral infection. Viral replicates are often found in the mitochondria after infection of cells with oncogenic viruses (see ref 1 for a summary of different studies). For example, replicates of Rous sarcoma virus were found to a high titre within mitochondria of infected cells.[61] Viral transformation can also elevate synthesis of mitochondrial DNA, RNA, or protein. For example, levels of mitochondrial mRNA increased in rat fibroblasts transformed with polyoma virus DNA or immortalized with the cellular *myc*, adenovirus E1A, or polyoma *plt* oncogenes.[62] Thus, oncogenic viruses could conceivably use mitochondria as carriers.[7] In doing so, the virus can disrupt mitochondrial gene expression.

Are mitochondrial mutations involved in neoplastic transformation? Some evidence is supportive

Although the mechanisms remain speculative, there is growing evidence of a relationship between mitochondrial mutations and cancer (reviewed in refs[2-10]). We summarize here some of the supporting evidence and proposed models.

1. *Mitochondrial DNA mutations or abnormalities propagate in transformed cells*

For example, circular dimer and catenated forms of mitochondrial DNA occur in leukocytes from all patients tested with granulocytic leukemia but not in mitochondria from normal leukocytes.[4] Restriction patterns were abnormal in mitochondrial DNA from leukemic leukocytes in 12 of 14 cases.[63] Another study on mitochondrial DNA from colon and brain tumors, however, did not reveal any abnormal restriction patterns.[64] Circular dimers of mitochondrial DNA appear in various other transformed cells and human solid tumors[e.g.,65] (reviewed in[1]). DNA sequences also differ in regions of the mitochondrial genome in some tumor cells compared with normal cells.[e.g.,66,67]

2. *Chemical carginogens accelerate mitochondrial mutations*

In yeast, various carginogens increase the frequency of petite mutations.[6-8] Such mutants result from deletions (*rho*$^-$ or *rho*^{0}) or mutations (*mit*$^-$ or *syn*$^-$) in the mitochondrial genome which produce respiration deficient mitochondria (see[68] for a review). Petite mutants share many properties of neoplastic cells, such as decreased respiratory capacity and increased glycolysis. Mutagens, many of which are known carcinogens, also produce heritable mitochondrial mutations in mammalian cell lines (reviewed in[10]). For example, mitochondrial mutants resistant to inhibitors of mitochondrial respiration, oxidative phosphorylation, or protein synthesis can be selected in mammalian cells exposed to mutagens.[69-75]

3. *Mitochondrial mutations produce changes in the cell surface*

Such mitochondrial lesions lead to changes in the yeast cell surface, reflected in reversal of flocculating

(clumping) cultures, increased cell agglutination by concanavalin A, decreased permeability to chlorimiprimine and cycloheximide, decreased uptake of some fermentable sugars such as galactose and maltose, and modifications in the pattern of plasma membrane proteins.[8] Mitochondrial mutations also modify expression of cell surface antigens in mouse cells.[76,77] Mitochondrial genetic lesions thus apparently influence the expression of nuclear genes involved in biogenesis of the plasma membrane and cell surface. These findings suggested a model for carcinogenesis in which mitochondrial DNA mutations alter expression of the mitochondrial inner membrane and disrupt regulatory influences of the mitochondria on nuclear expression of cell surface components (perhaps by an effect on calcium regulation).[2,8] Cell division and segregation of mitochondrial genotypes would eventually lead to neoplastic transformation.[2,8] Along with changes leading to unrestricted cell growth, mitochondrial mutation-induced changes to the cell surface may allow descendent cells to collaborate to enhance survival and to compete with normal cells for resources.[78]

4. *Mathematical models of carcinogenesis support extrachromasomal inheritance of some contributing events*

In one mathematical model, alteration of the mitochondrial genome leads to accumulation of the lesion in selected daughter cells through a stochastic distribution of pathological mitochondria during cellular and mitochondrial divisions.[7] Replacement of normal mitochondria by pathological mitochondria eventually leads in some way to neoplastic transformation.[7]

5. *Mitochondrial contributions can explain the latent period of cancer*

A mechanism involving oncogenes alone does not explain well the "lag time," or promotional phase of cancer.[10] The time for mitochondrial mutations to propagate better explains this phase.[10] A model in which some mitochondrial mutations provide a growth advantage to the cell when accumulated under selective environmental conditions (analogous to the increase in chloramphenicol-resistant mitochondria that occurs in chloramphenicol selection after mutation) is consistent with the need for a latent period.[10]

6. *Fusion of cancer cells with cytoplasts from nontumorigenic cells suppresses the tumorigenic phenotype*

Hybrid fusion of normal fibroblasts with tumor cells often suppresses tumorigenicity, despite full expression of activated oncogenes.[79–84] Moreover, cybrid fusion of tumor cells with cytoplasts from normal cells can suppress the tumor phenotype.[85–94] For example, cybrid transfer of mitochondrial DNA (mtDNA) from relatively normal HT1080 cells to HeLa cells retards cell growth (the converse was not true).[95] The HT1080 mtDNA codes for a chloramphenicol-sensitive, growth-retarding, phenotype that HeLa mtDNA lacks. An abnormal mitochondrial protein from HeLa was identified and localized to a specific mitochondrial gene (ND3). Conversely, cytoplasts from transformed cells reportedly immortalized B and T lymphocytes.[96] Cybrid transfer experiments thus suggest that in some cancer cells mutations to mitochondrial DNA change cell physiology to induce growth.[10,95] One model suggests that mitochondria produce a factor important for suppression of tumorigenicity which is lost when deleterious mitochondrial mutations cause a decline in mitochondria.[10]

7. *Rearranged and normal segments of mitochondrial DNA appear in nuclear genomes of various cell species*[97–102]

Mitochondrial DNA sequences can apparently enter nuclear DNA during mitochondrial breakdown or during normal cycles of organelle fusion/segregation.[9] Incorporated mitochondrial plasmids might then provoke activation of oncogenes.[9] For example, such plasmids could lead to control of an oncogene by a new promoter by encouraging "breakpoints" and somatic rearrangements or by inserting a mtDNA replication or transcription origin.[9] The observation of 16 times more oxidized bases (8-hydroxydeoxygaunosine) in mitochondrial DNA than in nuclear DNA[57] suggests that oxidative stress may cause appreciable strand scission and fragmentation of mitochondrial DNA.[11] Oxidatively generated fragments of mitochondrial DNA released from mitochondria might then incorporate into nuclear DNA to activate oncogenes or inactivate growth control genes.[11]

MITOCHONDRIAL MUTATIONS MAY INCREASE GENERATION OF ACTIVE OXYGEN

The potential for involvement of mitochondria in carcinogenesis is apparent, but what mechanism might link mitochondrial genome damage to neoplastic transformation is less clear. Disrupted calcium regulation or transfer of mitochondrial DNA fragments to the nucleus provide potential mechanisms. We now outline a mechanism by which minor damage to mitochondrial genes might amplify damage to the nuclear genome and produce changes in cellular phenotype.

Mitochondrially coded components minimize autoxidation of respiratory intermediates

Most injuries to the mitochondrial genome can predispose to increased generation of reactive species of oxygen. This predisposition occurs because the components of the respiratory chain coded for by mitochondrial genes remove reduced intermediates. The mitochondrial genome codes for cytochrome *b*, three large hydrophobic subunits of cytochrome oxidase, two subunits of the F_0F_1-ATPase, two rRNA components of the mitochondrial ribosomes, all mitochondrial tRNAs (see[68,103-106] for reviews), and, in mammals but not yeasts, seven hydrophobic subunits of NADH dehydrogenase.[107,108] Damage to the mitochondrial genome thus results in impaired function of cytochrome oxidase, ubiquinol:cytochrome *c* oxidoreductase (contains cytochrome *b*), the F_0F_1-ATPase complex, or NADH dehydrogenase. The loss of absorption bands for cytochrome *b* and cytochrome oxidase in yeast cells exposed to chemical carcinogens[8] is consistent with this prediction. The mitochondrial respiratory chain normally releases small amounts of $O_2\cdot^-$ and H_2O_2 through autoxidation of one or more reduced species of flavin, iron-sulphur complex, and ubiquinone[a] generated by succinate, NADH, and other ubiquinone reducing dehydrogenases (see[109] for a review).

Many of the mitochondrial components which produce autoxidizable intermediates are coded extramitochondrially

Except for some subunits of NADH dehydrogenase (in mammalian cells), flavoprotein dehydrogenases are synthesized extramitochondrially and thus not necessarily lost with mitochondrial mutations.[118] For example, *rho*$^-$ or *rho*^{0} mutants of yeasts, which lack segments or all of the mitochondrial genome, contain reasonably normal levels of flavoprotein dehydrogenases and other nuclear coded enzymes.[119-125] Ubiquinone is also available in normal amounts in petite mutants.[126-127] Components of the respiratory chain which contain mitochondrially synthesized subunits bear responsibility for oxidizing the reduced flavins (indirectly), ubisemiquinone and ubiquinol, and for reducing oxygen safely to water. Damage to the mitochondrial genome which mutates or deletes any of the cytochrome components should increase the steady-state concentration of these reduced intermediates and so increase free radical products of their autoxidation. In evidence of this supposition, inhibition of cytochrome oxidase (CN^-) or of cytochrome *b* oxidation (antimycin) stimulates generation of H_2O_2 and $O_2\cdot^-$ by mitochondria or submitochondrial particles.[112,117,125,128,129] Mutant mitochondria deficient in normal respiration may thus release abnormally high levels of reactive oxygen species and lipid peroxidation by-products to the cytosol and expose the nucleus and other cellular components to cytotoxic species. This situation gives a mechanism for mitochondrial mutations to promote cellular aging and cancer.

[a]Autoxidation of ubisemiquinone intermediates are likely a major source,[109-115] although autoxidation of cytochrome b_{566} may also be possible.[116] Flavin semiquinone or iron-sulphur centers are likely the major source of $O_2\cdot^-$ and H_2O_2 with isolated NADH dehydrogenase.[117]

SMALL CHANGES IN THE MITOCHONDRIAL GENOME MAY HAVE SEVERE CONSEQUENCES

The mammalian mitochondrial genome is highly organized. This genome has one origin of replication, one promoter for transcription, and heavy strand sequences coding for mRNAs, rRNAs, and tRNAs immediately contiguous to each other (see[104-106] for reviews). The single large transcript is processed by precise endonucleolytic cleavages before and after the tRNA regions which separate each of the rRNA and mRNA regions. Point mutations in any of the mRNA genes lead to abnormal subunits of the cytochrome oxidase, cytochrome b-c_1, NADH dehydrogenase, or ATPase complexes. Point mutations in the rRNA or tRNA sequences affects synthesis of all these proteins. Single-strand breaks disrupt the integrity of the genome, as evidenced by the formation of circular dimer or catenated forms.[e.g.,49] Even with intervening sequences and multiple promotor regions in their mitochondrial DNA, yeasts lose mitochondrial proteins (cytochromes *b* and *a*) and form petite mutants when exposed to mutagens.[8]

PROLIFERATION AND ASSEMBLY OF MITOCHONDRIA DO NOT REQUIRE AN INTACT MITOCHONDRIAL GENOME

Mitochondria containing a mutated genome continue to replicate, because the enzymes for DNA replication are coded by the nucleus and imported from the cytoplasm (see[103-105,118] for reviews of mitochondrial biogenesis). Membrane lipids, structural proteins and other enzymes needed for proliferation of mitochondria are also produced in the cytoplasm. Cells of *rho*$^-$ mutants of yeast lacking mitochondrial protein synthesis or even *rho*^{0} mutants lacking mitochondrial DNA still produce mitochondria. Although these mitochondria cannot respire normally and are fewer, they still contain flavoprotein dehydrogenases.[119-125] Because mitochondria grow and divide independent of cell division and even of mitochondrial genome

replication,[118,130-132b] mutated mitochondria accumulate in cells and may eventually predominate over normal mitochondria in certain daughter cells of a dividing tissue (as described mathematically in[7]). As demonstrated in yeasts, *rho*$^-$ mitochondria containing a truncated mitochondrial genome or tandem repeats of mitochondrial genes (a frequent occurrence) often gain a selective advantage over normal mitochondrial genomes due to faster replication and transcription.[58,134] Mitochondrial DNA polymorphisms between individuals,[135] mitochondrial myopathies in humans,[e.g.,136,cf.135,137] and mitochondrial inhibitor-resistant mutants in yeast or mammalian cell cultures[e.g.,74] show the somatic heritability of mitochondrial mutations. If these mutated mitochondria release pathological levels of reactive oxygen species, the probability of nuclear genetic damage and damage to other cellular constituents increases.

MITOCHONDRIA PRODUCE REACTIVE SPECIES OF OXYGEN DURING NORMAL OR CYTOCHROME-INHIBITED RESPIRATION

Production of $O_2\cdot^-$ and H_2O_2 by mitocondria accounts for about 1–2% of oxygen uptake under physiological conditions.[112,138] In intact mitochondria, the rate of H_2O_2 generation is higher in state IV (up to 4% of oxygen uptake), where the absence of ADP gives slow oxygen consumption and mostly reduced respiratory chain components. The rate is lower in state III or in the presence of an uncoupler, where oxygen uptake is fast and the respiratory components are mostly oxidized. Antimycin (which blocks oxidation of cytochrome *b*) or CN^- (which blocks cytochrome oxidase) stimulate release of H_2O_2 or $O_2\cdot^-$ in mitochondria or submitochondrial particles due to accumulation and autoxidation of reduced components (flavins and quinones) prior to the block.[c] Mutations to, or loss of any of the cytochromes should have similar effects.

[b]Mitochondria grow in mass by incorporation of new lipids and proteins into existing organelles (rather than by *de novo* formation) and in number by a dynamic fragmentation and fusion process. Mitochondrial DNA replicates throughout the cell cycle. The formation of new mitochondria by growth and fission of existing mitochondria, plus random partition of mitochondria at cell division, allow random changes in gene frequencies and vegetative segregation of alleles.[131]

[c]Addition of CN^- to antimycin-supplemented submitochondrial particles however *decreases* release of $O_2\cdot^-$, presumably due to decreased formation of ubisemiquinone in the presence of both inhibitors (see Q cycle (Fig. 1)).[110,112] Addition of an uncoupler to antimycin-supplemented submitochondrial particles further stimulates release of $O_2\cdot^-$ (probably from an effect of membrane potential on the rate of electron transfer between ubiquinol and cytochrome c_1).[139]

DEFECTIVE FUNCTION OF MITOCHONDRIAL GENE PRODUCTS INCREASES GENERATION OF ACTIVE OXYGEN

At least some heritable mutations to mitochondrial gene products are likely to increase generation of $O_2\cdot^-$ and H_2O_2. The effects of respiratory inhibitors and of respiration state support this premise. We now consider the consequences of impaired function of any of the mitochondrial gene products.

Loss of cytochrome oxidase increases superoxide yield

Cytochrome oxidase allows the electron transport chain to reduce oxygen safely to water. The cytochrome *c*/cytochrome oxidase complex also confers superoxide oxidase activity to mitochondria. (This activity results from the ability of ferricytochrome *c* to react with $O_2\cdot^-$, while cytochrome oxidase regenerates ferricytochrome *c* from the resulting ferrocytochrome *c*.) Alteration or deletion of cytochrome oxidase increases the steady state concentration of oxygen in the membrane and simultaneously accelerates autoxidation of the reduced electron transport components which accumulate.

Loss of cytochrome b increases levels of autoxidizable quinones

Similarly, alteration or deletion of cytochrome *b* impairs oxidation of the reduced species of flavin and ubiquinone by the cytochrome *b* complex and leaves them susceptible to attack by oxygen (or other cytotoxic electrophiles). Apparent from the ubiquinone ("Q") cycle[139-143] (Fig. 1), loss of function of cytochrome *b* could be a dangerous alteration. Other (nuclear encoded) subunits can still oxidize ubquinol to the more autoxidizable ubisemiquinone species. Such an alteration is equivalent to adding antimycin. Absence of cytochrome *b* does not affect incorporation of cytochrome c_1 (a nuclear gene product)[157] or formation of "centre *o*," the myxothiazol binding site.[158] However, absence of cytochrome *b* impairs assembly of the iron-sulphur protein into complex III[159,160] and thus might avoid ubisemiquinone formation at this site. A mutation to the cytochrome *b* gene (i.e., *mit*$^-$) which impairs the ability of the product to transfer electrons, but does not affect assembly of complex III however, would not avoid ubisemiquinone formation. Mitochondrial antibiotic-resistance (*ant*^{R}) mutations which lower affinity for an antibiotic such as antimycin or oligomycin but do not otherwise alter function of the

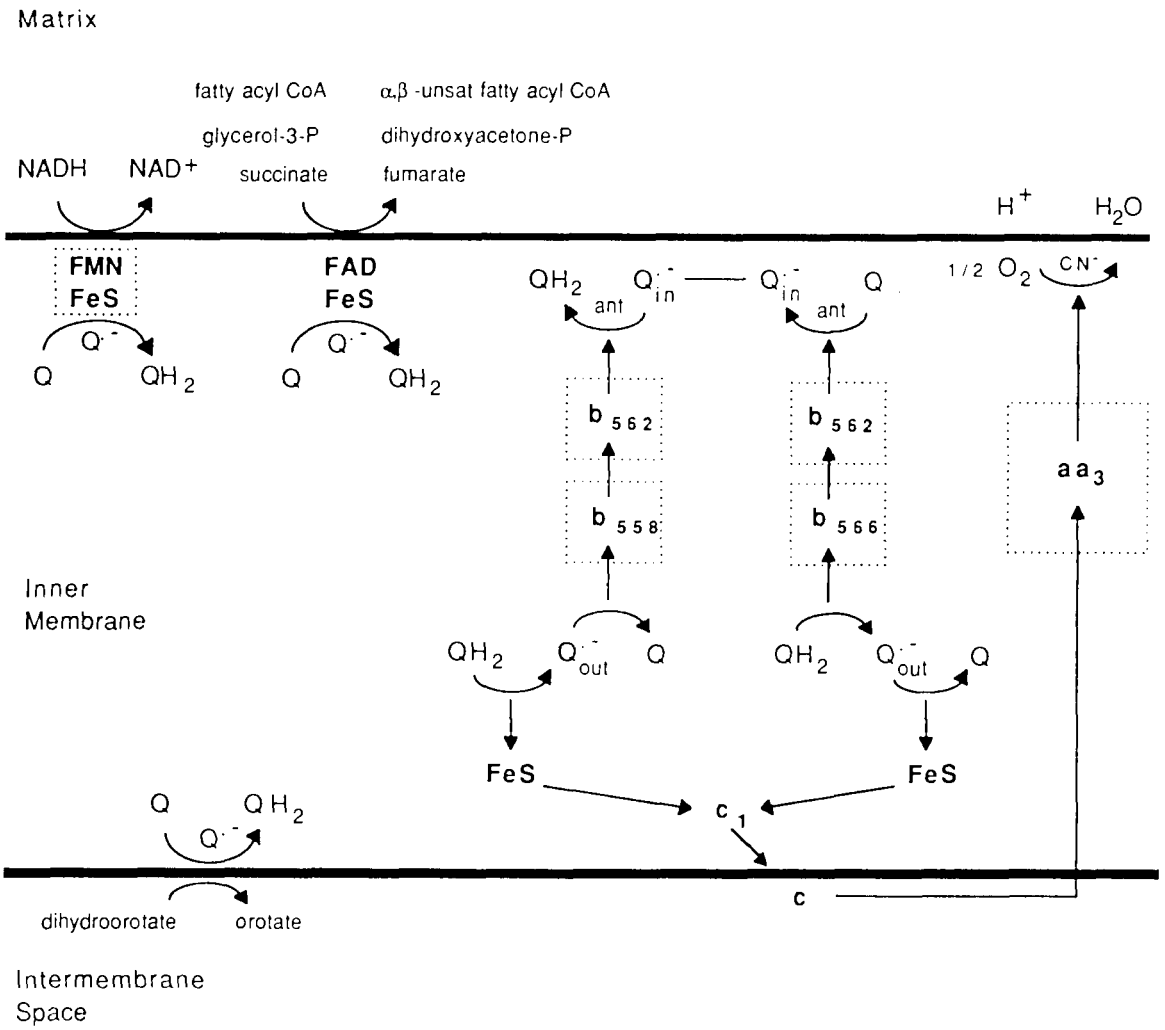

Fig. 1. The mitochondrial respiratory chain and "Q cycle." The "double Q cycle" model,[141,143,144] where the cytochrome bc_1 complex operates in a dimer is shown here. Some details of the mechanism of electron transfer through QH_2:cytochrome *c* oxidoreductase are yet to be firmly established,[145–152] so a somewhat differing model has also evolved.[153] The need for an oxidant of FeS/cytochrome c_1 for reduction of cytochrome *b* is well established however, and other models share major features (oxidation of QH_2 by FeS/cytochrome c_1; oxidation of $Q^{\cdot-}_{out}$ and reduction of $Q^{\cdot-}_{in}$ by *b* cytochromes). Assuming that unstable outer ubisemiquinone species ($Q^{\cdot-}_{out}$) are major autoxidizable species,[113,150,154–156] the double Q cycle model readily explains observations that CN^- stimulates $O_2^{\cdot-}$ and H_2O_2 release but inhibits antimycin-stimulated release.

Reactions catalyzed by NADH dehydrogenase and other ubiquinone-reducing flavoproteins are shown at the surfaces of the inner mitochondrial membrane. Subunits encoded by mitochondrial genes are shown by dotted boxes and sites of inhibition by CN^- and antimycin (ant) are indicated.

component affected[e.g.,161,162,cf.68] show the wide potential for functional changes.

Loss of F_0 ATPase function causes state IV-like generation of $O_2^{\cdot-}$ and H_2O_2

Alteration or deletion of the F_0ATPase complex would also increase $O_2^{\cdot-}$ and H_2O_2 generation (in addition to decreasing ATP production for the cell). The mitochondria would only be capable of state IV-like respiration if unable to dissipate the proton gradient through the ATPase complex.

Loss of NADH dehydrogenase subunits doesn't necessarily decrease $O_2^{\cdot-}$ generation

Loss or alteration of a mitochondrially coded subunit of the NADH dehydrogenase complex presumably removes this site of ubiquinone reduction and $O_2^{\cdot-}$ generation (the two main sites of $O_2^{\cdot-}$ generation are complex I, NADH dehydrogenase; and complex III, ubiquinol-cytochrome *c* oxidoreductase). Other dehydrogenases capable of reducing ubiquinone remain however (e.g., succinate dehydrogenase, dihydroorotate dehydrogenase, electron transferring flavoprotein,

glycerol-3-phosphate dehydrogenase, acyl CoA dehydrogenase, NADPH/NADH transhydrogenase). For example, glycerol-3-phosphate dehydrogenase or isolated dihydroorotate dehydrogenase themselves produce $O_2\cdot^-$ and H_2O_2.[111,163,164–167] Also, loss or mutation of a subunit of NADH dehydrogenase does not necessarily abolish $O_2\cdot^-$ generation from this site. One function of these subunits is to presumably help create an environment appropriate for the catalytic moities.[108,168] Thus, loss of a subunit, or particularly a mutation which does not otherwise affect assembly of the complex, might conceivably destabilize the flavosemiquinone or ubisemiquinone intermediates and increase $O_2\cdot^-$ release. For example, a small isoform of NADH dehydrogenase without mitchondrial encoded subunits assembles in *Neurospora* treated with chloramphenicol, which has the same electron transfer activity, but a lower affinity for ubiquinone.[169] (Similarly, a mutation to one of the subunits of cytochrome oxidase might conceivably destabilize the $O_2\cdot^-$ or H_2O_2 enzyme intermediates and lead to release of reactive species.) Such specific mutations may occur with less probability, but would be dangerous nonetheless.

Loss of function of more than one mitochondrial gene product also predicts increased generation of $O_2\cdot^-$

If cytochrome *b* plus one or more subunits of cytochrome oxidase were mutated, $O_2\cdot^-$ generation at site I would increase. For example, blocking cytochrome *b* and cytochrome oxidase with antimycin plus CN^- increases NADH-supported generation of $O_2\cdot^-$ over that in absence of inhibitors.[128] $O_2\cdot^-$ generation at site III would depend on the rate of autoxidation of reduced species of ubiquinone. This rate may be substantial at the increased oxygen tension which develops. Loss of cytochrome *b* plus subunit 8 of the F_1ATPase complex (which may control coupling of ATP synthesis) is equivalent to adding antimycin plus uncoupler; a situation which produces the highest rates of H_2O_2 generation in mitochondria (10–30-fold greater than that released in normal respiration). If the whole mitochondrial genome is lost, the rate of $O_2\cdot^-$ generation depends partly on the degree of assembly of complex III in the absence of cytochrome *b*, but ubiquinol is generated by succinate dehydrogenase and dehydrogenases other than NADH dehydrogenase. In addition to increasing univalent reduction of oxygen, mutations to mitochondrial genes can increase reduction of cytotoxic quinones or xenobiotics.

CYTOCHROME DEPLETED MITOCHONDRIA SUPPORT GENERATION OF ACTIVE OXYGEN

A cytochrome *c*-deficient yeast mutant had increased levels of both cytosolic and mitochondrial forms of superoxide dismutase (both are induced by oxidative stress in yeasts) and had increased CN^--resistant respiration.[170,171] These changes occurred with or without oxidative stress induced by paraquat, suggesting increased generation of $O_2\cdot^-$ and increased ability to reduce cytotoxic agents. Also, a cytochrome *b*-deficient yeast mutant contained at least normal amounts of mitochondrial superoxide dismutase, despite being deficient in normal respiration.[172] In this study, mitochondrial superoxide dismutase was highest in the log phase of yeast growth on glucose, before appearance of cytochromes (subject to glucose repression) and respiration. Flavoprotein dehydrogenases are less subject to glucose repression and were derepressed earlier than cytochrome oxidase. Petite (*rho*$^-$) yeast mutants also express immunoprecipitable mitochondrial superoxide dismutase[173] plus catalase[174] and peroxidase,[175] though lacking cytochromes and normal respiration.

Extraction of cytochrome *c* from rat heart mitochondria did not substantially alter the rate of succinate supported H_2O_2 generation per mg protein (in absence of antimycin).[114] The H_2O_2 yield per oxygen consumed (oxygen consumption was decreased 70%) increased 3-fold (comparison of Figs. 2, 4, and 5 in[114]). Also, dihydroorotate produced the same amount of $O_2\cdot^-$ after separation of the dehydrogenase from cytochrome *b* and other cytochromes as in the presence of cytochromes plus antimycin.[111,166]

Increased CN^--resistant respiration, presumably representing some autoxidizable component,[176] also develops in respiratory deficient cells of several eukaryotic microorganisms. These cells include respiration deficient mutants of *S. pombe*[176] and *N. crassa*,[58,177] *rho*$^-$ or *rho*^{0} mutants of *S. cerevisiae*,[176] and yeast cells grown anaerobically (and thus lacking mitochondrial cytochromes but not flavoprotein dehydrogenases).[178] Sonicated cells from anaerobically, but not aerobically grown *S. cerevisiae* spontaneously (endogenous substrate) generated $O_2\cdot^-$.[179]

MITOCHONDRIAL MUTATIONS PROVIDE AN AMPLIFIED ROUTE TO PATHOLOGY

The above analysis shows the number of possibilities for increasing generation of reactive oxygen species with injury to the mitochondrial genome. It seems almost certain that mutations causing increased free

radical generation occur at least occasionally. Whether such mutations manifest as overt pathology depends, of course, on a variety of other factors (e.g., protection, lethality, heritability). Presumably, most often they do not.

Although mitocondria often have more than one copy of the genome, mutation to one copy of gene still produces abnormal subunits. Also, the possibility that each copy of a gene receives mutations is not remote. Further, mutated genomes containing deletions sometimes gain a replicative or transcriptional advantage (as in petite (*rho*$^-$) mutants of yeasts[134]).

While mutations to nuclear genes coding for mitochondrial subunits occur with less frequency,[60] a mutation to a nuclear-encoded subunit of cytochrome oxidase, ubiquinol:cytochrome *c* oxidoreductase, or F_0F_1ATPase, (or an enzyme needed for mitochondrial protein synthesis) could similarly increase oxidative stress. In this case, the mutation would affect the whole mitochondrial population of the cell. Mitochondria would thus act as an adjuvant to nuclear mutations, increasing the steady state concentration of oxidative species.

MITOCHONDRIAL MUTATIONS CAUSING ACTIVATION OF OXYGEN CAN AMPLIFY CARCINOGENESIS

Multiple pathways lead to neoplastic transformation. These pathways include responses to chronic inflammation, or direct actions of carcinogens or their biotransformation products on the nuclear genome. Mutations to mitochondria leading to release of reactive oxygen provide one additional pathway. In this pathway, the initial event is the interaction of a mutegen with the mitochondrial genome, alone, or simultaneously with the nuclear genome. Lipophilic mutagens which can be activated by enzymes in the mitochondrial membranes, or which cooperate with activated oxygen preferentially damage mitochondrial DNA. Injury to the mitochondrial (or nuclear) genome impairs synthesis of one or more inner membrane products. Mutant mitochondria may then develop which release reactive species of oxygen and products of mitochondrial membrane degeneration (e.g., lipid peroxides, reactive aldehydes), and have increased ability to reduce reactive quinones. Such mitochondria continuously expose the nucleus (and other mitochondria) to genotoxic agents. Exposure might occur in an acute burst, as a mitochondria degenerates, or more insidiously, where mitochondria release moderately elevated levels of oxygen radicals for a substantial time before they inactivate. Within this time, the mutant mitochondrial genome may have replicated. The mutagenic process may be intracellularly amplified as genotoxic damage spreads from mutant mitochondria to adjacent normal mitochondria. Not only do such mutations increase generation of cytotoxic agents, they may also result in release of mitochondrial DNA fragments (and also decrease functional respiration and oxidative phosphorylation). By increasing oxidative stress, carcinogen-induced mutations to mitochondria can thus amplify exposure of the nucleus to genotoxins. This intracellularly amplified damage increases the likelihood of oncogene activation or growth-suppressor gene inactivation.

Mitochondrial mutations which increase oxidative stress can also influence transformation and cellular proliferation through effects on calcium storage or other intracellular signals. The enhanced proliferation of fibroblasts observed on exposure to low concentrations of H_2O_2[15] might reflect such effects of oxidative stress on intracellular signals. Hydroperoxides, or agents which induce oxidative stress, impair calcium sequestration and cause release of calcium from mitochondria and other intracellular stores (e.g.,[180–182] see[183] for a review). Moreover, peroxide or prooxidant-induced release of calcium from mitochondria and other stores causes cytoskeletal changes evident in blebbing of the cell surface of hepatocytes.[183–186] Increases in cytosolic calcium stimulate DNA synthesis and cell proliferation,[e.g.,187,188] and intracellular stores contribute to these processes.[e.g.,189–192,cf.193,194] Impaired calcium storage can activate calcium-sensitive proteases, phospholipases, and protein kinases, and the arachidonic acid cascade. Thus, mutant mitochondria might act intracellularly in a manner analogous to oncogenic growth factors which cause influx of calcium across the cell membrane (EGF) or release calcium from endoplasmic reticulum through turnover of phosphatidyl inositol (PDGF).

In contrast to most other models, a mitochondrial mutation/oxygen activation pathway theoretically allows just one, or a few, mutant mitochondria to instigate oncogenesis. Consistent with other models however, a latent period (promotional phase?) may ensue while cell division and mitochondrial proliferation allow for the segregation and accumulation of mutant mitochondria in selected daughter cells. The length of the latent period depends on the initial dose and potency of the mutagen, the number of mitochondria affected, the nature of the mutation, the lability of the mutated mitochondria, and the rate of cell division of

[d]Changes in calcium-buffering activity of mitochondria formed the basis of a previous model for the sustained growth of tumors.[195]

the tissue. In addition, time is needed for the multistep activation of oncogenes and inactivation of growth suppressor genes usually required for transformation.[195,196]

SOME TUMOR CELL CHARACTERISTICS ARE CONSISTENT WITH MITOCHONDRIAL RELEASE OF ACTIVE OXYGEN

Changes seen in tumor cells and tumor mitochondria do not necessarily reflect carcinogenic mechanisms because i) they are after the fact, and differences may be coincidental or even compensatory, ii) the mitochondrial population may not be homogeneous, and iii) mitochondria may not always be involved. Some changes may give indirect support, however. Several observations are consistent with involvement of mitochondrial mutations and of active oxygen release in cancer.

Products of mitochondrial genes are sometimes abnormal in tumor mitochondria where nuclear gene products are unaffected

Respiration of tumor cells is often severely impaired. The cells have fewer mitochondria, and those present are structurally abnormal (see[1] for a review). Consistent with decreased respiration, levels of cytochromes aa_3 and b (and less so, c_1) were decreased in hepatoma or virus-transformed cells.[197,198] Levels of cytochrome c (a nuclear gene product) were increased however, increasing the cytochrome c/cytochrome oxidase ratio. These observations, plus abnormal DNA seen in tumor mitochondria,[cf. 1] suggest impairment of mitochondrial gene products is more prevalent than impairment of mitochondrial proteins derived from nuclear genes.

Mitochondria from tumor cells generate normal or increased $O_2\cdot^-$

Mitochondria or submitochondrial particles from some tumor cells show normal or increased antimycin-induced generation of $O_2\cdot^-$ when compared with normal tissues.[199-201] In one case, hepatoma mitochondria reportedly generated more $O_2\cdot^-$ without antimycin than normal liver mitochondria.[200]

Despite ability of the mitochondria for normal or increased generation of $O_2\cdot^-$, tumor cells generally contain less mitochondrial superoxide dismutase (MnSOD) and sometimes less cytosolic superoxide dismutase (CuZnSOD).[199,200,202-204] Some contradictory reports appear, however.[204-207] The location and local environment (e.g., oxygenation) of the tumor cells can influence the expression of superoxide dismutase and other oxidative enzymes.[205]

Neoplastic transformation exhausts lipid peroxidation

Tumor cells and mitochondria produce less lipid peroxide in vitro.[208-214] Tumor mitochondrial membranes generally have decreased polyunsaturated phospholipids and increased monounsaturated phospholipids[e.g.,215] (see refs[1,214] for reviews). These changes in membrane composition suggest previously peroxidized lipids.[214,216]

Aldehydic products of lipid peroxidation (e.g., alkenals) are cytostatic, inhibiting chromosomal replication and transcription,[cf.214,217-221] as well as cytotoxic, inducing mutations[222] and triggering heat shock genes.[223] Addition of polyunsaturated fatty acids at sufficient concentration inhibits proliferation of tumor cells (or regenerating normal cells).[212,224-226] Chain-breaking antioxidants such as alpha-tocopherol or butylated-hydroxytoluene promote cell proliferation.[212,222] Levels of lipid peroxidation thus relate inversely with rates of cell proliferation.[212,224,227] Loss of the moderating influence of peroxidation on cell division may stimulate the proliferation of tumor cells.[201,202,204,205,228]

Thus, release of reactive oxygen species (oxygen radicals, peroxides, aldehydes, etc.) by mitochondria may contribute to initiation and promotion before cell transformation, while subsequent exhaustion of peroxidative ability may contribute to proliferation of transformed cells. In the broadest interpretation these views accomodate both the respiratory deficiency theories of Otto Warburg[229] and the bioelectronic (keto-aldehyde) theories of Albert Szent-Gyorgyi.[230-232]

MITOCHONDRIA FROM AGING ANIMALS RELEASE MORE ACTIVE OXYGEN

A link between mitochondrial mutations and oxygen activation adds to the current mitochondrial model of senesence by more accurately predicting accelerated aging on exposure to environmental mutagens or toxins. Thus, damage to the genome either from exposure to a mitochondrial genotoxin or from endogenous generation of oxygen radicals during respiration can lead to loss of normal respiration and further release of toxic oxygen species. In evidence mitochondria and submitochondrial particles isolated from the hearts of old rats release about 34% more H_2O_2 and $O_2\cdot^-$ than those from young rats.[36] This increase with age is relatively insignificant in liver, but is more substantial in rat brain

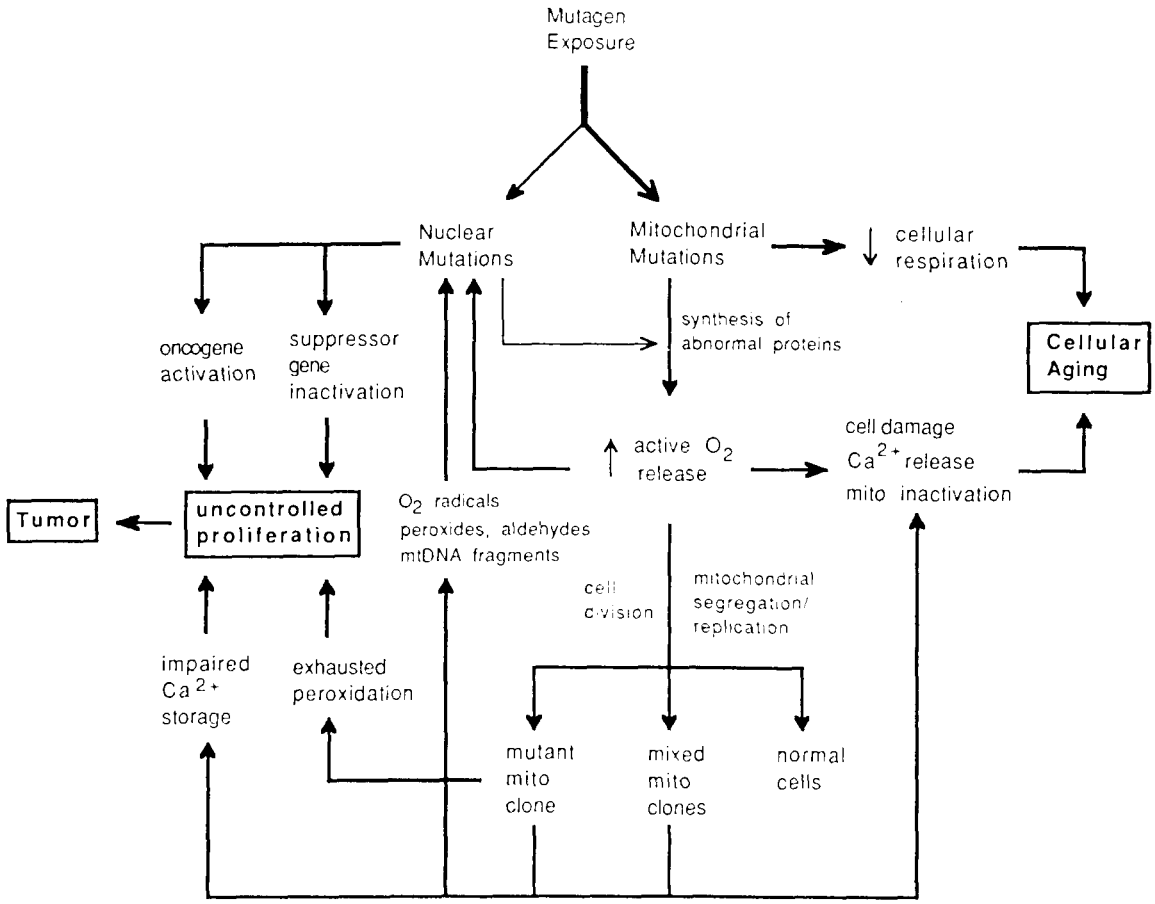

Fig. 2. Mitochondrial amplified pathways to cellular aging and tumorigenesis. Mitochondrial mutations can accelerate cellular aging by decreasing functional respiration and ATP production and by leading to amplified damage from those mitochondrial mutations which increase release of active oxygen. Propagation of such mutations in cellular and mitochondrial division can further amplify damage. Mitochondrial mutations which increase release of active oxygen also amplify exposure of the nucleus to mutagens. Propagation of these mitochondrial mutations chronically amplifies nuclear mutations. Chronic exposure of the nucleus to mutagens increases the possibility of mutations which activate oncogenes or inactivate supressor genes, and release the cell from normal growth controls. Additional mechanisms for mitochondrial mutations and increased oxygen activation to stimulate cellular proliferation include peroxide-induced release of calcium and exhausted release of peroxides and keto-aldehydes. These influences may combine to cause neoplastic transformation and tumor formation.

than in heart.[37] In houseflies, antimycin-resistant respiration increased 50% and mitochondrial generation of $O_2^{\cdot -}$ increased more than two-fold with aging.[38] Increased release of reactive oxygen species allows further damage to the mitochondrial genome and other mitochondrial structures, eventually causing loss of regenerative ability and a decline in normally functioning mitochondria. This amplification further explains the decreased respiratory rates, respiratory control ratios, and P/O ratios observed in mitochondria isolated from older animals.[e.g.,22] Such mitochondrial dysfunction causes damage to the mitochondria, and also to other cellular constituents through release of oxygen metabolites to the cytosol. Accumulation of mitochondrial genetic damage with age[e.g.,40] thus not only contributes to aging, but may also increase risk of cancer.

CELLS LEAST SUSCEPTIBLE TO CANCER ARE MOST SUSCEPTIBLE TO AGING

Those cells most susceptible to cellular aging (e.g., muscle and nerve)[29,233] are precisely those fixed, post-mitotic cells least susceptible to cancer.[234] So, a common mechanism of mitochondrial mutagenesis could contribute to cancer in dividing cells (e.g., fibroblasts, leukocytes, bone marrow), to aging in terminally differentiated cells, and to both aging and cancer in intermitotic cells (e.g., liver, kidney, adrenal gland). Fig. 2 provides a summary of the potential involvement of mitochondrial mutations in carcinogenesis and aging.

CONCLUSIONS

The tight conservation of mitochondrial encoded proteins, but labile mitochondrial DNA must reflect

the strong evolutionary pressures on this organelle. The high mutation rate of mitochondrial DNA allowed it to evolve a highly efficient and reasonably safe system for reducing oxygen. To reduce oxygen safely is a difficult task, and these evolutionary pressures remain. To allow beneficial mutations but safeguard against detrimental ones, nature apparently adopted a strategy where detrimental mutations self-destruct. By leaving the mitochondrial genome unprotected, any mutation which increases release of active oxygen should eventually destroy that copy of the genome. Sources of reactive intermediates such as those produced by NADH dehydrogenase or a mutated cytochrome b-c_1 complex thus would eventually be eliminated. This system helps limit long-term damage to the rest of the cell.

The criticism that respiratory deficiency may be a result rather than a cause of the cancer has limited wide acceptance of the respiratory deficiency model. If mitochondrial mutations which increase release of active oxygen prove to contribute to carcinogenesis and aging, this criticism falls aside, since the respiratory damage is both a result of carcinogen exposure and a cause of transformation. This intracellularly amplified pathway accommodates both mitochondrial and nuclear events, such as activation of oncogenes or inactivation of growth-suppressor genes, and the involvement of active oxygen.

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REFERENCES

1. Pedersen, P. L. Tumor mitochondria and the bioenergetics of cancer cells. *Prog. Exp. Tumor Res.* **22**:190–274; 1978.
2. Wilkie, D.; Evans, I. H.; Egilsson, V.; Diala, E. S.; Collier, D. Mitochondria, cell surface, and carcinogenesis. *Int. Rev. Cytol.* **15**(supp):157–189; 1983.
3. Warburg, O. Oxygen, the creator of differentiation. In: Mills, A. K.; Krebs, H., eds. *Aspects of yeast metabolism*. Oxford: Blackwell Scientific Publications; 1967:327–337.
4. Clayton, D. A.; Vinograd, J. Complex mitochondrial DNA in leukemic and normal human myeloid cells. *Proc. Nat. Acad. Sci. USA* **62**:1077–1084; 1969.
5. Wilkie, D.; Egilsson, V.; Evans, I. H. Mitochondria in oncogenesis. *Lancet* **1**:697–699; 1975.
6. Egilsson, V.; Evans, I. H.; Wilkie, D. Toxic and mutagenic effects of carcinogens on the mitochondria of Saccharomyces cerevisiae. *Molec. Gen. Genet.* **174**:39–46; 1979.
7. Neubert, D.; Hopfenmuller, W.; Fuchs, G. Manifestation of carcinogenesis as a stochastic process on the basis of an altered mitochondrial genome. *Arch. Toxicol.* **48**:89–125; 1981.
8. Wilkie, D.; Evans, I. Mitochondria and the yeast cell surface: implications for carcinogenesis. *TIBS* **7**:147–151; 1982.
9. Reid, R. A. Can migratory mitochondrial DNA activate oncogenes? *TIBS* **8**:190–191; 1983.
10. Shay, J. W.; Werbin, H. Genetic mechanisms in tumor initiation and progression.6. Are mitochondrial-DNA mutations involved in the carcinogenic process. *Mutation Res.* **186**:149–160; 1987.
11. Richter, C. Do mitochondrial-DNA fragments promote cancer and aging? *FEBS Lett* **241**:1–5; 1988.
12. Nohl, H.; De Silva, D.; Summer, K-H. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin induces oxygen activation associated with cell respiration. *Free Radical Biol. Med.* **6**:369–374; 1989.
13. Sohal, R. S.; Allen, R. G.; Nations, C. Oxygen free radicals play a role in cellular differentiation: an hypothesis. *J. Free Radical Biol. Med.* **2**:175–181; 1986.
14. Allen, R. G.; Balin, A. K. Oxidative influence on development and differentiation: an overview of a free radical theory of development. *Free Radical Biol. Med.* **6**:631–661; 1989.
15. Burdon, R. H.; Rice-Evans, C. Free radicals and the regulation of mammalian cell proliferation. *Free Radical Res. Comm.* **6**:345–358; 1989.
16. Burdon, R. H.; Gill, V.; Rice-Evans, C. Cell proliferation and oxidative stress. *Free Radical Res. Comm.* **7**:149–159; 1989.
17. Ashburner, M. Patterns of puffing activity in the salivary gland chromosomes of *Drosophila*. V. Responses to environmental treatments. *Chromosoma* **31**:356–376; 1970.
18. Leenders, H. J.; Berendes, H. D. The effect of changes in the respiratory metabolism upon genome activity in *Drosophila*. I. The induction of gene activity. *Chromosoma* **37**:433–444; 1972.
19. Leenders, H. J.; Beckers, P. J. A. The effect of changes in the respiratory metabolism upon genome activity. *J. Cell Biol.* **55**:257–265; 1972.
20. Ellgaard, E. G. Similarities in chromosomal puffing induced by temperature shocks and dinitrophenol in *Drosophila*. *Chromosoma* 37:417–422; 1972.
21. Sin, Y. T. Induction of puffs in *Drosophila* salivary glands by mitochondrial factor(s). *Nature* **258**:159–160; 1975.
22. Harman, D. The biologic clock: the mitochondria? *J. Am. Geriat. Soc.* **20**:145–147; 1972.
23. Nohl, H.; Hegner, D. Do mitochondria produce oxygen radicals in vivo? *Eur. J. Biochem.* **82**:563–567; 1978
24. Miquel, J.; Economos, A. C.; Fleming, J.; Johnson, J. E., Jr. Mitochondrial role in cell aging. *Exp. Gerontol.* **15**:575–591; 1980.
25. Harman, D. The aging process. *Proc. Nat. Acad. Sci. USA* **78**:7124–7128; 1981.
26. Harman, D. Free radical theory of aging: consequences of mitochondrial aging. *Age* **6**:86–94; 1983.
27. Harman, D. Free radicals and the origination, evolution, and present status of the free radical theory of aging. In: Armstrong, D., Sohal, R. S.; Cutler, R. G.; Slater, T. F., eds. *Free radicals in molecular biology, aging, and disease*. New York: Raven Press; 1984:1–12.
28. Miquel, J.; Fleming, J. E. An integrated theory of cell and organismic aging as the result of mitochondrial differentiation and intrinsic mt DNA mutagenesis. *Age* **6**:145–146; 1983.
29. Miquel, J.; Fleming, J. E. A 2-step hypothesis on the mechanisms of in vitro cell aging: cell-differentiation followed by intrinsic mitochondrial mutagenesis. *Exp. Gerontol.* **19**:31–36; 1984.
30. Sohal, R. S.; Allen, R. G. Relationship between metabolic rate, free radicals, differentiation and aging: a unified theory. In: Woodhead, A.; Blackett, A. D.; Hollaender, A., eds. *Molecular biology of aging*. Brookhaven Symposium, Vol. 35. New York: Plenum Press; 1985:75–104.
31. Sohal, R. S.; Allen, R. G. Relationship between oxygen metabolism, aging and development. *Adv. Free Radical Biol. Med.* **2**:117–160; 1986.
32. Cutler, R. G. Antioxidants, aging, and longevity. In: Prior, W. A., ed. *Free radicals in biology*. Vol. IV. Orlando: Academic Press; 1984:371–428.
33. Horrum, M. A.; Harman, D.; Tobin, R. B. Free radical theory of aging: effects of antioxidants on mitochondrial function. *Age* **10**:58–61; 1987.
34. Miquel, J.; Oro, J.; Bensch, K. G.; Johnson, J. E. Jr. Lipofuscin: fine-structural and biochemical studies. In: Prior, W. A., ed. *Free radicals in biology*. Vol. III. Orlando: Academic Press; 1977:133–181.

35. Koobs, D. H.; Schultz, R. L.; Jutzy, R. V. The origin of lipofuscin and possible consequences to the myocardium. *Arch. Pathol. Lab. Med.* **102**:66–68; 1978.
36. Spoerri, P. E. Mitochondrial alterations in ageing mouse neuroblastoma cells in culture. *Monogr. Dev. Biol.* **17**:210–220, 1984.
37. Nohl, H.; Breuninger, V.; Hegner, D. Influence of mitochondrial radical formation on energy-linked respiration. *Eur. J. Biochem.* **90**:385–390; 1978.
38. Sawada, M.; Carlson, J. C. Changes in superoxide radical and lipid peroxide formation in the brain, heart and liver during the lifetime of the rat. *Mech. Ageing Dev.* **41**: 125–137; 1987.
39. Farmer, K. J.; Sohal, R. S. Relationship between superoxide anion radical generation and aging in the housefly, *Musca domestica*. *Free Radical Biol. Med.* **7**:23–29; 1989.
40. Fleming, J. E.; Miquel, J.; Cottrell, S. F.; Yengoyan, L. S.; Economos, A. C. Is cell aging caused by respiration-dependent injury to the mitochondrial genome. *Gerontology* **28**:44–53; 1982.
41. Piko, L.; Hougham, A. J.; Bulpitt, K. J. Studies of sequence heterogeneity of mitochondrial DNA from rat and mouse tissues: evidence for an increased frequency of deletions/additions with aging. *Mech. Ageing Dev.* **43**:279–293; 1988.
42. Graffi, A. Intracellular benzpyrene accumulation in living normal- and tumor-cells. *Z. Krebsforsch* **50**:196–219; 1940.
43. Wunderlich, V.; Schutt, M.; Bottger, M.; Graffi, A. Preferential alkylation of mitochondrial deoxyribonucleic acid by N-methyl-N-nitrosourea. *Biochem. J.* **118**:99–109; 1970.
44. Allen, J. A.; Coombs, M. M. Covalent binding of polycyclic aromatic compounds to mitochondrial and nuclear DNA. *Nature* **287**:244–245; 1980.
45. Nagatani, Y. Cytological studies on the affinity of the carcinogenic azo dyes for cytoplasmic components. *Int. Rev. Cytol.* **10**:243–313; 1960.
46. Takayama, S.; Murumatsu, M. Incorporation of tritiated dimethylnitrosamine into subcellular fractions of mouse liver after long-term administration of dimethylnitrosamine. *Z. Krebsforsch* **73**:172–180; 1969.
47. Backer, J. M.; Weinstein, I. B. Mitochondrial DNA is a major cellular target for a dihydrodiol-epoxide derivative of benzo(*a*)pyrene. *Science* **209**:297–299; 1980.
48. Wunderlich, V.; Tetzlaff, I.; Graffi, A. Studies on nitrosodimethylamine: preferential methylation of mitochondrial DNA in rats and hamsters. *Chem. Biol. Interactions* **4**:81–89; 1971.
49. Wilkinson, R.; Hawks, A.; Pegg, A. E. Methylation of rat liver mitochondrial deoxyribonucleic acid by chemical carcinogens and associated alterations in physical properties. *Chem. Biol. Interactions* **9**:157–167; 1975.
50. Miyaki, M.; Yatagai, K.; Ono, T. Strand breaks of mammalian mitochondrial DNA induced by carcinogens. *Chem. Biol. Interactions* **17**:321–329; 1977.
51. Niranjan, B. G.; Bhat, N. K.; Avadhani, N. G. Preferential attack of mitochondrial DNA by aflotoxin B_1 during hepatocarcinogenesis. *Science* **215**:73–75; 1981.
52. Rossi, S. C.; Wetterhahn, K. E. The carcinogen chromium(VI) targets mitochondrial-DNA. *Abstracts Of Papers Of The American Chemical Society* **195**:407(INOR) abstr. (1988).
53. Rossi, S. C.; Gorman, N.; Wetterhahn, K. E. Mitochondrial reduction of the carcinogen chromate: formation of chromium(V). *Chem. Res. Toxicol.* **1**:101–107; 1988.
54. Tomasi, A.; Albano, E.; Banni, S.; Botti, B.; Corongiu, F.; Dessi, M. A.; Iannone, A.; Vannini, V.; Dianzani, M. U. Free-radical metabolism of carbon tetrachloride in rat liver mitochondria. *Biochem. J.* **246**:313–317; 1987.
55. Levy, G. N.; Brabec, M. J. Binding of carbon tetrachloride metabolites to rat hepatic mitochondrial DNA. *Toxicol. Lett.* **22**:229–234; 1984.
56. Hruszkewycz, A. M. Evidence for mitochondrial DNA damage by lipid peroxidation. *Biochem. Biophys. Res. Comm.* **153**:191–197; 1988.
57. Richter, C.; Park, J. W.; Ames, B. N. Normal oxidative damage to mitochondrial and nuclear-DNA is extensive. *Proc. Natl. Acad. Sci. USA* **85**:6465–6467; 1988.
58. Lloyd, D. *The mitochondria of microorganisms*. London: Academic Press; 1974.
59. Brown, W. M.; George, M.; Wilson, A. C. Rapid evolution of animal mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* **76**:1967–1971; 1979.
60. Wallace, D. C.; Ye, J.; Neckelmann, S. N.; Singh, G.; Webster, K. A. Sequence analysis of cDNAs for the human and bovine ATP synthase *B* subunit: mitochondrial genes sustain seventeen times more mutations. *Curr. Genet.* **12**:81–90; 1987.
61. Kara, J.; Mach, O.; Cerna, H. Replication of Rous Sarcoma Virus and the biosynthesis of the oncogenic subviral ribonucleoprotein particles ("virosomes") in the mitochondria isolated from Rous sarcoma tissue. *Biochem. Biophys. Res. Comm.* **44**:162–170; 1971.
62. Glaichenhaus, N.; Leopold, P.; Cuzin, F. Increased levels of mitochondrial gene expression in rat fibroblast cells immortalized or transformed by viral and cellular oncogenes. *EMBO J.* **5**:1261–1265; 1986.
63. Gianni, A. M.; Favera, R. D.; Polli, E. Restriction enzyme analysis of human leukemic mitochondrial DNA. *Leukemic Res.* **4**:155–160; 1980.
64. Welter, C.; Blin, N. Observation of mitochondrial DNA in malignant human tumors. *J. Exp. Clin. Cancer Res.* **7**:13–20; 1988.
65. Hudson, B.; Vinograd, J. Catenated circular DNA molecules in HeLa cell mitochondria. *Nature* **216**:647–652; 1967.
66. Koike, K.; Taira, M.; Kochino, Y.; Yaginuma, K.; Sekuguchi, T.; Kobayashi, M. Mutation of rat mitochondrial genome. In: Schweyen, R. J.; Wolf, K.; Kaudewitz, F., eds. *Mitochondria 1983: nucleo-mitochondrial interactions*. Berlin: Walter de Gruyter; 1983:371–387.
67. Taira, M.; Yoshida, E.; Kobayashi, M.; Yaginuma, K.; Koike, K. Tumor-associated mutations of rat mitochondrial transfer RNA genes. *Nucleic Acids Res.* **11**:1635–1643; 1983.
68. Tzagoloff, A. *Mitochondria*. New York: Plenum Press; 1982.
69. Spolski, C. M.; Eisenstadt, J. M. Chloramphenicol-resistant mutants of human HeLa cells. *FEBS Lett.* **25**:319–324; 1972
70. Harri, M. Cytoplasmic transfer of resistance to antimycin A in Chinese hamster cells. *Proc. Natl. Acad. Sci. USA* **75**:5604–5608; 1978.
71. Kearsey, S. E.; Craig, I. W. Altered ribosomal RNA genes in mitochondria from animal cells with chloramphenicol resistance. *Nature* **290**:607–608; 1981.
72. Blanc, H.; Wright, C. T.; Bibb, M. J.; Wallace, D. C.; Clayton, D. A. Mitochondrial DNA of chloramphenicol-resistant mouse cells contains a single nucleotide change in the region encoding the 3' end of the large ribosomal RNA. *Proc. Natl. Acad. Sci. USA* **78**:3789–3793; 1981.
73. Breen, G. A. M.; Miller, D. L.; Holmans, P. L.; Welch, G. Mitochondrial DNA of two independent oligomycin-resistant Chinese hamster ovary cell lines contains a single nucleotide change in the ATPase 6 gene. *J. Biol. Chem.* **261**:11680–11685; 1986.
74. Howell, N.; Nalty, M. S. Mitochondrial chloramphenicol-resistant mutants can have deficiencies in energy metabolism. *Somatic Cell Mol. Genet.* **14**:185–193; 1988.
75. Howell, N.; Whitfield, C. Mutational analysis of the mitochondrial cytochrome *b* gene. *In Vitro Cell. Dev. Biol.* **23**:73A abstr. (1987).
76. Lindahl, K. F.; Hausmann, B.; Chapman, V. M. A new *H*-2-linked class I gene whose expression depends on a maternally inherited factor. *Nature* **306**:383–385; 1983.
77. Smith, R. (III); Huston, R. N.; Jenkin, R. N.; Huston, D.; Rich, R. R. Mitochondria control expression of a cell surface antigen. *Nature* **306**:599–601; 1983.
78. John, P. Mitochondrial regulation of cell surface components in relation to carcinogenesis. *J. Theor. Biol.* **110**:377–381; 1984.
79. Harris, H. Cell fusion and the analysis of malignancy: the Croonian lecture. *Proc. Royal Soc. London* **179**:1–20; 1971.

80. Stanbridge, E. J. Suppression of malignancy in human cells. *Nature* **260**:17–20; 1976.
81. Marshall, C. J.; Dave, H. Suppression of the transformated phenotype in somatic cell hybrids. *J. Cell Sci.* **33**:171–190; 1978.
82. Klinger, H. P. Suppression of tumorigenicity. *Cytogenet. Cell Genet.* **32**:68–84; 1982.
83. Benedict, W. F.; Weissman, B.; Mark, C.; Stanbridge, E. J. Tumorigenicity of human HT1080 fibrosarcoma X normal fibroblast hybrids; chromosome dosage dependency. *Cancer Res.* **44**:3471–3479; 1984.
84. Sager, R. Genetic suppression of tumor formation. *Cancer Res.* **44**:45–68; 1985.
85. Jonasson, J.; Harris, H. The analysis of malignancy by cell fusion. VIII. Evidence for the intervention of an extrachromosomal element. *J. Cell Sci.* **24**:255–263; 1977.
86. Howell, A. N.; Sager, R. Tumorigenicity and its suppression in cybrids of mouse and Chinese hamster cell lines. *Proc. Nat. Acad. Sci. USA* **75**:2358–2362; 1978.
87. Howell, A. N.; Sager, R. Genetic analysis of tumorigenesis VIII: suppression of SV_{40} transformation in cell hybrids and cytoplasmic transferants. *Cytogenet. Cell Genet.* **31**:214–227; 1981.
88. Coon, H. G. Tumorigenicity of cybrids. *J. Cell Biol.* **83**:499A abstr. (1979).
89. Shay, J. W.; Lorkowski, G.; Clark, M. A. Suppression of tumorigenicity in cybrids. *J. Supramol. Struct. Cell Biochem.* **16**:75–82; 1981.
90. Giguere, L.; Morais, R. On suppression of tumorigenicity in hybrid and cybrid mouse cells. *Somatic Cell Mol. Genet.* **7**:457–471; 1981.
91. Koura, M.; Isaka, H.; Yoshida, M. C.; Tosu, M.; Sekiguchi, T.; Kobayashi, M. Suppression of tumorigenicity in interspecific reconstituted cells and cybrids. *Gann* **73**:574–580; 1982.
92. Israel, B. A.; Schaeffer, W. I. Cytoplasmic hybrids (cybrids) and reconstructed cells beween normal and tumorigenetic rat liver cells: suppression of the tumorigenic phenotype. *In Vitro* **18**:284A abstr. (1982).
93. Israel, B. A.; Schaeffer, W. I. Cytoplasmic suppression of malignancy. *In Vitro Cell. Develop. Biol.* **23**:627–631; 1987.
94. Shay, J. W.; Yinong, L.; Werbin, H. Cytoplasmic suppression of tumor progression in reconstituted cells. *Somatic Cell Molec. Genet.* **14**:345–350; 1988.
95. Wallace, D. C.; Hodge, J. A.; Lott, M. T.; Neckelmann, S. N.; Singh, G.; Ye, J.; Zheng, X. Mitochondrial genes and cancer. *In Vitro Cell. Develop. Biol.* **23**:A73 abstr. (1987).
96. Abken, H.; Jungfer, H.; Albert, W. H. W.; Willecke, K. Immortalization of human lymphocytes by fusion with cytoplasts of transformed mouse L cells. *J. Cell Biol.* **103**:795–805; 1986.
97. Vierny, C.; Keller, A-M.; Begel, O. A sequence of mitochondrial DNA is associated with the onset of senescence in a fungus. *Nature* **297**:157–159; 1982.
98. Farrelly, F.; Butow, R. A. Rearranged mitochondrial genes in the yeast nuclear genome. *Nature* **301**:296–301; 1983.
99. Gellissen, G.; Bradfield, J. Y.; White, B. N.; Wyatt, G. R. Mitochondrial DNA sequences in the nuclear genome of a locust. *Nature* **301**:631–634; 1983.
100. Jacobs, H. T.; Posakony, J. W.; Davidson, E. H. Mitochondrial DNA sequences in the nuclear genome of *Strongylocentrotus purpuratus*. *J. Mol. Biol.* **165**:609–632; 1983.
101. Wright, R. M.; Cummings, D. J. Integration of mitochondrial gene sequences within the nuclear genome during senescence in a fungus. *Nature* **302**:86–88; 1983.
102. Gellison, G.; Michaelis, G. Gene transfer. Mitochondria to nucleus. *Ann. NY Acad. Sci.* **503**:391–401; 1987.
103. Schatz, G. The biogenesis of mitochondria. In: Lee, C. P.; Schatz, G.; Gallner, G., eds. *Mitochondria and microsomes*. Reading: Addison-Wesley Publishing Co.; 1981:45–66.
104. Attardi, G. Organization and expression of the mammalian mitochondrial genome: a lesson in economy. *TIBS* **6**:86–89; 1981.
105. Attardi, G. Organization and expression of the mammalian mitochondrial genome: a lesson in economy. *TIBS* **6**:100–103; 1981.
106. Rosamond, J. The molecular biology of the mitochondrion. *Biochem. J.* **202**:1–8; 1982.
107. Chomyn, A.; Mariottini, P.; Cleeter, M. W. J.; Ragan, C. I.; Matsunoyagi, A.; Hatefi, Y.; Doolittle, R. F.; Attardi, G. 6 Unidentified reading frames of human mitochondrial-DNA encode components of the respiratory-chain NADH dehydrogenase. *Nature* **314**:592–597; 1985.
108. Chomyn, A.; Cleeter, M. W. J.; Ragan, C. I.; Riley, M.; Doolittle, R. F.; Attardi, G. Urf6, last unidentified reading frame of human mtDNA, codes for an NADH dehydrogenase subunit. *Science* **234**:614–618; 1986.
109. Trumpower, B. L.; Simmons, Z. Diminished inhibition of mitochondrial electron transfe. from succinate to cytochrome *c* by thenoyltrifluoroacetone induced by antimycin. *J. Biol. Chem.* **254**:4608–4616; 1979.
110. Cadenas, E.; Boveris, A. Enhancement of hydrogen peroxide formation by protophores and ionophores in antimycin-supplemented mitochondria. *Biochem. J.* **188**:31–37; 1980.
111. Forman, H. J. The role of ubisemiquinone in superoxide production by dihydroorotate dehydrogenase: a proposed mechanism. In: Bannister, J. V.; Hill, H. A. O., eds. *Chemical and biochemical aspects of superoxide and superoxide dismutase*. Vol. 1. Amsterdam: Elsevier; 1980:76–83.
112. Forman, H. J.; Boveris, A. Superoxide radical and hydrogen peroxide in mitochondria. In: Prior W. A., ed. *Free radicals in biology*. Vol. V. Orlando: Academic Press; 1982:65–90.
113. Ksenzenko, M.; Konstantinov, A. A.; Khomutov, G. B.; Tikhonov, A. N.; Ruuge, E. K. Effect of electron transfer inhibitors on superoxide generation in the cytochrome bc_1 site of the mitochondrial respiratory chain. *FEBS Lett.* **155**:19–24; 1983.
114. Turrens, J. F.; Alexandre, A.; Lehninger, A. L. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch. Biochem. Biophys.* **237**:408–414; 1985.
115. Sugioka, K.; Nakano, M.; Totsunenakano, H.; Minakami, H.; Terokubota, S.; Ikegami, Y. Mechanism of O_2^- generation in reduction and oxidation cycle of ubiquinones in a model of mitochondrial electron-transport systems. *Biochim. Biophys. Acta* **936**:377–385; 1988.
116. Nohl, H.; Jordan, W. The mitochondrial site of superoxide formation. *Biochem. Biophys. Res. Comm.* **138**:533–539; 1986.
117. Turrens, J. F.; Boveris, A. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem. J.* **191**:421–427; 1980.
118. Tzagoloff, A.; Myers, A. M. Genetics of mitochondrial biogenesis. *Ann. Rev. Biochem.* **55**:249–285; 1986.
119. Linnane, A. W.; Still, J. L. A re-examination of some problems of "petite" yeast. *Australian J. Sci.* **18**:165; 1956.
120. Gregolin, C.; Ghiretti-Magaldi, A. The lactic dehydrogenases of yeast. II. The identification of the L(+) lactic dehydrogenase of "petite" yeast as cytochrome b_2. *Biochim. Biophys. Acta* **54**:62–66; 1961.
121. Schatz, G.; Tuppy, H.; Klima, J. Separation and characterization of cytoplasmic particles from normal and respiratory-deficient baker's yeast. A contribution to the question of the genetic continuity of the mitochondria of *Saccharomyces cerevisiae*. *Z. Naturforsch* **18B**:145–153; 1963.
122. Mahler, H. R.; Mackler, B.; Grandchamp, S.; Slonimski, P. P. Biochemical correlates of respiratory deficiency. I. The isolation of a respiratory particle. *Biochemistry* **3**:668–677; 1964.
123. Perlman, P. S.; Mahler, H. R. Formation of yeast mitochondria III: biochemical properties of mitochondria isolated from a cytoplasmic petite mutant. *Bioenergetics* **1**:113–138; 1970.
124. Schatz, G.; Groot, G. S. P.; Mason, T.; Rouslin, W.; Wharton, D. C.; Saltzgaber, J. Biogenesis of mitochondrial inner membranes in bakers yeast. *Fed. Proc.* **31**:21–29; 1972.
125. Packer, L.; Williams, M. A.; Criddle, R. S. Freeze-fracture

studies on mitochondria from wild-type and respiratory-deficient yeasts. *Biochim. Biophys. Acta* **292**:92–104; 1973.
126. Mahler, H. R.; Neiss, G.; Slonimski, P. P.; Mackler, B. Biochemical correlates of respiratory deficiency. III. The level of some unsaponifiable lipids in different strains of baker's yeast. *Biochemistry* **3**:893–895; 1964.
127. Gordon, P. A.; Stewart, P. R. Ubiquinone formation in wild-type and petite yeast: the effect of catabolite repression. *Biochim. Biophys. Acta* **177**:358–360; 1969.
128. Boveris, A.; Turrens, J. F. Production of superoxide anion by the NADH-dehydrogenase of mammalian mitochondria. In: Bannister, J. V.; Hill, H. A. O., eds. *Chemical and biochemical aspects of superoxide and superoxide dismutase*. Vol. 1. Amsterdam: Elsevier; 1980:84–91.
129. Turrens, J. F.; Freeman, B. A.; Levitt, J. G.; Crapo, J. D. The effect of hyperoxia on superoxide production by lung submitochondrial particles. *Arch. Biochem. Biophys.* **217**:401–410; 1982
130. Williamson, D. H.; Moustacchi, E. The synthesis of mitochondrial DNA during the cell cycle in the yeast *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Comm.* **42**:195–201; 1971.
131. Bogenhagen, D.; Clayton, D. A. Mouse L cell mitochondrial DNA molecules are selected randomly for replication throughout the cell cycle. *Cell* **11**:719–727; 1977.
132. Clayton, D. A. Replication of animal mitochondrial DNA. *Cell* **28**:693–705; 1982.
133. Birky, C. W. Jr. Relaxed cellular controls and organelle heredity. *Science* **222**:468–475; 1983.
134. Bernardi, G. The petite mutation in yeast. *TIBS* **4**:197–201, 1979.
135. Wallace, D. C. Maternal genes: mitochondrial diseases. *Birth Defects* **23**:137–190; 1987.
136. Tanaka, M.; Nishikimi, M.; Ozawa, T. Lack of subunit II of cytochrome *c* oxidase in a patient with mitochondrial myopathy. *Ann. NY Acad. Sci.* **488**:503–504; 1986.
137. Carafoli, E. Mitochondrial pathology: an overview. *Ann. NY Acad. Sci.* **488**:1–18; 1986.
138. Boveris, A.; Oshino, N.; Chance, B. The cellular production of hydrogen peroxide. *Biochem. J.* **128**:617–630; 1972.
139. Mitchell, P. Possible molecular mechanisms of the protonmotive function of cytochrome systems. *J. Theor. Biol.* **62**:327–367; 1976.
140. Trumpower, B. L. Evidence for a protonmotive Q cycle mechanism of electron transfer through the cytochrome b-c_1 complex. *Biochem. Biophys. Res. Comm.* **70**:73–80; 1976.
141. De Vries, S.; Albracht, S. P. J.; Berden, J. A.; Slater, E. C. The pathway of electrons through QH_2:cytochrome *c* oxidoreductase studied by pre-steady-state kinetics. *Biochim. Biophys. Acta* **681**:41–53; 1982.
142. Slater, E. C. The Q cycle, an ubiquitous mechanism of electron transfer. *TIBS* 7:239–242; 1983.
143. De Vries, S. The pathway of electron transfer in the dimeric QH_2:cytochrome *c* oxidoreductase. *J. Bioenerg. Biomemb.* **18**:195–224; 1986.
144. De Vries, S.; Albracht, S. P. J.; Berden, J. A.; Marres, C. A. M.; Slater, E. C. The effect of pH, ubiquinone depletion and myxothiazol on the reduction kinetics of the prosthetic groups of uhiquinol:cytochrome c oxidoreductase. *Biochim. Biophys. Acta* **723**:91–103; 1983.
145. Mitchell, P.; Moyle, J. The role of ubiquinone and plastoquinone in chemiosmotic coupling between electron transfer and proton translocation. In: Lenaz, G., ed. *Coenzyme Q*. John Wiley & Sons Ltd.; 1985:145–163.
146. Rieske, J. S.; Ho, S. H. K. Respiratory complex III: structure–function relationships. In: Lenaz, G., ed. *Coenzyme Q*. John Wiley & Sons Ltd.; 1985:337–363.
147. Berry, E. A.; Trumpower, B. L. Pathways of electrons and protons through the cytochrome bc_1 complex of the mitochondrial respiratory chain. In: Lenaz, G., ed. *Coenzyme Q*. John Wiley & Sons Ltd.; 1985:365–389.
148. Rich, P. R. A perspective on Q-cycles. *J. Bioenerg. Biomemb.* **18**:145–156; 1986.
149. Rieske, J. S. Experimental observations on the structure and function of mitochondrial complex III that are unresolved by the protonmotive ubiquinone-cycle hypothesis. *J. Bioenerg. Biomemb.* **18**:235–257; 1986.
150. Von Jago, G.; Link, T. A.; Ohnishi, T. Organization and function of cytochrome *b* and ubiquinone in the cristae membrane of beef heart mitochondria. *J. Bioenerg. Biomemb.* **18**:157–179; 1986.
151. Rich, P. R.; Wikstrom, M. Evidence for mobile semiquinone in the redox cycle of the mammalian cytochrome bc_1 complex. *FEBS Lett.* **194**:176–182; 1986.
152. De Vries, S.; Vanhoek, A. N.; Berden, J. A. The oxidation-reduction kinetics of cytochrome-*b*, cytochrome-c_1 and cytochrome-*c* in initially fully reduced mitochondrial-membranes are in agreement with the Q-cycle hypothesis. *Biochim. Biophys. Acta* **935**:208–216; 1988.
153. Wikstrom, M.; Krab, K. The semiquinone cycle. A hypothesis of electron transfer and proton translocation in cytochrome *bc*-type complexes. *J. Bioenerg. Biomemb.* **18**:181–193; 1986.
154. Ohnishi, T.; Trumpower, B. L. Differential effects of antimycin on ubisemiquinone bound in different environments in isolated succinate cytochrome *c* reductase complex. *J. Biol. Chem.* **255**:3278–3284; 1980.
155. De Vries, S.; Berden, J. A.; Slater, E. C. Properties of a semiquinone anion located in the QH_2:cytochrome *c* oxidoreductase segment of the mitochondrial respiratory chain. *FEBS Lett.* **122**:143–148; 1980.
156. De Vries, S.; Albracht, S. P. J.; Berden, J. A.; Slater, E. C. A new species of bound ubisemiquinone anion in QH_2:cytochrome *c* oxidoreductase. *J. Biol. Chem.* **256**: 11996–11998; 1981.
157. Sen, K.; Beattie, D. S. Decreased amounts of core proteins I and II and the iron-sulfur protein in mitochondria from yeast lacking cytochrome *b* but containing cytochrome c_1. *Arch. Biochem. Biophys.* **242**:393–401; 1985.
158. Zhu, Q. S.; Sprague, S. G.; Beattie, D. S. Reduction of exogenous quinones and 2,6-dichlorophenol indophenol in cytochrome-*b*-deficient yeast mitochondria: a differential effect on center-*i* and center-*o* of the cytochrome-b-c_1 complex. *Arch. Biochem. Biophys.* **265**:447–453; 1988.
159. Sen, K.; Beattie, D. S. Cytochrome *b* is necessary for the effective processing of core protein I and the iron-sulfur protein of complex III in the mitochondria. *Arch. Biochem. Biophys.* **251**:239–249; 1986.
160. Japa, S.; Beattie, D. S. Cytochrome *b* is necessary for the assembly of subunit VII in the cytochrome b-c_1 complex of yeast mitochondria. *Arch. Biochem. Biophys.* **264**:231–237; 1988.
161. Weber, S.; Wolf, K. 2 Changes of the same nucleotide confer resistance to diuron and antimycin in the mitochondrial cytochrome-*b* gene of *Schizosaccharomyces-pombe*. *FEBS Lett.* **237**:31–34; 1988.
162. Howell, N.; Gilbert, K. Mutational analysis of the mouse mitochondrial cytochrome-*b* gene. *J. Molec. Biol.* **203**:607–618; 1988.
163. Zoccarato, F.; Cavallini, L.; Deana, R.; Alexandre, A. Pathways of hydrogen-peroxide generation in guinea-pig cerebral-cortex mitochondria. *Biochem. Biophys. Res. Commun.* **154**: 727–734; 1988.
164. Forman, H. J.; Kennedy, J. A. Role of superoxide radical in mitochondrial dehydrogenase reactions. *Biochem. Biophys. Res. Comm.* **60**:1044–1050; 1974.
165. Forman, H. J.; Kennedy, J. Superoxide production and electron transport in mitochondrial oxidation of dihydroorotic acid. *J. Biol. Chem.* **250**:4322–4326; 1975.
166. Forman, H. J.; Kennedy, J. Dihdyroorotate-dependent superoxide production in rat brain and liver. A function of the primary dehydrogenase. *Arch. Biochem. Biophys.* **173**:219–224; 1976.
167. Forman, H. J.; Kennedy, J. Mammalian dihydroorotate dehydrogenase: physical and catalytic properties of the primary

enzyme. *Arch. Biochem. Biophys.* **191**:23–31; 1978.
168. Ragan, C. I. The molecular organization of NADH dehydrogenase. *Subcellular Biochem.* **7**:267–307; 1980.
169. Friedrich, T.; Hofhaus, G.; Ise, W.; Nehls, U.; Schmitz, B.; Weiss, H. A small isoform of NADH-ubiquinone oxidoreductase without mitochondrially encoded subunits is made in chloramphenicol-treated *Neurospora-crassa*. *Eur. J. Biochem.* **180**:173–180; 1989.
170. Lee, F-J.; Hassan, H. M. Biosynthesis of superoxide dismutase in *Saccharomyces cerevisiae*: effects of paraquat and copper. *Free Radical Biol. Med.* **1**:319–325; 1985.
171. Lee, F-J.; Hassan, H. M. Biosynthesis of superoxide dismutase and catalase in *Saccharomyces cerevisiae*: effects of oxygen and cytochrome *c* deficiency. *J. Indust. Microbiol.* **1**:187–193; 1986.
172. Westerbeek-Marres, C. A. M.; Moore, M. M.; Autor, A. P. Regulation of manganese superoxide dismutase in *Saccharomyces cerevisiae*. The role of respiratory chain activity. *Eur. J. Biochem.* **174**:611–620; 1988.
173. Weisiger, R. A.; Fridovich, I. Mitochondrial superoxide dismutase. *J. Biol. Chem.* **248**:4793–4796; 1973.
174. Ruis, H. The biosynthesis of catalase. *Can J Biochem* **57**:1122–1130; 1979.
175. Reid, G. A.; Schatz, G. Import of proteins into mitochondria. Yeast cells grown in the presence of carbonyl cyanide *m*-chlorophenylhydrazone accumulate massive amounts of some mitochondrial precursor proteins. *J. Biol. Chem.* **257**:13056–13061; 1982.
176. Goffeau, A.; Crosby, B. A new type of cyanide-insensitive, azide-sensitive respiration in the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. In: Bacilla, M.; Horecker, B. L.; Stoppani, A. O. M., eds. *Biochemistry and genetics of yeasts*. New York: Academic Press; 1978:81–96.
177. Tissieres, A.; Mitchell, H. K.; Haskins, F. A. Studies on the respiratory system of the poky strain of *Neurospora*. *J. Biol. Chem.* **205**:423–433; 1953.
178. Criddle, R. S.; Schatz, G. Promitochondria of anaerobically grown yeast. 1. Isolation and biochemical properties. *Biochemistry* **8**:322–334; 1969.
179. Autor, A. P. Regulation of manganese superoxide dismutase by oxygen in *Saccharomyces cerevisiae*. *Biochem. Soc. Transactions* **10**:78–85; 1982.
180. Lotscher, H. R.; Winterhalter, K. H.; Carafoli, E.; Richter, C. Hydroperoxide-induced loss of pyridine nucleotides and release of calcium from rat liver mitochondria. *J. Biol. Chem.* **255**:9325–9330; 1980.
181. Bellomo, G.; Jewell, S. A.; Thor, H.; Orrenius, S. Regulation of intracellular calcium compartmentation: studies with isolated hepatocytes and *t*-butyl hydroperoxide. *Proc. Natl. Acad. Sci. USA* **79**:6842–6846; 1982.
182. Jewell, S. A.; Bellomo, G.; Thor, H.; Orrenius, S. Bleb formation in hepatocytes during drug metabolism is caused by disturbances in thiol and calcium ion homeostasis. *Science* **217**:1257–1259; 1982.
183. Richter, C.; Frei, B. Ca^{2+} release from mitochondria induced by prooxidants. *Free Radical Biol. Med.* **4**:365–375; 1988.
184. Thor, H.; Hartzell, M. T.; Bellomo, G.; Jewell, S. A.; Orrenius, S. The metabolism of menadione (2-methyl-1,4-napthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells. *J. Biol. Chem.* **257**:12419–12425; 1982.
185. Bellomo, G.; Martino, A.; Richelmi, P.; Moore, G. A.; Jewell, S. A.; Orrenius, S. Pyridine nucleotide oxidation, Ca^{2+} cycling and membrane damage during *tert*-butyl hydroperoxide metabolism by rat-liver mitochondria. *Eur. J. Biochem.* **140**:1–6; 1984.
186. Smith, T.; Thor, H.; Jewell, S. A.; Bellomo, G.; Sandy, M. S.; Orrenius, S. Free radical-induced changes in the surface morphology of isolated hepatocytes. In: Armstrong, D.; Sohal, R. S.; Cutler, R. G.; Slater, T. F., eds. *Free radicals in molecular biology, aging, and disease*. Aging Vol 27. New York: Raven Press; 1984:103–118.
187. Whitfield, J. F.; MacManus, J. P.; Rixon, R. H.; Boynton, A. L.; Youdale, T.; Swierenga, S. The positive control of cell proliferation by the interplay of calcium ions and cyclic nucleotides. A review. *In Vitro* **12**:1–18; 1976.
188. Whitfield, J. F.; Boynton, A. L.; MacManus, J. P.; Rixon, R. H.; Sikroska, M.; Tsang, B.; Walker, P. R.; Swierenga, S. H. H. The role of calcium and cyclic AMP in cell proliferation. *Ann NY Acad Sci* **339**:216–240; 1980.
189. Chambers, E. L. Fertilization and cleavage of eggs of the sea urchin *Letechinus variegatus* in Ca^{2+}-free sea water. *Eur. J. Cell Biol.* **22**:476 abstr.; 1980.
190. Wolniak, S. M.; Hepler, P. K.; Jackson, W. T. Ionic changes in the mitotic apparatus at the metaphase/anaphase transition. *J. Cell Biol.* **96**:598–605; 1983.
191. Poenie, M.; Alderton, J.; Tsien, R. Y.; Steinhardt, R. A. Changes of free calcium levels with stages of the cell division cycle. *Nature* **315**:147–149; 1985.
192. Steinhardt, R. A.; Alderton, J. A. Intracellular free calcium rise triggers nuclear envelope breakdown in the sea urchin embryo. *Nature* **332**:364–366; 1988.
193. Moolenaar, W. H.; Defize, L. H. K.; Tilly, B. C.; Bierman, A. J.; De Laat, S. W. Transmembrane signalling by growth factors. *Ann. NY Acad. Sci.* **488**:491–502; 1986.
194. Cantley, L. C.; Whitman, M.; Chahwala, S.; Fleischman, L.; Kaplan, D. R.; Schaffhausen, B. S.; Roberts, T. M. Oncogenes and phosphatidylinositol turnover. *Ann. NY Acad. Sci.* **488**:481–490; 1986.
195. Sager, R. Tumor suppressor genes: the puzzle and the promise. *Science* **246**:1406–1512; 1989.
196. Marx, J. Many gene changes found in cancer. *Science* **246**:1386–1388; 1989.
197. Sato, N.; Chance, B.; Kato, K.; Kleitman, W. Comparative study of cytochromes between virus-transformed and untransformed cells. *Biochim. Biophys. Acta* **305**:493–502; 1973.
198. Sato, N.; Hagihara, B. Spectrophotometric analyses of cytochromes in Ascites hepatomas of rats and mice. *Cancer Res.* **30**:2061–2068; 1970.
199. Dionisi, O.; Galeotti, T.; Terranova, T.; Azzi, A. Superoxide radicals and hydrogen peroxide formation in mitochondria from normal and neoplastic tissues. *Biochim. Biophys. Acta* **403**:292–300; 1975.
200. Bize, I. B.; Oberley, L. W.; Morris, H. P. Superoxide dismutase and superoxide radical in Morris hepatomas. *Cancer Res.* **40**:3686–3693; 1980.
201. Konstantinov, A. A.; Peskin, A. V.; Popova, E. Y.; Khomutov, G. B.; Ruuge, E. K. Superoxide generation by the respiratory chain of tumor mitochondria. *Biochim. Biophys. Acta* **894**:1–10; 1987.
202. Oberley, L. W.; Buettner, G. R. Role of superoxide-dismutase in cancer: review. *Cancer Res.* **39**:1141–1149; 1979.
203. Bannister, W. H.; Federici, G.; Heath, J. K.; Bannister, J. V. Antioxidant systems in tumour cells: the levels of antioxidant enzymes, ferritin, and total iron in a human hepatoma cell line. *Free Radical Res. Comm.* **1**:361–367; 1986.
204. Oberley, L. W.; McCormick, M. L.; Sierra-Rivera, E.; Kasemset-St. Clair, D. Manganese superoxide dismutase in normal and transformed human embryonic lung fibroblasts. *Free Radical Biol. Med.* **6**:379–384; 1989.
205. Westman, N. G.; Marklund, S. L. Copper and zinc-containing superoxide dismutase and manganese-containing superoxide dismutase in human tissues and human malignant tumors. *Cancer Res.* **41**:2962–2966; 1981.
206. Marklund, S. L.; Westman, N. G.; Lundgren, E.; Roos, G. Copper and zinc-containing superoxide dismutase, manganese-containing superoxide dismutase, catalase, and glutathione peroxidase in normal and neoplastic cell lines and normal human tissues. *Cancer Res.* **41**:1955–1961; 1982.
207. Del Maestro, R.; McDonald, W.; Anderson, R. Superoxide dismutases, catalase and glutathione peroxidase in experimental and human brain tumors. In: Greenwald, R. A.; Cohen, G., eds. *Oxy radicals and their scavenger systems*. Vol II. Amsterdam: Elsevier; 1983:28–35.

208. Utsumi, K.; Yamamoto, G.; Inaba, K. Failure of Fe^{2+}-induced lipid peroxidation and swelling in the mitochondria isolated from ascites tumor cells. *Biochim. Biophys. Acta* **105**:368–371; 1965.
209. Player, T. J.; Mills, D. J.; Horton, A. A. NADPH-dependent lipid peroxidation in mitochondria from livers of young and old rats and from hepatoma D30. *Biochem. Soc. Transactions* **5**:1506–1508; 1977.
210. Bartoli, G. M.; Galeotti, T. Growth-related lipid peroxidation in tumour microsomal membranes and mitochondria. *Biochim. Biophys. Acta* **574**:537–541; 1979.
211. Benedetti, A.; Malvaldi, G.; Fulceri, R.; Comporti, M. Loss of lipid peroxidation as a histochemical marker for preneoplastic hepatocellular foci of rats. *Cancer Res.* **44**:5712–5717; 1984.
212. Cornwell, D. G.; Morisaki, N. Fatty acid paradoxes in the control of cell proliferation: prostaglandins, lipid peroxides, and cooxidation reactions. In: Pryor, W. A., ed. *Free radicals in biology*. Vol IV. Orlando: Academic Press; 1984:95–148.
213. Cheeseman, K. H.; Collins, M.; Proudfoot, K.; Slater, T. F.; Burton, G. W.; Webb, A. C.; Ingold, K. U. Studies on lipid peroxidation in normal and tumour tissues. *Biochem. J.* **235**:507–514; 1986.
214. Masotti, L.; Casali, E.; Gaelotti, T. Lipid peroxidation in tumour cells. *Free Radical Biol. Med.* **4**:377–386; 1988.
215. Canuto, R. A.; Biocca, M. E.; Muzio, G.; Dianzani, M. U. Fatty acid composition of phospholipids in mitochondria and microsomes during diethylnitrosamine carcinogenesis in rat liver. *Cell Biochem. & Function* **7**:11–19; 1989.
216. Galeotti, T.; Borrello, S.; Minotti, G. Membrane alterations in cancer cells. The role of oxy radicals. *Ann. NY Acad. Sci.* **488**:469–480; 1986.
217. Schauenstein, E.; Esterbauer, H. Formation and properties of reactive aldehydes. In: *Submolecular biology and cancer*. Ciba Found Symp 67. Amsterdam: Excerpta Medica; 1979:225–244.
218. Esterbauer, H. Aldehydic products of lipid peroxidation. In: McBrien, D. C. H.; Slater, T. F., eds. *Free radicals, lipid peroxidation and cancer*. London: Academic Press; 1982: 101–128.
219. Dianzani, U. Biochemical effects of saturated and unsaturated aldehydes. In: McBien, D. C. H.; Slater, T. F., eds. *Free radicals, lipid peroxidation and cancer*. London: Academic Press; 1982:129–158.
220. Schauenstein, E. Effects of low concentrations of aldehydes on tumour cells and tumour growth. *Free radicals, lipid peroxidation and cancer*. In: McBrien, D. C. H.; Slater, T. F., eds. London: Academic Press; 1982:159–172.
221. Slater, T. F.; Cheeseman, K. H.; Proudfoot, K. Free radicals, lipid peroxidation, and cancer. In: Armstrong, D.; Sohal, R. S.; Cutler, R. G.; Slater, T. F., eds. *Free radicals in molecular biology, aging, and disease*. Aging Vol. 27. New York: Raven Press; 1984:293–306.
222. Vaca, C. E.; Wilhelm, J.; Harms-Ringdahl, M. Interaction of lipid peroxidation products with DNA. A review. *Mutation Res.* **195**:137–149; 1988.
223. Cajone, F.; Bernelli-Zazzera, A. The action of 4-hydroxynonenal on heat shock expression in cultured hepatoma cells. *Free Radical Res. Comm.* **7**:189–194; 1989.
224. Begin, M. E. Effects of polyunsaturated fatty acids and of their oxidation products on cell survival. *Chem. Phys. Lipids* **45**:269–313; 1987.
225. Das, U. N.; Begin, M. E.; Ells, G.; Huang, Y. S.; Horrobin, D. F. Polyunsaturated fatty acids augment free radical generation in tumour cells in vitro. *Biochem. Biophys. Res. Comm.* **145**:15–24;1987.
226. Das, U. N.; Huang, Y.-S.; Begin, M. E.; Ells, G.; Horrobin, D. F. Uptake and distribution of *cis*-unsaturated fatty acids and their effect on free radical generation in normal and tumor cells in vitro. *Free Radical Biol. Med.* **3**:9–14; 1987.
227. Player, T. J. Lipid peroxidation in rat liver, hepatomas and regenerating liver. In: McBrien, D. C. H.; Slater, T. F., eds. *Free radicals, lipid peroxidation and cancer*. London: Academic Press; 1982:173–192.
228. Duchesne, J. A unifying biochemical theory of cancer, senescence and maximal life span. *J. Theor. Biol.* **66**:137–145; 1977.
229. Warburg, O. On the origin of cancer cells. *Science* **123**:309–314; 1956.
230. Szent-Gyorgyi, A.; Egyud, L. G.; McLaughlin, J. A. Ketoaldehydes and cell division. *Science* **155**:539–541; 1967.
231. Szent-Gyorgyi, A. Electronic biology and cancer. In: Kaminer, B., ed. *Search and discovery: a tribute to Albert Szent-Gyorgyi*. New York: Academic Press; 1977:329–335.
232. Szent-Gyorgyi, A. The living state and cancer. *Submolecular biology and cancer*. Ciba Foundation Symposium 67. Amsterdam: Excerpta Medica; 1979:3–18.
233. Miquel, J.; Economos, A. C.; Bensch, K. G.; Atlan, H.; Johnson, E., Jr. Review of cell aging in *Drosophila* and mouse. *Age* **2**:78–88; 1979.
234. Pierce, G. B. Differentiation of normal and malignant cells. *Fed. Proc.* **29**:1248–1254; 1970.

Chapter 4.

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Interactions between Metals, Ligands, and Oxygen in the Autoxidation of 6-Hydroxydopamine: Mechanisms by which Metal Chelation Enhances Inhibition by Superoxide Dismutase[1]

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Transition metal ions and superoxide participate in different autoxidations to a variable extent. In the reaction of 6-hydroxydopamine (6-OHDA) with oxygen at pH 7.0 or 8.0, addition of 5 to 300 U/ml superoxide dismutase inhibited autoxidation by up to 96% at the highest concentrations. Superoxide dismutase at concentrations of 5-20 U/ml inhibited by less than 40% when present alone, but inhibited by over 99% in the presence of desferrioxamine or histidine. EDTA also enhanced the inhibition by 20 U/ml superoxide dismutase to 86%, even though EDTA *accelerated* the autoxidation of 6-OHDA when present alone or with desferrioxamine. In contrast, other ligands, such as ADP or phytic acid, had little or no effect on inhibition by superoxide dismutase. Proteins such as albumin, cytochrome oxidase, or denatured superoxide dismutase also enhanced inhibition by active superoxide dismutase from less than 40% to over 90%. Evidently, in the presence of redox active metals, autoxidation occurs by inner sphere electron transfer, presumably within a ternary 6-OHDA · metal · oxygen complex. This mechanism does not involve free $O_2^{\cdot}$ and is not inhibited by superoxide dismutase. On the other hand, the presence of certain ligands (including proteins) diminishes the ability of trace metals to exchange electrons with 6-OHDA or oxygen by an inner sphere mechanism. These ligands render autoxidation dependent on propagation by $O_2^{\cdot}$ and therefore inhibitable by superoxide dismutase. Previously conflicting reports that superoxide dismutase alone inhibits 6-OHDA autoxidation are thus explicable on the basis that at sufficient concentration the apoprotein coordinates trace metals in such a way to preclude inner sphere metal catalysis.

Interactions between metals, their ligands, and oxygen must be considered when investigating biological autoxidations. The autoxidation of catecholamines such as 6-hydroxydopamine (6-OHDA),[3] or other autoxidizable compounds is stimulated by added metal ions such as Cu^{2+} or Fe^{2+} (1-4). In addition, since the direct reaction of oxygen with organic reductants is spin restricted, many "autoxidations" require transition metal catalysis (5). Even so reactive a compound as 6-OHDA does not reduce molecular oxygen directly (6), since the reaction reportedly requires a ternary 6-OHDA · metal-oxygen complex (6, 7).

Superoxide acts as an autocatalytic intermediate in the aerobic oxidation of 6-OHDA, and hence, superoxide dismutase inhibits this autoxidation by 93-96% (6-9). However, Sullivan and Stern report that superoxide dismutase failed to inhibit 6-OHDA autoxidation except in the presence of the metal chelators EDTA or DE-

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[3] Abbreviations used: 6-OHDA, 6-hydroxydopamine; SOD, superoxide dismutase.

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TAPAC (4). Moreover, addition of iron (EDTA chelate) accelerated autoxidation and rendered it insensitive to superoxide dismutase. Thus O_2^- does not appear to contribute to oxidation of 6-OHDA in the presence of transition metal ion.

The catalytic efficiency of a particular metal and its reactivity with a reductant or with oxygen depend largely on its coordination structure and thus on the nature of available ligands. For example, desferrioxamine forms a stable octahedral coordination complex with ferric ion by virtue of its six oxygen donor atoms and prevents the reduction of iron (10). Thus, desferrioxamine inhibits iron-catalyzed autoxidation of 6-OHDA (2) as well as iron-catalyzed · OH formation or lipid peroxidation (11–13). EDTA, on the other hand, is often found to accelerate these reactions (4, 11, 14). Crystallography of the Fe^{2+} · EDTA complex reveals a hexadentate complex which displays an unusual seventh coordination site occupied by water (15). This coordinated H_2O can be readily displaced by ligands such as azide, H_2O_2 or oxygen, allowing the chelated iron to participate in redox reactions (16). While EDTA stabilizes Fe^{3+} and thus slows its reduction, it does not *prevent* the reduction of iron (as evidenced by reduction of Fe^{3+} · EDTA, but not Fe^{3+} · DETAPAC or Fe^{3+} · desferrioxamine, by O_2^-) (17, 18). In addition to the interaction between reductant and metal, the stability of reduced metal, metal · oxygen, or metal · superoxide complexes depends in turn on the nature of other ligands present (19–21). With the hope of providing a more systematic and complete understanding of the roles of ligands in the interactions between reductants, metals, and oxygen we investigated the effects of various added ligands on the rate of autoxidation and on the ability of superoxide dismutase to inhibit autoxidation of 6-OHDA.

MATERIALS AND METHODS

Reagents. 6-OHDA hydrobromide (Sigma Chemical Co.) was prepared anaerobically in H_2O immediately before use. Substantial exclusion of oxygen from stock solutions was confirmed by the absence of significant concentrations of the *p*-quinone oxidation product at the start of each assay. Superoxide dismutase (bovine blood), EDTA, phytic acid, ADP, cytochrome *c* (type III), bovine serum albumin, and potassium phosphate buffer salts were also from Sigma. Desferrioxamine (Desferal Mesylate) was a gift from CIBA. Histidine was from Coleman, Matheson, and Bell. Cytochrome oxidase was prepared in this laboratory by the method of Wainio *et al.* (22) and was assayed to have an activity of 0.231 s^{-1} mg^{-1} ml at an initial ferrocytochrome *c* concentration of 20 μM. Catalase, from Boehringer Manheim, was assayed for superoxide dismutase activity (according to the method of (23)) and was found to be free of superoxide dismutase activity even at 12500 U/ml of catalase activity. Sigma catalase on the other hand was confirmed to have substantial superoxide dismutase activity and was not used in these experiments.

Water, buffers, and metal ion contamination. Distilled, deionized water was used for preparation of reagent and buffer solutions. Transition metal ions were present largely as a result of trace contaminants in the buffer salts. In view of the ineffectiveness of the customary precautions to remove all traces of transition metal ions and because trace metals were desired in these experiments, no further attempts were made to lower the level of trace metals in the buffer solutions. These experiments should thus represent standard laboratory conditions.

Assay procedures and reaction conditions. Experiments were conducted at both pH 7.0 and pH 8.0 in 2.5 ml final volume air-saturated, 50 mM, potassium phosphate buffer; 25°C. Ligands were added to a concentration of 1 mM. This concentration was presumed to be sufficiently above the initial 6-OHDA concentration that the added ligand could compete effectively with the catechol hydroxyls of 6-OHDA for coordination of metals. The concentrations of any added proteins or enzymes are indicated in the legends to figures. Reactions were initiated upon addition by gas-tight syringe of a 25-μl aliquot of 6-OHDA stock solution to give an initial 6-OHDA concentration of 200 μM, just below that of dissolved oxygen. Formation of the *p*-quinone product of autoxidation was monitored spectrophotometrically at 490 nm. Data were collected on both a linear strip chart recorder and a microcomputer equipped with an analog-digital converter. The data were transferred to an IBM mainframe computer and analyzed using APL programmes. The initial rate constant of *para*-quinone formation was used as the measure of maximal reaction velocity. For those reactions which displayed an induction period, the subsequent linear phase of *p*-quinone formation was used to measure the rate constant at maximum velocity.

The activity of stock superoxide dismutase solutions was assayed by the method of McCord and Fridovich (23). Based on the observed dose-response

curves for inhibition of 6-OHDA autoxidation by superoxide dismutase (Fig. 5) a concentration of superoxide dismutase which inhibited by close to 40% was chosen for comparison of the effects of different ligands on the inhibition of 6-OHDA autoxidation by superoxide dismutase.

Since the contribution of cytochrome *c* to absorbance at 490 nm decreases as it becomes reduced, data were collected in comcomitant reactions monitored at 490 and 550 nm for those reactions involving cytochrome *c*. Simultaneous solutions for three equations (A_{490}, A_{550}, total cytochrome *c* concentration) in three unknowns (*p*-quinone concentration, ferrocytochrome *c* concentration, ferricytochrome *c* concentration) were then solved using molar absorbtivities determined specifically for these reaction conditions. This allowed calculation of the true rate of 6-OHDA autoxidation. The correction was not large for the reactions reported here (e.g., 10% of the A_{490} rate constant) since at 10 μM cytochrome *c* (added to both sample and reference cuvettes) the change in its contribution to absorbance at 490 nm was relatively small.

Data analyses. All reactions were performed at least in duplicate. Since the variability between duplicates was roughly proportional to the rate of the reaction, the relative standard error for the control reaction of a particular experiment (performed a number of times) was used to estimate the standard error for the other conditions. These estimates are represented by the error bars in the figures.

RESULTS

Effect of Ligands or Proteins Alone

The addition of ligands to the reaction mixture markedly influenced the rate of 6-OHDA autoxidation (Fig. 1a). At pH 7.0 desferrioxamine or histidine inhibited the reaction by over 50%. Addition of EDTA accelerated the rate of autoxidation to over twice that of 6-OHDA alone, while phytic acid or ADP had little or no effect. The results were similar at pH 8.0 (not shown) except that desferrioxamine was less inhibitory, EDTA less stimulatory, and phytic acid was somewhat stimulatory (40%). While the autoxidation of 6-OHDA is more rapid at pH 8.0 (with a rate constant of 8.7×10^{-3} for 6-OHDA alone versus 3.1×10^{-3} at pH 7.0) the effects of added ligands on the reaction rate were proportionately diminished. Thus the acceleration at increased pH is largely attributable to an increased contribution of *metal independent* pathways.

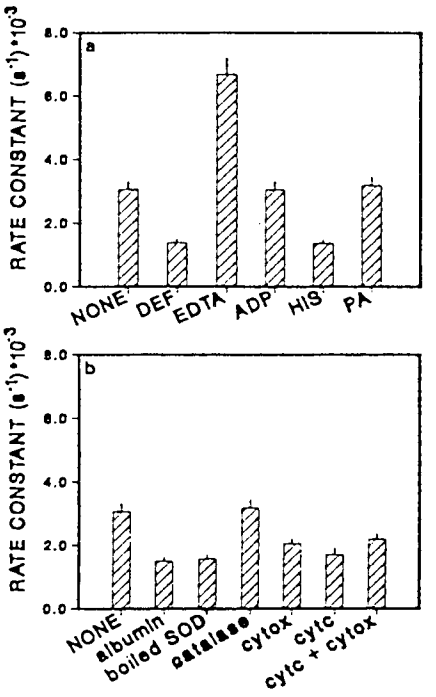

FIG. 1. (a) Effect of added ligands on the autoxidation of 6-OHDA. Reactions were carried out in air-saturated 50 mM phosphate buffer (pH 7.0) at 25°C. 6-OHDA (200 μM) was used to initiate the reaction and *p*-quinone formation was followed spectrophotometrically at 490 nm. Ligands present in the reaction mixture (at 1 mM) are indicated on the *x* axis. Abbreviations used: NONE, no ligand added; DEF, desferrioxamine; PA, phytic acid; HIS, histidine. Error bars represent estimates of standard deviation from the mean. (b) Effect of added proteins on the autoxidation of 6-OHDA. Reaction conditions as in (a). Proteins present are indicated on the *x* axis: albumin, bovine serum albumin (130 μg/ml); catalase (100 U/ml); boiled superoxide dismutase (100 μg/ml); cytox, cytochrome oxidase (100 μg/ml); cytc, cytochrome *c* (10 μM).

With a few exceptions, proteins (enzymic or nonenzymic) substantially inhibited 6-OHDA autoxidation (Fig. 1b). Thus, albumin (0.13 mg protein/ml), boiled superoxide dismutase (0.10 mg protein/ml), and cytochrome oxidase (0.1 mg/ml), all inhibited by 30-45% at either pH 7.0 or pH 8.0. Inhibition then, appears to be a general effect of added proteins, presumably mediated by chelation of trace metals. Moreover, albumin and to some extent cytochrome oxidase caused an induction pe-

riod before the maximal, linear phase of oxidation. This latency persisted after addition of catalase and was thus not due to accumulation of H_2O_2. There were some notable exceptions to the inhibitory effects of added proteins. Very low concentrations, such as the catalytic amounts of catalase (added to 1.5 μg protein/ml), were ineffective. Also, at pH 8.0 cytochrome *c* did not display the general inhibitory effect of added protein and, in the presence of cytochrome oxidase, actually accelerated the rate of 6-OHDA autoxidation over that of control (not shown). Cytochrome *c* is rapidly reduced by 6-OHDA under aerobic or anaerobic conditions (24) and cytochrome oxidase increases the contribution of this process by recycling ferricytochrome *c*.

Competing Effects of Ligands and Proteins on Inhibition by Desferrioxamine

At pH 7.0, ADP or phytic acid had little or no effect on inhibition by desferrioxamine (Fig. 2a). Histidine augmented the inhibition slightly. In contrast, EDTA not only removed the inhibition afforded by desferrioxamine but actually accelerated the rate of autoxidation in the presence of desferrioxamine to 27% greater than that of 6-OHDA alone. EDTA must therefore compete effectively with desferrioxamine for binding of one or more metals which, when coordinated with EDTA, actively catalyze this autoxidation. At pH 8.0 (not shown), desferrioxamine was less inhibitory and either histidine or ADP augmented the inhibition. EDTA again reversed the inhibition. Phytic acid was timulatory at this pH and, like EDTA, reversed inhibition by desferrioxamine.

Among the proteins tested, ablumin, catalase, boiled superoxide dismutase (at pH 7.0) or cytochrome oxidase had little or no effect on the inhibition afforded by desferrioxamine (Fig. 2b), although albumin did produce an induction period. At pH 8.0, boiled superoxide dismutase enhanced the inhibition by desferrioxamine (from 24 to 87%). It should be noted however, that we were not able to completely inactivate the superoxide dismutase preparation by boiling. Even with extensive

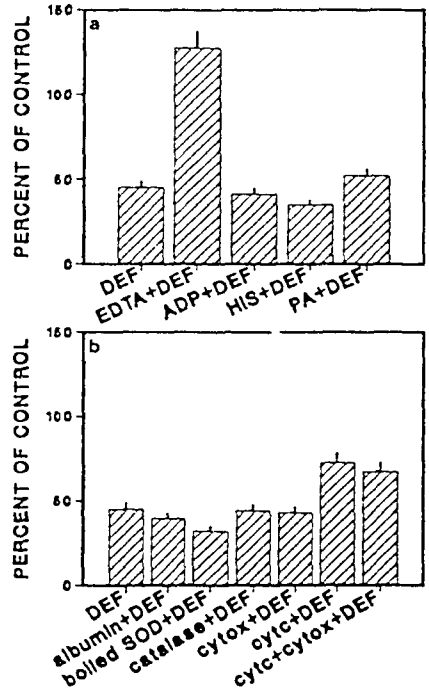

FIG. 2. (a) Inhibition by desferrioxamine: Effect of additional ligand. Reaction conditions and abbreviations as in Fig. 1a. The data are expressed as percentages of control (6-OHDA alone) rate constant (0.00306 s^{-1}). (b) Inhibition by desferrioxamine: Effect of added protein. Reaction conditions and abbreviations as in Fig. 1b. The data are expressed as percentages of control (6-OHDA alone) rate constant (0.00306 s^{-1}).

boiling we were only able to decrease the superoxide dismutase activity from 3200 to 50 U/mg. Thus, by adding 0.1 mg/ml of a boiled superoxide dismutase preparation we may be adding up to 5 U/ml of superoxide dismutase activity. Cytochrome *c*, either in the presence or the absence of cytochrome oxidase, removed approximately 50% of the inhibition provided by desferrioxamine alone at pH 7.0 and, at pH 8.0, accelerated the reaction over that of 6-OHDA alone. Cytochrome *c* thus provides a redox-active metal center which is inaccessable to coordination by desferrioxamine.

Inhibition by Superoxide Dismutase: Effects of Added Ligands or Proteins

The various ligands (including proteins) were tested for their ability to augment or

relieve inhibition of 6-OHDA autoxidation caused by a concentration of superoxide dismutase which alone inhibited by 40%. As has been found for other autoxidations (e.g., epinephrine, (25)) superoxide dismutase was much more inhibitory at higher pH. Thus 20 U/ml superoxide dismutase was used at pH 7.0 while only 5 U/ml was required at ph 8.0. Of the ligands tested, phytic acid or ADP had little or no effect on the inhibition by superoxide dismutase (Fig. 3a). At the other extreme, the presence of desferrioxamine plus superoxide dismutase inhibited 6-OHDA autoxidation almost completely (99.7%). As little as 5 U/ml superoxide dismutase was sufficient even at ph 7.0 to provide 99.5% inhibition in the presence of desferrioxamine (not shown). Histidine plus superoxide dismutase also afforded very substantial (e.g., 95% at pH 7.0) inhibition.

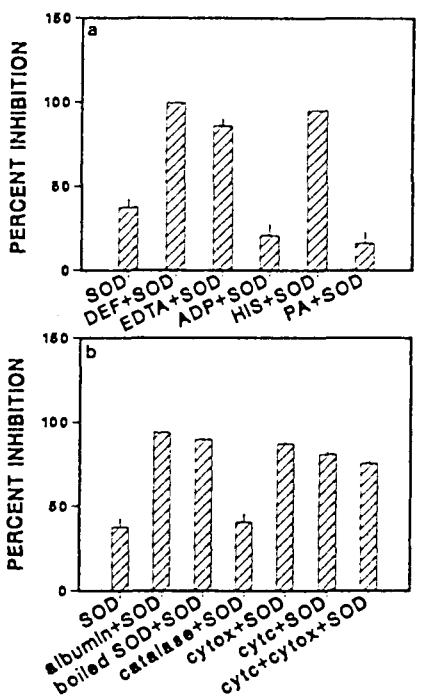

FIG. 3. (a) Inhibition by superoxide dismutase: Effect of added ligands. Reaction conditions and abbreviations as in Fig. 1a. The data are expressed as percentages of inhibition from the 6-OHDA alone condition. (b) Inhibition by superoxide dismutase: Effect of added proteins. Reacton conditions and abbreviations as in Fig. 1b. The data are expressed as percentages of inhibition from the 6-OHDA alone condition.

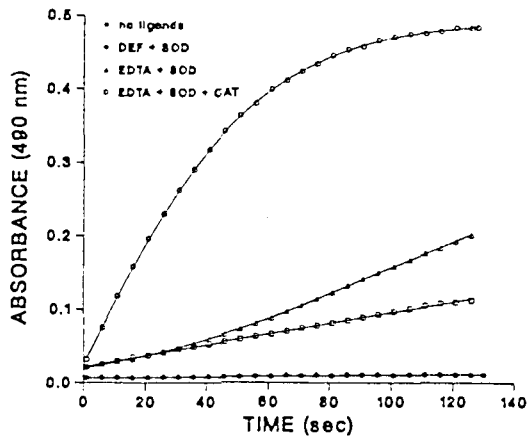

FIG. 4. Inhibition by EDTA + superoxide dismutase: Effect of catalase on the latent period. Reaction conditions as in Fig. 1a at pH 8.0 with superoxide dismutase present at 5 U/ml and catalase (CAT) present at 100 U/ml.

One of the more remarkable observations is that while EDTA accelerates 6-OHDA autoxidation when present alone or even with desferrioxamine (Figs. 1a and 2a), it dramatically augments inhibition of 6-OHDA autoxidation by superoxide dismutase (Fig. 3a). EDTA also changed the kinetics of the reaction in the presence of superoxide dismutase, producing a significant induction period. (The inhibition by superoxide dismutase + EDTA shown in Fig. 3a is calculated from the maximal rate, i.e., *after* the induction period.) This induction period could be abolished and net inhibition enhanced by futher addition of catalase (Fig. 4). Thus, in the presence of superoxide dismutase plus EDTA, accumulated H_2O_2 does contribute to the net rate of autoxidation.

Histidine produced a similar but briefer induction period in the presence of superoxide dismutase (not shown). With histidine plus superoxide dismutase, however, the induction period could not be abolished by further addition of catalase. In this case some species other than H_2O_2 (such as the semiquinone or paraquinone) is accumulating as a propagator of the autoxidation.

Several proteins also enhanced the inhibition of 6-OHDA autoxidation induced by superoxide dismutase (Fig. 3b). Albumin,

cytochrome oxidase, and boiled superoxide dismutase were the most effective, providing 87-93% inhibition in the presence of active superoxide dismutase. Note that although the boiled superoxide dismutase preparation contained some residual superoxide dismutase activity, the inhibition by active superoxide dismutase was enhanced to a much greater extent by 0.1 mg/ml boiled superoxide dismutase than the equivalent 5 U/ml superoxide dismutase activity which it provides (compare Fig. 3b with Fig. 5). Albumin plus superoxide dismutase again caused an induction period which was not abolished by catalase (not shown). Synergism between the inhibitory actions of superoxide dismutase and cytochrome *c* (or cytochrome *c* plus cytochrome oxidase) was less than with the other proteins tested (particularly at pH 8.0). This lessened effect is presumably due to stimulatory effects of the redox-active iron of cytochrome *c*. Catalytic quantities of catalase, on the other hand, did not enhance inhibition by superoxide dismutase.

Increasing the concentration of superoxide dismutase alone led to progressive increases in inhibition (Fig. 5). However, since in the presence of desferrioxamine only 5 U/ml superoxide dismutase was required for almost 100% inhibition, and since even inactivated superoxide dismutase enhanced the inhibition by active superoxide dismutase (Fig. 3b), much of the effect of increased superoxide dismutase concentration can be attributed to metal chelating effects of the apoprotein (0.33 μg protein/U superoxide dismutase activity). In the absence of a metal chelator it is difficult to say how much inhibition by superoxide dismutase is caused by removal of O_2^- and how much is caused by chelation of metals by the protein. One must be cautious therefore in interpreting results from experiments with superoxide dismutase added alone. In the presence of metal chelators on the other hand, the inhibitory effect of catalytic amounts of superoxide dismutase is consistent and dramatic (e.g., Fig. 3a).

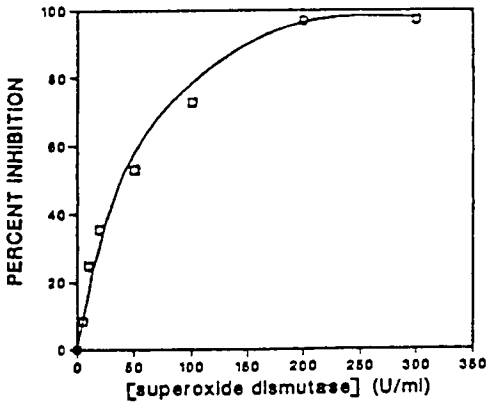

FIG. 5. Inhibition by superoxide dismutase: Dose-response curve. Reaction conditions as in Fig. 1a with no ligands added. Superoxide dismutase was present at the indicated concentration before initiating the reaction with 6-OHDA.

Inhibition by Superoxide dismutase Plus Desferrioxamine: Effect of Competing Ligands or Proteins

EDTA was unique among the ligands tested in that it relieved (by 25%) the inhibition provided by superoxide dismutase plus desferrioxamine (Fig. 6a). Thus EDTA must compete effectively with desferrioxamine for one or more redox-active metals and allows some autoxidation to occur even in the presence of superoxide dismutase. Further addition of catalase lessened this effect of EDTA (not shown).

None of the proteins except cytochrome *c* removed any of the inhibition by superoxide dismutase plus desferrioxamine (Fig. 6b). Cytochrome *c* removes 8% of the inhibition at pH 7.0 and 26% at pH 8.0. As before (Fig. 2b) cytochrome *c* acts as a metal center which is capable of directly oxidizing 6-OHDA but remains inaccessable to chelation by desferrioxamine. Predictably, in the presence of cytochrome oxidase this stimulatory action of cytochrome *c* is enhanced (Fig. 6b).

DISCUSSION

Metal Chelation Is Required for Inhibition of 6-OHDA Autoxidation by Superoxide Dismutase

The current results resolve a conflict between studies which showed substantial (93-96%) inhibition by superoxide dismutase alone (2, 6, 7) and those which found

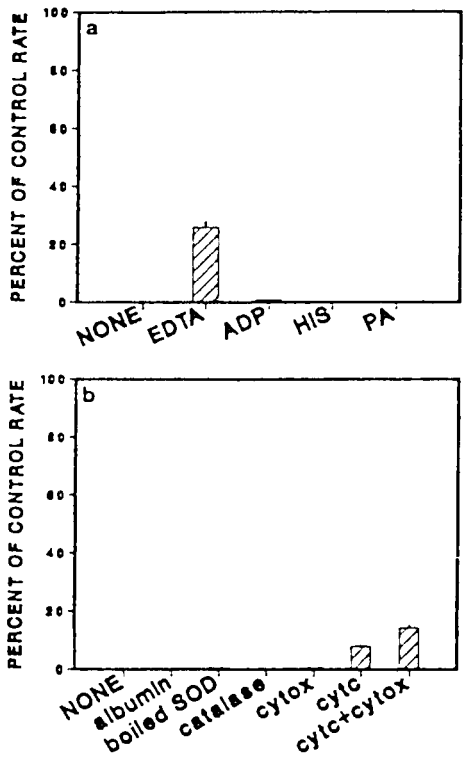

FIG. 6. (a) Inhibition by desferrioxamine + superoxide dismutase: Effect of additional ligands. Reaction conditions and abbreviations as in Fig. 1a. Desferrioxamine (1 mM) and superoxide dismutase (20 U/ml) were present in all cases. The additional ligands present are listed on the x axis. The data are expressed as percentages of control (6-OHDA alone) rate. (b) Inhibition by desferrioxamine + superoxide dismutase: Effect of additional proteins. Reaction conditions and abbreviations as in Fig. 1b. Desferrioxamine (1 mM) and superoxide dismutase (20 U/ml) were present in all cases. The additional ligands present are listed on the x axis.

no inhibition by superoxide dismutase except in the presence of EDTA or DETAPAC (4). High concentrations of superoxide dismutase (200 U/ml (66 μg/ml) or more) do indeed inhibit 6-OHDA autoxidation by over 90% (Fig. 5). On the other hand, 5 U/ml (1.67 μg/ml) had little effect alone (at pH 7.0) but inhibited by over 99% in the presence of desferrioxamine or histidine. Proteins such as albumin, cytochrome oxidase, or denatured SOD (at 100–130 μg protein/ml) also render the autoxidation inhibitable by 5–20 U/ml superoxide dismutase. Thus, the almost complete inhibition by 200 U/ml superoxide dismutase alone involves not only removal of $O_2^{\cdot-}$ but also metal chelation. The differences between the findings of previous investigators of 6-OHDA autoxidation, then, lie in the differing amount of superoxide dismutase added (relative to the concentration of trace metals present). Those studies which found greater than 90% inhibition by superoxide dismutase alone had added 250 U/ml (82 μg/ml) (6) to 300 U/ml (100 μg/ml) (8) at pH 7.0. Those studies which reported little or no inhibition by catalytic quantities of superoxide dismutase except in the presence of additional metal chelation had used 2.1 U/ml (0.7 μg/ml) (4) to 5 U/ml (1.7 μg/ml) (current study).

Roles of Metals in the Autoxidation of 6-OHDA

Trace metals serve as the primary initiating species in the autoxidation of 6-OHDA. This follows from the findings that removal of metal catalysis and $O_2^{\cdot-}$ with desferrioxamine plus 5 U/ml superoxide dismutase inhibit by almost 100% (therefore either metals or $O_2^{\cdot-}$ initiate) and that desferrioxamine alone (but not 5 U/ml superoxide dismutase alone) inhibits by over 50%. In the presence of a metal chelator such as desferrioxamine, then, $O_2^{\cdot-}$ serves as a major propagating species. Catalase, on the other hand, had no effect on the aerobic oxidation of 6-OHDA either in the presence or in the absence of desferrioxamine. Thus while H_2O_2 is capable of oxidizing 6-OHDA (26, 27) it does not contribute significantly to the process of autoxidation (unless $O_2^{\cdot-}$ is removed by superoxide dismutase). A reaction scheme consistent with the results might be as follows:

Initiation:

$$M^{n+} + \text{6-OHDA} \rightarrow sQ^{\cdot-} + M^{(n-1)+} \quad [1]$$

Propagation:

$$M^{(n-1)+} + O_2 \rightarrow M^{n+} O_2^{\cdot-} \quad [2]$$

$$sQ^{\cdot-} + O_2 \rightarrow pQ + O_2^{\cdot-} \quad [3]$$

$$O_2^{\cdot-} + \text{6-OHDA} \rightarrow sQ^{\cdot-} + H_2O_2 \quad [4]$$

(Further elaborations of this scheme could show participation of metals in Step [3], and dismutation of the semiquinone.)

Effects of Desferrioxamine

Desferrioxamine, which binds strongly to ferric ions (Ka = 10^{31} for Fe^{3+} versus Ka = 10^{10} for Fe^{2+}, (10)) and prevents their reduction (11, 12, 17), slows the net autoxidation (Fig. 1a), as well as the iron-catalyzed oxidation, of 6-OHDA (2, 28) due to inhibition of initiation. Thus desferrioxamine decreased (by 42%) the amount of 6-OHDA semiquinone detectable with ESR under anaerobic conditions (29), presumably by inhibiting participation of iron (16, 29). While desferrioxamine binds most strongly to iron, it also prevents stimulation of dopamine autoxidation by added copper (2) and of 6-OHDA autoxidation by added copper or manganese (in preparation).

In the presence of desferrioxamine, the initiating event (i.e., which allows 50% of the control rate at pH 7.0), is either oxidation by extremely low levels of unchelated metals, oxidation by a chelated metal, or direct oxidation by molecular oxygen. Any of these potentially initiating reactions occurs only very slowly, because when propagation by O_2^- is also removed (with superoxide dismutase) the autoxidation is inhibited 99.7%.

Effects of Histidine

Histidine also inhibited 6-OHDA autoxidation (in fact slightly more strongly than desferrioxamine at pH 8.0) and enhanced inhibition by superoxide dismutase from less than 40 to 86% or more (depending on pH). While ligands with oxygen donor atoms show greater affinity for Fe^{3+} than Fe^{2+}, ligands (like histidine) which contain nitrogen donor atoms preferentially bind Fe^{2+} over Fe^{3+} and thus stabilize Fe^{2+}, slowing its autoxidation (Reaction [2]) (5, 30, 31). Thus we would not expect that histidine should sensitize the reaction to superoxide dismutase by effects exerted on iron. On the other hand, such nitrogen-donating ligands bind more strongly to Cu^{2+} than to Cu^{+} (e.g., Ka = 10^{18} for $[Cu(Hist_2)]^{2+}$ versus Ka = 10^{7} for $[Cu(HistH_2)_2]^{+}$) (31). Consistent with the expected effects of a nitrogen donor ligand on the autoxidation of 6-OHDA, histidine had little effect on catalysis of 6-OHDA autoxidation by added iron, but it inhibited catalysis by added copper or manganese (in preparation). Also consistent with cooper and iron selectivities, histidine augmented inhibition of 6-OHDA autoxidation by desferrioxamine (particularly at pH 8.0).

Effects of EDTA

The observations that EDTA accelerates 6-OHDA autoxidation, but also greatly potentiates inhibition by superoxide dismutase (4) (Figs. 1a and 3a) suggest, of course, that EDTA changes the mechanism of autoxidation in such a way that chain propagation is more dependent on O_2^-. EDTA has similarly been found to sensitize the autoxidations of sulphite (32), of epinephrine (25), and of pyrogallol (33) to inhibition by superoxide dismutase, although EDTA alone did not stimulate but in fact slowed these autoxidations.

Since EDTA accelerates the autoxidation of 6-OHDA in the presence or absence of added iron (2, 4) (Fig. 1a), but inhibits stimulation of 6-OHDA autoxidation by added copper (1) or manganese (in preparation), we presume that the stimulatory effect of EDTA in the present system results predominately from its binding to traces of iron. EDTA is an oxygen donor ligand with a higher affinity for Fe^{3+} than Fe^{2+} (Ka = 10^{25} for Fe^{3+} versus 10^{14} for Fe^{2+}) (34) and therefore EDTA lowers the reduction potential of iron from 0.77 to 0.12 V (34, 35), accelerating the autoxidation of Fe^{2+} (36–38). The ability of EDTA to change the reduction potential of iron in this way explains the ability of EDTA *both* to *accelerate* autoxidation of strong electron donors (such as in the current system) and to *slow* autoxidation of less strong reductants (such as diphenols). The accelerated autoxidation of 6-OHDA in the presence of EDTA is evidence that (due to the strong reducing capacity of 6-OHDA) reduction of iron·EDTA by 6-OHDA is not rate limiting. Thus, slowing

of this step is more than compensated for by acceleration of the (rate-limiting) reoxidation of Fe^{2+} (recycling Fe^{3+} and stimulating O_2^- production). In contrast, the autoxidations of those weaker reductants which are inhibited by EDTA (dopamine, norepinephrine, epinephrine, or 6-aminodopamine) (2, 4, 25) are slowed because reduction of the metal is the rate-limiting process and is rendered even slower by the presence of EDTA.

Unexpectedly, EDTA added in the presence of desferrioxamine not only removed inhibition by desferrioxamine but also accelerated 6-OHDA autoxidation over that of 6-OHDA alone. This was unexpected since desferrioxamine has a much greater affinity for Fe^{3+} than EDTA ($Ka = 10^{31}$ versus 10^{25}) and since both desferrioxamine and EDTA *inhibit* catalysis of 6-OHDA oxidation by copper or manganese (1, in preparation). Since EDTA inhibits catalysis by redox-active metals other than iron, the $1:10^6$ of the iron bound by EDTA must be sufficient to stimulate autoxidation.

Effects of ADP

ADP had little or no effect on the rate of 6-OHDA autoxidation when added alone or with SOD and therefore does not affect redox cycling of the active metals. Similarly, ADP-iron participates in the Haber-Weiss reaction (e.g., Ref. (19)) or in iron-catalyzed lipid peroxidation (20, 21). On the other hand ADP augmented the inhibition of 6-OHDA autoxidation by desferrioxamine (only slightly at pH 7.0, but twofold at pH 8.0). As in the case of histidine, perhaps the nitrogenous donor atoms of the adenosine further chelate copper or other trace metals not well bound by desferrioxamine.

Effects of Phytic Acid

Phytic acid (inositol hexaphosphate), which offers binding through phosphate but lacks the abovementioned nitrogenous donor groups, had no effect on 6-OHDA autoxidation at pH 7.0 but stimulated autoxidation at pH 8.0. In the presence of desferrioxamine, phytic acid had little effect at pH 7.0 but dramatically removed inhibition by desferrioxamine and even accelerated autoxidation over that of 6-OHDA at pH 8.0. Thus at pH 8.0, but not at pH 7.0, phytate shares this property of EDTA. Ferrous chelates of polyphosphates appear to autoxidize similarly to $Fe^{2+} \cdot$ EDTA (38, 39), which may explain the stimulatory effects of phytate at pH 8.0. However, in contrast to EDTA the iron-phytate chelate lacks a free H_2O coordination site (at pH 7.4 at least) and was unreactive in the Haber-Weiss reaction (16). Also, unlike EDTA, phytate did not enhance inhibition by superoxide dismutase in the present study.

Why Does Superoxide Dismutase at Catalytic Concentrations Not Inhibit the Autoxidation of 6-OHDA?

The reaction between freely hydrated metals and 6-OHDA must occur at a substantial rate when the metal is allowed to undergo redox cycling (Reactions [1] and [2]). This follows from the observation that catalytic quantities of superoxide dismutase (e.g., 5 U/ml or less) only inhibit to a limited extent (if at all) in the absence of effective metal chelation. In the presence of a chelator which stabilizes the oxidized form of the metal (thus slowing initiation of 6-OHDA oxidation but accelerating autoxidation of the metal) propagation by O_2^- should be relatively more significant and net inhibition by superoxide dismutase would be enhanced.

We must also include the possibility that in the absence of metal chelation (other than by 6-OHDA itself), autoxidation occurs through inner sphere electron transfer within a ternary 6-OHDA · metal · oxygen complex (in agreement with (6)). In this case, the present data imply that 6-OHDA sequentially passes two electrons to oxygen (reaction [5]), resulting in direct formation of H_2O_2; thus precluding net inhibition by superoxide dismutase.

$$\text{6-OHDA} + M^{n+} + O_2 \rightarrow [\text{6-OHDA} \cdot M^{n+} \cdot O_2] \rightarrow pQ + M^{n+} + H_2O_2 \quad [5]$$

Such a mechanism would involve formation of a complex, transfer of one electron

from 6-OHDA to the metal and oxygen, electronic rearrangement of the semiquinone, and transfer of the second electron.

While catecholamines such as 6-OHDA readily complex metals (40-42), the nature and quantity of competing ligands determines the extent to which metal coordination by 6-OHDA occurs. Strongly binding ligands which contain multiple donor groups (such as desferrioxamine or EDTA) form complexes stable enough to prevent metal coordination by catecholamines (42). Other ligands can form mixed catecholamine · metal · ligand complexes (42), and may enhance oxidation more effectively than the metal ions alone (43) (particularly if it does not preclude simultaneous binding of oxygen and produces an appropriate redox potential).

Superoxide dismutase-sensitizing effects of EDTA in other systems have been explained on the basis that EDTA changes the mechanism from inner sphere electron transfer to outer sphere electron transfer (25, 32). However, the presence of a freely displacable seventh coordination site on EDTA - iron complexes (15, 16) allows for an inner sphere mechanism. In either case, the implication in the current system is that EDTA prevents the formation of a ternary reductant · metal · oxygen complex, and thus forces reduction of oxygen to proceed by single electron steps with release of O_2^-.

Ligands not only determine the ability of oxidized metals to form ternary 6-OHDA - metal · oxygen complexes or mixed 6-OHDA · metal - ligand complexes, but also affect the stability of reduced metal · oxygen complexes (5). For example, some ligands stabilize an $Fe^{2+} \cdot O_2$ complex, permitting its reaction with another Fe^{2+}, and resulting in H_2O_2 formation by a concerted two-electron transfer (5). Those ligands such as ADP or phytic acid, which did not enhance the ability of superoxide dismutase to inhibit 6-OHDA autoxidation, may act in this manner (or may allow inner sphere two-electron transfer from 6-OHDA as previously discussed). Other ligands destablize the $Fe^{2+} \cdot O_2$ complex, thus resulting in the formation of O_2^-. Ligands such as desferrioxamine and EDTA which enhance the inhibition of 6-OHDA autoxidation by superoxide dismutase presumably act in this manner. Histidine, which stabilizes Cu^{2+} and would destabilize a $Cu^+ \cdot O_2$ complex, may act in a similar manner to enhance inhibition by superoxide dismutase. We can therefore expand Reaction [2] to include the possibility of reduced metal · oxygen complexes (as in (44)):

$$M^{(n-1)+} + O_2 \leftrightarrow [M^{(n-1)+} \cdot O_2] \quad [2a]$$

$$[M^{(n-1)+} \cdot O_2] \rightarrow M^{n+} + O_2^- \quad [2b]$$

$$[M^{(n-1)+} \cdot O_2] + M^{(n-1)+} \rightarrow 2M^{n+} + H_2O_2 \quad [6]$$

In summary, we have the alternative possibilities of formation of ternary 6-OHDA · metal · oxygen complexes, mixed 6-OHDA · metal · ligand · oxygen complexes, and reduced metal · oxygen complexes which allow electron transfer without release of free (kinetically accessable) O_2^-. Any of these mechanisms precludes inhibition by superoxide dismutase. Whether such complexes form depends on the binding properties of the ligands present.

CONCLUSIONS

From the effects of different ligands on the autoxidation of 6-OHDA, trace metals serve as the primary initiating species and metal chelation is clearly required for inhibition by superoxide dismutase. In the absence of metal chelators, electron transfer to oxygen likely occurs by sequential two-electron transfer within a ternary 6-OHDA · metal · oxygen complex, and results in formation of H_2O_2 without release of free O_2^-. Ligands such as ADP or phytic acid then allow the formation of mixed 6-OHDA · metal · ligand · oxygen complexes or reduced metal · ligand · oxygen complexes since they do not sensitize the autoxidation to superoxide dismutase. Other ligands such as desferrioxamine, EDTA, and histidine sensitize the autoxidation to inhibition by superoxide dismutase by changing the mechanism of autoxidation in such a way that it is dependent on propagation by O_2^-. These ligands thus prevent the formation of ternary 6-OHDA · metal · oxygen complexes and

destabilize reduced metal·oxyen complexes, so that electron transfers occur in single steps which release $O_2^{\cdot-}$. Proteins such as albumin, cytochrome oxidase, or even boiled superoxide dismutase also destabilize such complexes and thus they sensitize the autoxidation of 6-OHDA to inhibition by catalytic quantities of active superoxide dismutase.

REFERENCES

1. HEIKKILA, R. E., AND CABBAT, F. S. (1978) *Life Sci.* 23, 33-38.
2. HEIKKILA, R. E., AND CABBAT, F. S. (1981) *Biochem. Pharmacol.* 30, 2945-2947.
3. WALLACE, K. B., AND AUTOR, A. (1980) *Pharmacology* 22, 173.
4. SULLIVAN, S. G., AND STERN, A. (1981) *Biochem. Pharmacol.* 30, 2279-2285.
5. AUST, S. D., MOREHOUSE, L. A., AND THOMAS, C. E. (1985) *J. Free Rad. Biol. Med.* 1, 3-25.
6. GEE, P., AND DAVISON, A. J. (1984) *Arch. Biochem. Biophys.* 231, 164-168.
7. GEE, P., AND DAVISON, A. J. (1984) *IRCS Med. Sci.* 12, 127.
8. HEIKKILA, R. E., AND COHEN, G. (1973) *Science* 181, 456-457.
9. COHEN, G., AND HEIKKILA, R. E. (1974) *J. Biol. Chem.* 249, 2447-2452.
10. KEBERLE, H. (1964) *Ann. N. Y. Acad. Sci. USA* 119, 758-768.
11. GUTTERIDGE, J. M., RICHMOND, R., AND HALLIWELL, B. (1979) *Biochem. J.* 184, 469-472.
12. HOE, S., ROWLEY, D. A., AND HALLIWELL, B. (1982) *Chem. Biol. Int.* 41, 75-81.
13. MOREHOUSE, L. A., THOMAS, C. E., AND AUST, S. D. (1984) *Arch. Biochem. Biophys.* 232, 366-377.
14. MCCORD, J. M., AND DAY, E. D., JR. (1978) *FEBS Lett.* 8, 139-142.
15. LIND, M. D., HAMOR, M. J., HAMOR, T. A., AND HOARD, J. L. (1964) *Inorg. Chem.* 3, 34-43.
16. GRAF, E., MAHONEY, J. R., BRYANT, R. G., AND EATON, J. W. (1984) *J. Biol. Chem.* 259, 3620-3624.
17. BUTLER, J., AND HALLIWELL, B. (1982) *Arch. Biochem. Biophys.* 218, 174-178.
18. BUETTNER, G. R., DOHERTY, T. P., AND PATTERSON, L. K. (1983) *FEBS Lett.* 158, 143-146.
19. FLOYD, R. A. (1983) *Arch. Biochem. Biophys.* 225, 263-270.
20. HOCHSTEIN, P., NORDNBRAND, K., AND ERNSTER, L. (1964) *Biochem. Biophys. Res. Commun.* 14, 323-328.
21. BUCHER, J. R., TIEN, M., AND AUST, S. D. (1983) *Biochem. Biophys. Res. Commuo.* 111, 777-784.
22. WAINIO, W. W. (1964) *J. Biol. Chem.* 239, 1402-1405.
23. MCCORD, J. M., AND FRIDOVICH, I. (1969) *J. Biol. Chem.* 244, 6049-6055.
24. DAVISON, A. J., AND GEE, P. (1984) *Arch. Biochem. Biophys.* 233, 164-168.
25. MISRA, H. P., AND FRIDOVICH, I. (1972) *J. Biol. Chem.* 247, 3170.
26. LIANG, Y. O., WIGHTMAN, R. M., AND ADAMS, R. N. (1976) *Eur. J. Pharmacol.* 36, 455-458.
27. GEE, P., AND DAVISON, A. J. (1985) *Biochim. Biophys. Acta* 838, 183-190.
28. GEE, P. (1984) Mechanisms of cytotoxicity of 6-hydroxydopamine: roles of oxygen free radicals and the effects of scavengers. Ph.D. Thesis, Department of Kinesiology, Simon Fraser University.
29. FLOYD, R. A., AND WISEMAN, B. B. (1979) *Biochim. Biophys. Acta* 586, 196-207.
30. SPIRO, T. G., AND SALTMAN, P. (1974) *in* Iron in Biochemistry and Medicine (Jacobs, A., and Worwood, M., Eds.), pp. 1-28, Academic Press, New York/London.
31. MARTELL, A. E., AND SMITH, R. M. (1974) Critical Stability Constants, Vol. 1, Plenum, New York.
32. MCCORD, J. M., AND FRIDOVICH, I. (1969) *J. Biol. Chem.* 244, 6056-6063.
33. MARKLUND, S., AND MARKLUND, G. (1974) *Eur. J. Biochem.* 47, 469-474.
34. SCHWARZENBACH, G., AND HELLER, J. (1951) *Helv. Chim. Acta* 34, 576-591.
35. KOLTHOFF, I. M., AND AUERBACH, C. (1952) *J. Amer. Chem. Soc.* 74, 1452-1456.
36. HARRIS, D. C., AND AISEN, P. (1973) *Biochim. Biophys. Acta* 329, 156-158.
37. LAMBETH, D. O., ERICSON, G. W., YOREK, M. A., AND RAY, P. D. (1982) *Biochim. Biophys. Acta* 719, 301-308.
38. KURIMURA, Y., OCHIAI, R., AND MATSUURA, N. (1968) *Bull. Chem. Soc. Japan* 41, 2234-2239.
39. KURIMURA, Y., AND KURIYAMA, H. (1969) *Bull. Chem. Soc. Japan* 42, 2238-2242.
40. BOGGESS, R. K., AND MARTIN, R. B. (1975) *J. Amer. Chem. Soc.* 97, 3076-3081.
41. MENTASTI, E., PELIZZETTI, E., AND BAIOCCHI, C. (1976) *J. Inorg. Nucl. Chem.* 38, 2017-2021.
42. GERGELY, A., AND KISS, T. (1979) *in* Metal Ions in Biological Systems (Sigel, H., Ed.), Vol. 9, pp. 144-172, Dekker, New York.
43. LOSSE, G., BARTH, A., AND LANGENBECK, W. (1961) *Chem. Ber.* 94, 2271-2277.
44. WEISS, J. (1952) *Experientia* 9, 61-62.

Chapter 5.
Relative Catalytic Effectiveness of Cu, Fe, Mn, and V in the Autoxidation of 6-Hydroxydopamine

by

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Catalysts of oxygen reduction

Keywords: 6-hydroxydopamine oxidation, dioxygen, transition metal catalysis, metal:ligand complexes, reduction potentials, iron, copper, manganese, vanadium, free radicals

ABSTRACT

Comparing the catalytic effectiveness of different transition metals in the autoxidation of 6-hydroxydopamine in HEPES buffer (pH 7.4), copper accelerated autoxidation 61 fold, iron 24 fold, manganese 7.3 fold, and vanadium 5.7 fold. Copper was thus the most effective catalyst despite being the weakest oxidant. EDTA, which decreases the reduction potential of Fe(III)/Fe(II), increased catalysis by iron to almost that of copper. Since EDTA decreased the catalytic effectiveness of each of the other metals, the effects of EDTA in buffer alone are apparently due to effects on trace iron. Desferrioxamine strongly inhibited catalysis by all of the metals. Histidine prevented catalysis by copper, accelerated catalysis by iron (43%), and had little effect on catalysis by manganese or vanadium. ADP and phytate inhibited catalysis by iron and manganese (50% or more), accelerated catalysis by vanadium (100%), and had no effect on catalysis by copper. Although steric effects obviously contribute to the effect of a ligand on catalysis, the effect of the metal complexes on catalysis of 6-hydroxydopamine autoxidation could be largely attributed to reduction potential. An intermediate potential suitable for facile reduction and reoxidation proved most effective.

INTRODUCTION

Traces of adventitious metals catalyse most "autoxidations",[1,2] and several researchers have described effects of metal chelators on the reactions of oxygen with 6-hydroxydopamine and other catecholamines.[3-11] Added iron or copper are often reported to stimulate these[3-5,7,12,13] and other[1,2] autoxidations. However, few systematic and detailed comparisons of catalysis by added metals and metal:ligand complexes are available.[cf. 12,13] One report[14] describes the effects of a range of metals on epinephrine oxidation, but only in the presence of EDTA. In ascorbate autoxidation, the effects of a range of ligands have been described, but only for catalysis by iron and copper.[15-17] In this study therefore, we extend our observations on effects of ligands on 6-hydroxydopamine autoxidation (trace metal catalysed)[10,11] to effects on catalysis by added transition metals.

The effects of desferrioxamine, diethylenetriaminepentaacetic acid (DTPA), and EDTA on oxidations catalysed by iron or copper have been well documented. Desferrioxamine and DTPA stabilize iron in its ferric state, and inhibit iron-catalysed oxidations of catecholamines and other reductants, such as ascorbate.[5,7,17] Similarly, desferrioxamine and DTPA inhibit copper-catalysed oxidation of 6-hydroxydopamine.[5] EDTA inhibits iron-catalysed oxidations of dopamine and norepinephrine,[5] and copper-catalysed oxidations of 6-hydroxydopamine, 6-aminodopamine, norepinephrine, and ascorbate,[4,17] but **stimulates** iron-catalysed oxidations of 6-hydroxydopamine and ascorbate.[7,17] With strong reductants, EDTA accelerates the otherwise rate-limiting reoxidation of Fe^{2+}. Contrasting effects of other ligands on catalysis by iron and copper, and the influence of ligands on oxidations catalysed by metals other than iron and copper have not been well characterized.

While some of the properties of metal complexes relevant to catalysis of autoxidations have been discussed,[1,2] systematic investigations of the roles of ligands in metal catalysed oxidations are surprisingly sparse. The influence of different metal catalysts on reduction of oxygen depend not only on the nature of the metal and the available ligands, but also on metal binding and thermodynamic properties of the reductant. Here we compare the effects

of a series of ligands (EDTA, desferrioxamine, ADP, histidine, and phytic acid) on catalysis by Fe, Cu, Mn, and V, in the autoxidation of one reductant, 6-hydroxydopamine.

Ligands can influence the reaction rate in two ways: 1) they may modify the reduction potential of the metal, making redox cycling more or less facile, 2) they may hinder access of oxygen and/or 6-hydroxydopamine to inner sphere electron transfers. In this analysis we ask what properties of a given metal-ligand combination determine its catalytic activity in the reduction of oxygen by 6-hydroxydopamine.

MATERIALS AND METHODS

Reagents

6-Hydroxydopamine, HEPES buffer, phytic acid, and ADP were from Sigma Chemical Company (St. Louis, MO). Histidine was from Matheson Coleman and Bell Manufacturing Chemists (Norwood, Ohio). Desferrioxamine (Desferal mesylate) was a gift from CIBA Pharmaceutical Co. (Summit, NJ). Sodium ortho vanadate, cupric acetate, and EDTA were from Fisher Scientific Co. (Fair Lawn, NJ). Manganese sulphate was from Mallinckrodt Inc. (Paris, Kentucky). Ferrous sulphate was from American Scientific and Chemical (Portland, Oregon). All solutions were made using deionized (Corning demineralizer 3508-A), distilled water.

Procedures

Nitrogen-saturated stock solutions of 6-hydroxydopamine were prepared by three repetitions of evacuation and flushing with nitrogen using a Virtis evacuator, and sealed under a positive pressure of nitrogen. Aliquots were removed through a rubber septum with a Hamilton gas-tight syringe. Experiments were conducted at pH 7.4, 25°C, in air-saturated 50 mM HEPES buffer. HEPES buffer was used in these experiments in order to avoid competing ligand effects of phosphate buffer. Metals, from freshly prepared stock solutions, were added to a final concentration of 50 μM. Ligands were added to 1 mM. Reactions were started by adding a 25 μl aliquot of 6-hydroxydopamine (to an initial concentration of 200 μM) and quickly mixing with a cuvette plunger. Formation of *p*-quinone oxidation product was followed spectrophotometrically at 490 nm.

Data Analyses

Because the rapidity of some reactions made determination of initial rates difficult from strip chart recordings, half completion times were used to calculate first order rate constants ($k=0.693/t_{½}$)[18]. Means and standard deviations were calculated from reactions run at least in triplicate.

RESULTS

In the absence of added metals, the ligands had effects similar to those observed in phosphate buffer in previous studies.[10,11] Desferrioxamine and histidine were inhibitory (13.6-21.6%), EDTA was stimulatory (4.3 fold), and ADP and phytate had little effect (Figure 1).

In the absence of added ligands, copper was the most active catalyst, accelerating 6-hydroxydopamine oxidation 61-fold (Figure 2). Iron alone accelerated the reaction 24-fold, while manganese and vanadium accelerated 7.3-fold and 5.7-fold.

EDTA inhibited catalysis by each of the metals except Fe, where it stimulated 74% (Figure 2). Stimulatory effects of EDTA alone therefore, in this and the previous studies, were likely due to effects on trace iron. EDTA coordinates most transition metal ions strongly (Table I), generally stabilizing the oxidized form, and thus slowing reduction. In the case of iron however, accelerated reaction of the reduced metal with oxygen apparently accelerates the overall reaction.

Desferrioxamine inhibited catalysis by each of the metals (Figure 2). With Fe, Cu, and Mn, desferrioxamine slowed 6-hydroxydopamine oxidation to less than the rate of the control reaction, likely due to coordination of additional trace metals present. Desferrioxamine slowed Mn-catalysed oxidation of 6-hydroxydopamine to less than 31% of the control in absence of metal, perhaps in part due to superoxide dismutase activity of Mn:desferrioxamine.[25] Vanadate was unique in retaining some catalytic activity in the presence of desferrioxamine.

Histidine prevented catalysis by Cu, mildly increased catalysis by Fe (43%), and did not significantly alter catalysis by Mn, or V (Figure 2). Histidine has fairly similar binding affinities for Fe^{3+} and Fe^{2+} (Table I) and so does not greatly influence the ability of iron to redox cycle. Histidine does form relatively strong mixed ligand complexes with iron

however (Table I), and could inhibit formation of a ternary 6-hydroxydopamine:Fe:O_2 complex. Consequently, inhibition of inner sphere electron transfers may explain the superoxide dismutase sensitizing effect of histidine.[10] The affinity of histidine for Mn is weaker than for Fe, and apparently does not strongly affect its ability to redox cycle. Copper was unique among the metals studied in that histidine blocked catalysis. Inhibitory actions of histidine alone are thus likely due to effects on trace copper. Histidine binds copper relatively strongly (Table I), stabilizing Cu^{2+} and inhibiting its activity as a redox catalyst.

The two phosphate-containing ligands, ADP and phytate, inhibited Fe- and Mn- catalysed oxidations (45-91%), and had no effect on catalysis by Cu or V. The stronger inhibition of Fe catalysis by phytate may be due to its ability to form a closed-shell hexadentate complex with iron.[26,27]

DISCUSSION

The most effective catalysts possess a reduction potential suitable for reduction by 6-hydroxydopamine and re-oxidation by oxygen.

The Cu(II)/Cu(I) couple had the lowest reduction potential (+0.153 V),[28-30] and copper was the most effective catalyst of 6-hydroxydopamine autoxidation. Iron, manganese, and vanadium were decreasingly effective, despite being much stronger oxidants ($E^{o}_{Fe(III)/Fe(II)}$ = 0.77 V, $E^{o}_{Mn(III)/Mn(II)}$ = 1.51 V, $E^{o}_{V(V)/V(IV)}$ = 0.991 V). For the aquo metals, the rate limiting process in the autoxidation of 6-hydroxydopamine is evidently the thermodynamically unfavourable one-electron reduction of oxygen ($E^{o}_{O_2/O_2^{\cdot-}}$ = -0.16 V) by the reduced metal. Thus, at catalytic concentrations, copper is more active than iron, which is more active than manganese or vanadium. Predictably, this order reverses if the metals are present in stoichiometric quantities.[31]

Ligands which influence the reduction potential of the metal (by preferential affinity for either the oxidized or reduced form) strongly influence the catalytic activity of the metal on 6-hydroxydopamine autoxidation. Thus, EDTA, which decreases the reduction potential of iron to 0.12 V [30,32] and accelerates autoxidation of ferrous ion,[33-36] stimulated catalysis by iron to almost that of copper. However, desferrioxamine, which has an even stronger preferential affinity for ferric ion and decreases the reduction potential of iron further (to approx. -0.45 V)[37,38], strongly inhibited catalysis by iron. A very strong reductant, such as the paraquat cation radical, can reduce ferrioxamine sufficiently for it to catalyse Fenton reactions.[37] In the current studies, desferrioxamine apparently limits reduction of Fe^{3+} (and other metals) by 6-hydroxydopamine sufficiently to prevent redox cycling catalysis.*

Similarly, ligands which influenced the reduction potential of copper strongly influenced its catalytic activity. Histidine, which binds Cu(II) more strongly than Cu(I), lowers the

* Since desferrioxamine inhibits, the $O_2^{\cdot-}$ produced during the accelerated preliminary autoxidation of 50 μM Fe(II) obviously did not contribute substantially to 6-hydroxydopamine oxidation.

reduction potential of copper to -0.17 V [39] and prevented catalysis of 6-hydroxydopamine oxidation. EDTA, which also has a strong affinity for Cu(II) (Table I) but lesser affinity for Cu(I),[40] also inhibited catalysis by copper.

The most effective catalysts had intermediate reduction potentials, presumably a function of the potentials of reducing and oxidising species involved in the reaction. Relevant reduction potentials include not only those of the added metal:ligand complexes, but also those of collision complexes or mixed-ligand complexes with 6-hydroxydopamine or oxygen. Nevertheless, a plot of the catalytic activity of metal complexes as a function of their standard reduction potentials shows a window of reduction potentials for effective redox cycling catalysis (Figure 3).

Steric effects of ligands reinforce or oppose effects on reduction potential

EDTA accelerated Fe-catalysed autoxidation of 6-hydroxydopamine despite preventing ternary 6-hydroxydopamine:Fe:O_2 complex formation. The EDTA-induced sensitization of 6-hydroxydopamine autoxidation to inhibition by superoxide dismutase[10] likely reflects steric considerations in the stereochemistry of the Fe:EDTA complex. Although the septacoordinate structure of Fe:EDTA [27,41] may allow inner sphere transfer of an electron to oxygen, it hinders access enough to force discrete one-electron steps, with release of free superoxide. Thus, while sterically preventing concerted 2-electron reduction of oxygen, EDTA accelerates the catalytic effectiveness of iron by optimizing its reduction potential. Desferrioxamine on the other hand, not only prevents inner sphere electron transfers by forming a closed-shell hexadentate complex,[42] but it decreases the reduction potential of iron to below the maximally effective range.

Histidine had contrasting effects on catalysis by iron and copper. With copper, histidine not only decreases the reduction potential to a catalytically ineffective value, but it forms a stable multi-ligand complex (Table I) to inhibit inner sphere electron transfers from

6-hydroxydopamine to oxygen. With iron, histidine also forms a fairly strong multi-ligand complex, inhibiting inner sphere electron transfers, but has relatively little effect on the reduction potential. Histidine thus allows iron to redox cycle but forces outer sphere electron transfers, as reflected in the susceptibility of 6-hydroxydopamine autoxidation to inhibition by superoxide dismutase.

Copper is unique in that redox participation requires a change in coordination number and geometry. Cu(II) generally adopts an octahedral geometry (or, less frequently, square pyramidal or square planar), while Cu(I) adopts a tetrahedral geometry.[43,44] Phytate and ADP, which did not hinder the catalytic activity of copper in 6-hydroxydopamine autoxidation, presumably allow this change. The ligands which inhibited catalysis by copper (desferrioxamine, histidine, and EDTA), have a stronger affinity for Cu(II) and maintain an octahedral geometry. These ligands then, not only hinder inner-sphere access to copper but influence the reduction potential, in part by an unfavorable entropy of reorganization required for reduction to Cu(I).

CONCLUSIONS

The effects of ligands on catalytic activities of metals in the autoxidation of 6-hydroxydopamine depend largely on reduction potential of the metal complex. The most active complexes had intermediate reduction potentials, appropriate for facile reduction and reoxidation. However, steric effects influence the reaction mechanism and the energetics, either reinforcing or opposing the influence on reduction potential. Steric effects include 1) decreased oxygen and/or reductant accessibility, resulting in a change in mechanism from inner sphere to outer sphere, and 2) revised entropy of transition state during electron transfer.

REFERENCES

1. Aust, S.D.; Morehouse, L.A.; Thomas, C.E. Role of metals in oxygen radical reactions. *J. Free Rad. Biol. Med.* **1**:3-25; 1985.

2. Miller, D.M.; Buettner, G.R.; Aust, S.D. (1990) Transition metals as catalysts of "autoxidation" reactions. *Free Rad. Biol. Med.* **8**:95-108.

3. Misra, H.P.; Fridovich, I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* 247:3170-3175; 1972.

4. Heikkila, R.E.; Cabbat, F.S. The stimulation of 6-hydroxydopamine autoxidation by bivalent copper: potential importance in the neurotoxic process. *Life Sciences* **23**:33-38; 1978.

5. Heikkila, R.E.; Cabbat, F.S. Inhibition of iron-stimulated catecholamine degradation by the iron-chelators DETAPAC and desferal. Potentially useful laboratory agents. *Biochem. Pharmacol.* **30**:2945-2947; 1981.

6. Floyd, R.A.; Wiseman, B.B. Spin-trapping free radicals in the autoxidation of 6-hydroxydopamine. *Biochim. Biophys. Acta* 586:196-207; 1979.

7. Sullivan, S.G.; Stern, A. Effects of superoxide dismutase and catalase on catalysis of 6-hydroxydopamine and 6-aminodopamine autoxidation by iron and ascorbate. *Biochemical Pharmacol.* **30**:2279-2285; 1981.

8. Gee, P.; Davison, A.J. 6-Hydroxydopamine does not reduce molecular oxygen directly, but requires a co-reductant. *Arch. Bioch. Biophys.* **231**:164-168; 1984.

9. Gee, P.; Davison, A.J. "Autoxidation" of 6-hydroxydopamine involves a ternary reductant-metal-oxygen complex. *IRCS Med. Sci.* **12**:127; 1984.

10. Bandy, B.; Davison, A.J. Interactions between metals, ligands, and oxygen in the autoxidation of 6-hydroxydopamine: Mechanisms by which metal chelation enhances inhibition by superoxide dismutase. *Arch. Bioch. Biophys.* **259:**305-315; 1987.

11. Gee, P.; Davison, A.J. Intermediates in the aerobic autoxidation of 6-hydroxydopamine: relative importance under different reaction conditions. *Free Rad. Biol. Med.* **6**:271-284; 1989.

12. Heacock, R.A. The chemistry of adrenochrome and related compounds. *Chem. Rev.* **59**:181-237; 1959.

13. Gergely, A.; Kiss, T. Coordination chemistry of L-dopa and related ligands. In: Sigel, H. ed.; *Metal Ions in Biological Systems.* New York: Marcel Dekker; 1977:143-172.

14. Ahuda, B.S.; Sudershan. Effect of different metal ions, inhibitors/metabolites & antibiotics on autoxidation of adrenaline to adrenochrome in alkaline medium. *Indian J. Biochem. Biophys.* **20**:309-310; 1983.

15. Khan, T.M.M.; Martell, A.E. Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. I. Cupric and ferric ion catalyzed oxidation *J. Amer. Chem. Soc.* 89:4176-4185; 1967.

16. Khan, T.M.M.; Martell, A.E. Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. II. Cupric and ferric chelate catalyzed oxidation. *J. Amer. Chem. Soc.* 89:7104-7111; 1967.

17. Buettner, G.R. Ascorbate autoxidation in the presence of iron and copper chelates. *Free Rad. Res. Comms.* **1**:349-353; 1986.

18. Jenks, W.P. *Catalysis in Chemistry and Enzymology.* McGraw Hill Book Company, New York, 1969.

19. Milazzo, G.; Caroli, S. *Tables of Standard Electrode Potentials.* John Wiley & Sons, Ltd.; 1978.

20. Martell, A.E.; Smith, R.M. *Critical Stability Constants*, Vol. 1: Amino Acids, Plenum, New York, 1974.

21. Martell, A.E.; Smith, R.M. *Critical Stability Constants*, Vol. 3: Other Organic Ligands, Plenum, New York, 1977.

22. Martell, A.E.; Smith, R.M. *Critical Stability Constants*, Vol. 5: First Supplement, Plenum, New York, 1982.

23. Smith, R.M.; Martell, A.E. *Critical Stability Constants*, Vol. 2: Amines, Plenum, New York, 1975.

24. Smith, R.M.; Martell, A.E. *Critical Stability Constants*, Vol. 6: Second Supplement, Plenum, New York, 1989.

25. Darr D., Zarilla K.A., and Fridovich I. A mimic of superoxide dismutase activity based upon desferrioxamine B and manganese (IV). *Arch. Biochem. Biophys.* **258**:351-355; 1987.

26. Leong, J.; Raymond, K. Coordination isomers of biological iron transport compounds. IV. Geometrical isomers of chromic desferrioxamine B. *J. Amer. Chem.* **97**:293-296; 1975.

27. Graf, E.; Mahoney, J.; Bryant, R.; Eaton, J. Iron-catalyzed hydroxyl radical formation. Stringent requirement for free iron coordination site. *J. Biol. Chem.* **295**:3620-3624; 1984.

28. Latimer, W.M. *The oxidation states of the elements and their potentials in aqueous solutions.* Prentice-Hall Inc., Englewood Cliffs, N.J., 1952.

29. Weast, R.C.; Astle, M.J.; Beyer, W.H. *CRC Handbook of Chemistry and Physics.* 64th Edition, CRC Press Inc. Boca Raton, Florida, USA; 1984.

30. Koppenol, W.H.; Butler, J. Energetics of interconversion reactions of oxyradicals. *Adv. Free Rad. Biol. Med.* **1**:91-131; 1985.

31. Steele, D. M.Sc. Thesis. Simon Fraser University, 1988.

32. Kolthoff, I.M.; Auerbach, C. Studies on the system iron-ethylenediamine tetraacetate. *J. Am. Chem. Soc.* **74**:1452-1456; 1952.

33. Kaden, T.; Fallab, S. Chelate catalysed autoxidation of iron(II) complexes. In: Kirschner, S., ed. *Advances in the Chemistry of Coordination Compounds.* New York: MacMillan Co.; 1961:654-663.

34. Harris, D.C.; Aisen, P. Facilitation of Fe(II) autoxidation by Fe(III) complexing agents. *Biochim. Biophys. Acta* **329**:156-158; 1973.

35. Lambeth, D.O.; Ericson, G.W.; Yorek, M.A.; Ray, P.D. Implications for *in vitro* studies of the autoxidation of ferrous ion and the iron-catalysed autoxidation of dithiothreitol. *Biochim. Biophys. Acta* **719**:301-308; 1982.

36. Burkitt, M.J.; Gilbert, B.C. The autoxidation of iron(II) in aqueous systems: The effects of iron chelation by physiological, non-physiological and therapeutic chelators on the generation of reactive species and the inducement of biomolecular damage. *Free Rad. Res. Comms.* **14**:107-123; 1991

37. Borg, D.C.; Schaich, K.M. Prooxidant action of desferrioxamine: Fenton-like production of hydroxyl radicals by reduced ferrioxamine. *J. Free Rad. Biol. Med.* **2**:237-243; 1986.

38. Cooper, S.R.; McArdle, J.V.; Raymond, K.N. Siderophore electrochemistry: Relation to intracellular iron release mechanism. *Proc. Natl. Acad. Sci. USA* **75**:3551-3554; 1978.

39. James, B.R.; Williams, R.J.P. The oxidation-reduction potentials of some copper complexes. *J. Chem. Soc.* **1961**:2007-2019; 1961.

40. Hemmerich, P. Model Studies on the binding of univalent and redox-active copper in proteins. In: Peisach, J.; Aisen, P.; Blumberg, W.E., eds. *The Biochemistry of Copper.* Academic Press; 1966:15-34.

41. Hoard, J.L.; Lind, M.; Silverton, J.V. The stereochemistry of the ethylenediaminetetraacetatoaquoferrate(III) ion. *J. Amer. Chem. Soc.* **83**:2770-2771; 1961.

42. Keberle, H. The biochemistry of desferrioxamine and its relation to iron metabolism. *Ann. N.Y. Acad. Sci. USA* **119**:758-768; 1964.

43. Zuberbuhler, A.D. Interaction of Cu(I) complexes with dioxygen. In: Sigel, H., ed. *Metal Ions in Biological Systems.* 5:325; 1976.

44. Jameson, R.F. Coordination chemistry of copper with regard to biological systems. In: Sigel, H., ed. *Metals Ions in Biological Systems.* **12**:1-3; 1981.

Table I. Stability constants (log Ka), reflecting affinity of a ligand for metal ion (data from [20-24]).

	EDTA	DEF	HIS	$(HIS)_2$	ADP	PA	Catechol	$(Catechol)_2$
Cu^{+}				7.0				
Cu^{2+}	18.7	14.2	10.2	18.1	5.9		13.62	24.94
Fe^{2+}	14.3	7.2	5.9	10.4			7.95	13.5
Fe^{3+}	25.0	30.6	4.7				20.0	34.7
Mn^{2+}	13.8		3.3	6.3	4.2		7.47	12.8
Mn^{3+}	25.3							
V^{2+}	12.7							
V^{3+}	25.3						18.3	
VO_2^{+}	18.8						17.7	
V^{5+}	18.1							

LEGENDS TO FIGURES

Figure 1. *Effects of ligands on the trace metal-catalysed autoxidation of 6-hydroxydopamine.* ADP, phytic acid (PA), EDTA, histidine (His), and desferrioxamine (DEF) were at 1 mM. 6-Hydroxydopamine autoxidation was monitored in air-saturated HEPES buffer, pH 7.4, 25°C, and first order rate constants were calculated. Error bars represent standard deviations from the mean of at least three replicates.

Figure 2. *Relative catalytic effectiveness of metals and metal complexes in the autoxidation of 6-hydroxydopamine.* Metals included were at 50 μM; ligands were at 1 mM. 6-Hydroxydopamine autoxidation was monitored as in figure 1, and the first order rate constants are plotted.

Figure 3. *Dependence of catalytic activity on the reduction potential of the metal complex.* Standard reduction potentials for Fe, Cu, Mn, and V, were from Milazzo and Caroli[19]. Standard reduction potentials have also been reported for Fe(III):EDTA (0.12 V)[19], Cu(II):histidine$_2$ (-0.17 V)[39], and Fe(III):DEF (-0.45 V)[37,38]. Other reduction potentials were estimated using the Nernst equation and the relative affinities of a ligand for the reduced and oxidized form of a metal (from Table I). The curve represents a sixth order polynomial fit through the data.

Figure 1. Effects of Ligands Alone on 6-OHDA Autoxidation

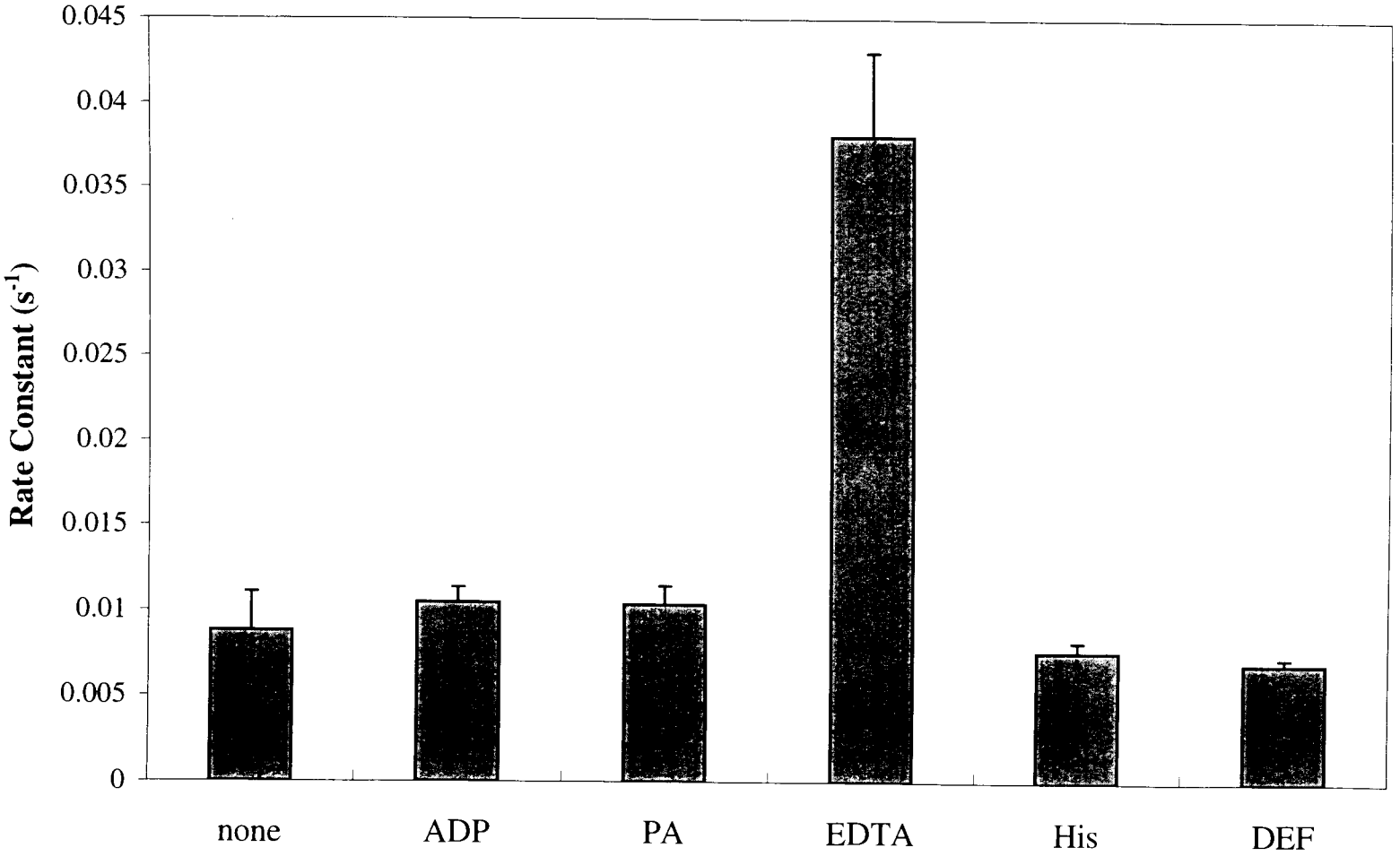


Figure 2. Catalysis by Metals and Metal Complexes

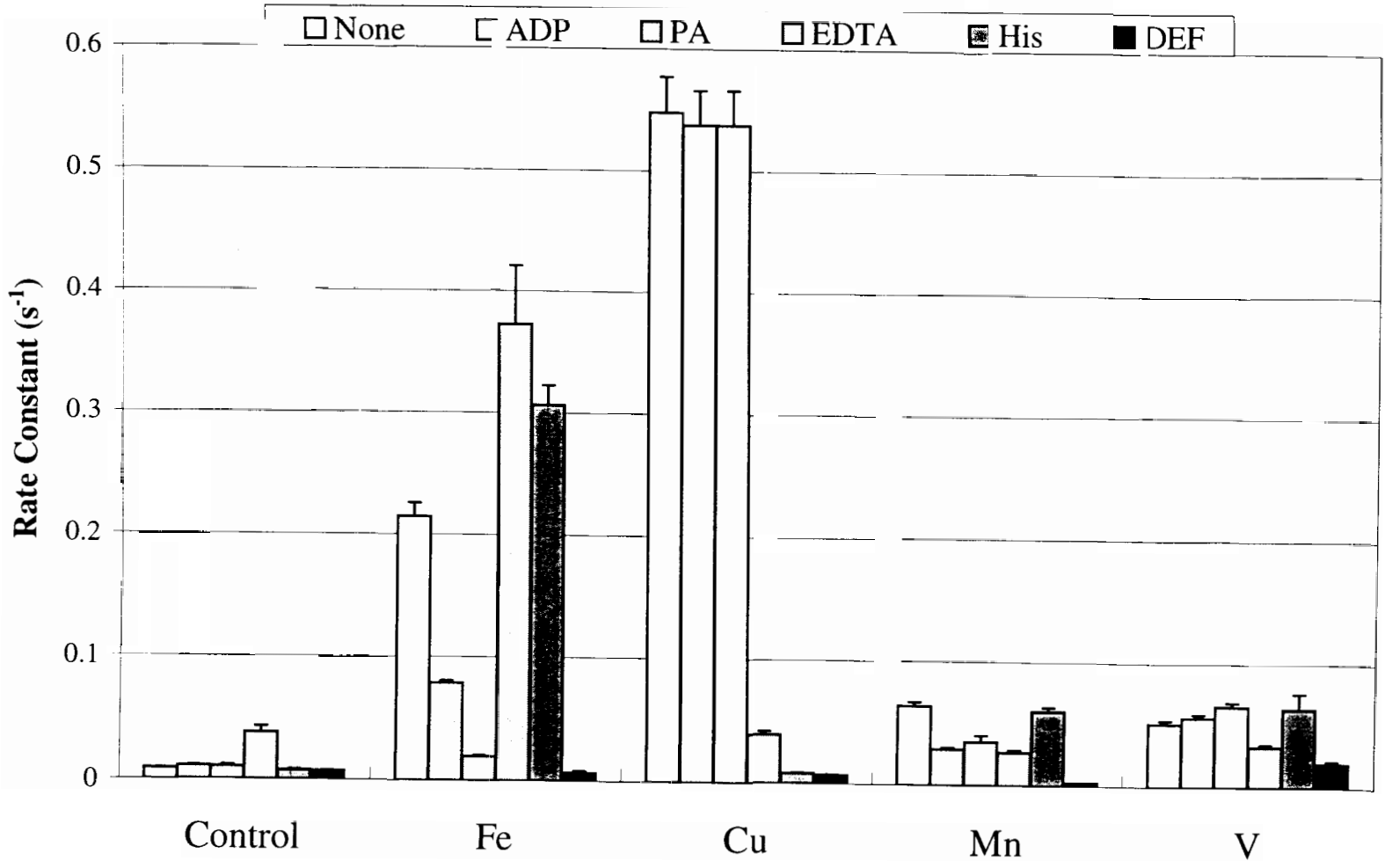
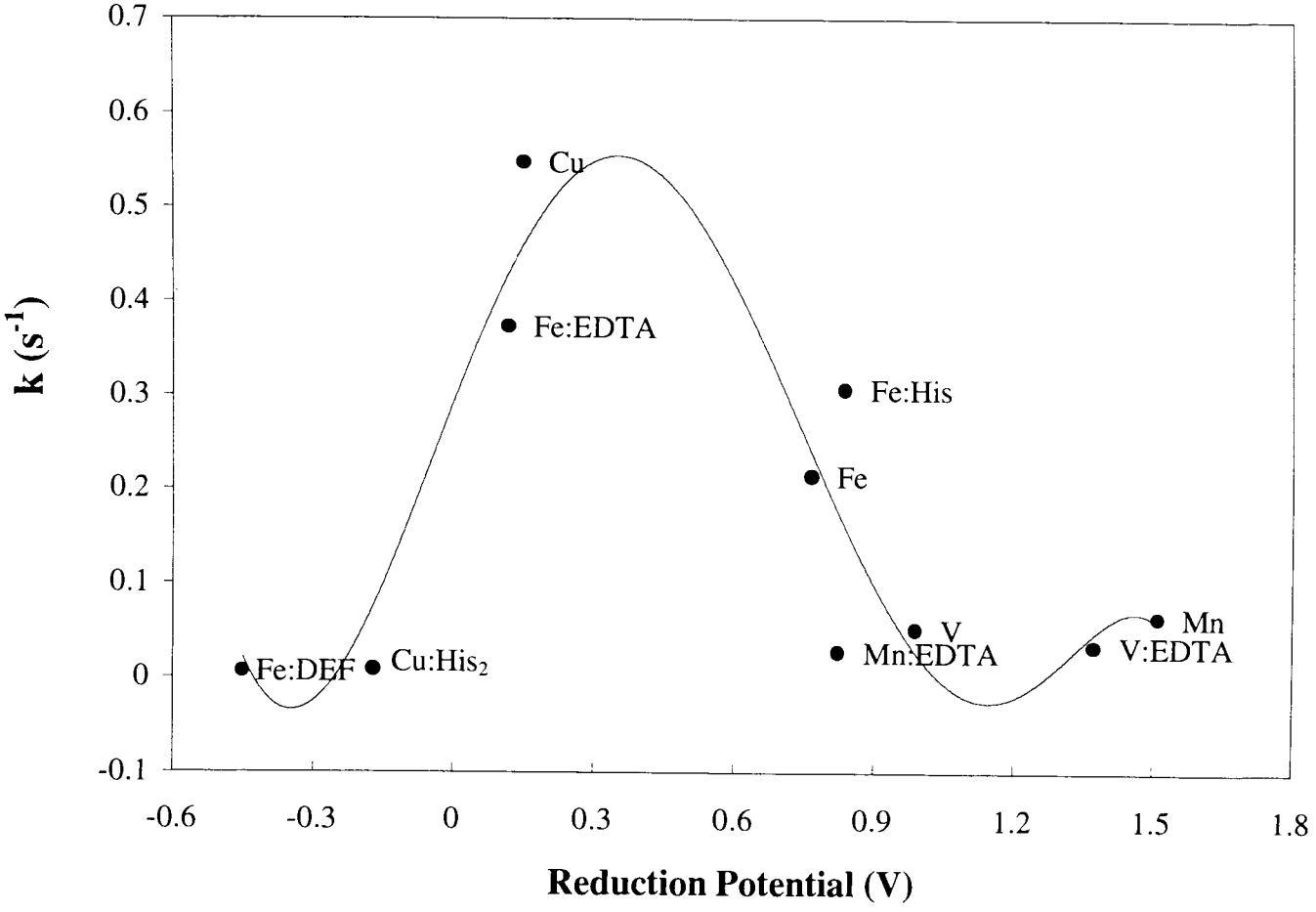


Figure 3. Dependence of Catalytic Activity on Reduction Potential



Chapter 6.

The Superoxide Dismutase Mimic Mn-desferrioxamine Accelerates rather than Inhibits 6-Hydroxydopamine Autoxidation

by

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ABSTRACT

The superoxide dismutase mimic, manganese-desferrioxamine (MnDF, green complex), inhibited reduction of cytochrome *c* by xanthine oxidase plus xanthine, but showed no superoxide dismutase activity in the autoxidation of 6-hydroxydopamine. In the presence of desferrioxamine, which strongly sensitizes the autoxidation to inhibition by superoxide dismutase activity, the MnDF complex did not inhibit. At 20-80 μM, MnDF mildly (14-21%) accelerated autoxidation of 200 μM 6-hydroxydopamine, and removed sigmoidal kinetics produced by desferrioxamine. Higher concentrations of MnDF (200 μM) accelerated oxidation further (146%), showing the ability of MnDF to oxidize 6-hydroxydopamine. Strong inhibition (98%) by superoxide dismutase (5 U/ml) in the presence of 80 μM MnDF showed that the MnDF-accelerated autoxidation was $O_2^{\cdot -}$-propagated. Clearly MnDF, unlike superoxide dismutase, does not compete effectively with 6-hydroxydopamine for superoxide.

INTRODUCTION

Superoxide dismutase has antioxidant and anti-inflammatory properties which make it potentially useful in treatment of oxidative stresses such as hyperoxia, reperfusion injury, organ transplantation, autoimmune diseases (eg. rheumatoid arthritis), spinal chord injury, and pro-oxidant cytotoxin exposure (eg. quinones). However, the usefulness of enzymic superoxide dismutase is limited by antigenicity, short extracellular lifetime, and lack of cell permeability. Development of a low molecular weight catalyst of superoxide dismutation has received considerable attention. Such a complex, if non-toxic, would be desirable for several pharmacologic and therapeutic applications.

Several low molecular weight complexes of copper, iron, or manganese possess substantial superoxide dismutase activity.[1-10] In most cases however, these complexes are not stable in the presence of physiological metal chelators such as albumin[10,11], or are cytotoxic[12,13].

Most recently, a mimic of superoxide dismutase activity based on Mn(IV) and desferrioxamine was developed which is non-toxic, and stable toward albumin.[11,14] The green complex which results from reacting MnO_2 with desferrioxamine exerted activity equivalent to 1 U superoxide dismutase at 1 μM Mn-desferrioxamine (MnDF) in the xanthine oxidase/cytochrome *c* or nitroblue tetrazolium photoreduction assays. The complex also protects cultured Chinese hamster ovary cells or green algae cells against the oxidative toxicities of paraquat or sulphite.[13,15] Formation of the green complex results from oxidation of one of the hydroxamate moieties on desferrioxamine by Mn(IV) to give a Mn(III)DF complex. A more active and more stable (toward EDTA) pink complex, in which all of the hydroxamate groups remain intact and ligated to Mn(III), is produced by including ascorbate in the reaction mixture with desferrioxamine and MnO_2.[16]

Except for a few stopped-flow or pulse radiolysis studies of $O_2^{\cdot-}$ disappearance, "superoxide dismutase activity" of metal complexes has been determined using probes for reductive actions of $O_2^{\cdot-}$ (cytochrome *c* or nitroblue tetrazolium). To be truly effective, a superoxide

dismutase mimic should also prevent oxidizing actions of $O_2^{\cdot -}$, and thus inhibit autoxidations in which $O_2^{\cdot -}$ serves as initiator or propagator. The sensitivity of 6-hydroxydopamine autoxidation in the presence of desferrioxamine to inhibition by superoxide dismutase activity[17] gives an ideal test for the anti-*oxidative* ability of an superoxide dismutase mimic based on MnDF. Since absorption at 490 nm, and the presence of dehydroascorbate in the pink complex preparation complicate interpretation of the results, we tested the effect of the green MnDF complex on the autoxidation of 6-hydroxydopamine.

MATERIALS AND METHODS

MnO_2 was from Fisher Scientific (New Jersey). Desferrioxamine was a gift from Ciba Pharmaceuticals. All other reagents were from Sigma. All aqueous solutions were made using deionized, distilled water. Buffer solutions were not purified further, so contained usual levels of trace metals.

The green MnDF complex was prepared essentially as described[11,16]. Desferrioxamine methansulphonate (50 mM) was dissolved in water, and a 25% molar excess of powdered MnO_2 added and stirred for 4 hours at 25°C. The resulting green solution was allowed to settle, decanted, and passed through a 0.22 μm Millex-GV syringe filter to remove unreacted MnO_2. The concentration of the green MnDF complex was determined from its absorbance at 640 nm (E_{640} = 1.09 X 10^2 M^{-1} cm^{-1})[16]. Activity of the MnDF complex in the xanthine oxidase/cytochrome *c* reduction assay was tested in the absence of EDTA (as described by Darr et al[11]).

6-Hydroxydopamine was prepared fresh before use in argon-saturated water and sealed under a slight positive pressure of argon. Aliquots were removed through a rubber septum with a Hamilton gas-tight syringe (initially purged with argon to prevent introduction of air to the vessel). Autoxidations were initiated by addition of 6-hydroxydopamine to air-saturated 50 mM phosphate buffer, pH 7.4, 25°C, and monitored at 490 nm for formation of *p*-quinone oxidation product.

RESULTS AND DISCUSSION

Figure 1 confirms inhibitory activity of the MnDF preparation in the xanthine oxidase/cytochrome *c* reduction assay for $O_2^{\cdot -}$. When tested on the autoxidation of 6-hydroxydopamine however, MnDF had little effect, or even accelerated oxidation at higher concentrations (Figure 2). Most significantly, in the presence of additional desferrioxamine to sensitize the autoxidation to inhibition by superoxide dismutase activity, the MnDF complex had no inhibitory effect. Superoxide dismutase activity of the complex was thus not evident in this assay system.

The acceleration of 6-hydroxydopamine autoxidation by high (stoichiometric) concentrations of the MnDF complex, and the removal of sigmoidal kinetics in the presence of desferrioxamine, suggest that the MnDF complex can itself catalyse the autoxidation. The MnDF complex has been shown to oxidize Fe(II) in the presence of excess desferrioxamine,[16] so is at least a weak oxidant ($E^{o'}_{Fe(III):DF/Fe(II):DF}$ = -0.45 V)[18,19]. However, the ability of the MnDF complex to act as a redox catalyst (other than in $O_2^{\cdot -}$ dismutation) has not been reported. The strong (98%) inhibition by 5 U/ml superoxide dismutase in the presence of MnDF (Figure 2) shows that MnDF-catalysed 6-hydroxydopamine autoxidation is largely $O_2^{\cdot -}$-propagated. Superoxide could propagate the reaction by oxidizing a new molecule of 6-hydroxydopamine, or by reoxidizing Mn(II) to Mn(III). The Mn^{2+}-catalysed oxidations of dihydroxyfumarate and NADH are similarly sensitive to inhibition by superoxide dismutase, and it was suggested that Mn^{2+} interacts with $O_2^{\cdot -}$ to produce an oxidizing species (Mn^{3+} or MnO_2^{+}).[20] In the current system however, desferrioxamine stabilizes Mn(III) over Mn(II) (as evidenced by rapid autoxidation of Mn^{2+} on addition of desferrioxamine)[16] and thus likely stimulates (outer sphere) oxidation of Mn(II) by O_2 to produce $O_2^{\cdot -}$. A possible reaction scheme may be as follows.

$$QH_2 + \text{Mn(III):DF} \xrightarrow{\textbf{slow}} Q^{\cdot -} + \text{Mn(II):DF} \qquad (1)$$

$$\text{Mn(II):DF} + O_2 \xrightarrow{\textbf{fast}} \text{Mn(III):DF} + O_2^{\cdot -} \qquad (2)$$

$$Q^{\cdot -} + O_2 \xrightarrow{\textbf{fast}} Q + O_2^{\cdot -} \qquad (3)$$

$$QH_2 + O_2^{\cdot -} \xrightarrow{\text{fast}} Q^{\cdot -} + H_2O_2 \qquad (4)$$

Superoxide dismutase would thus inhibit the autoxidation in the presence of MnDF by preventing reaction 4.

The current results raise the question of whether the MnDF complex gives apparent superoxide dismutase activity in reductive assays for $O_2^{\cdot -}$ (eg. cytochrome *c* reduction) by acting as an oxidant for the probe. The rapid oxidation of Fe^{2+} (which is a weaker reductant than ferrocytochrome *c*) by the green MnDF complex[16] shows the possibility for oxidation of ferrocytochrome *c*. However, the stability of 550 nm absorption after addition of excess MnDF to the xanthine oxidase/cytochrome *c* reduction assay (Figure 1) argues against this possibility.

These results with 6-hydroxydopamine autoxidation show that the green MnDF complex does not eliminate oxidizing actions of $O_2^{\cdot -}$, at least toward 6-hydroxydopamine. 6-Hydroxydopamine (or its semiquinone) can apparently reduce the Mn(III)DF complex, since MnDF accelerates autoxidation. Moreover, since enzymic superoxide dismutase inhibits the autoxidation in the presence of MnDF, the Mn(II)DF complex does not compete effectively with 6-hydroxydopamine for $O_2^{\cdot -}$.

The search for an effective low molecular weight mimic of superoxide dismutase continues. To be a true and effective catalyst, the mimic should inhibit both reducing and oxidizing actions of $O_2^{\cdot -}$.

REFERENCES

1. Brigelius, R.; Spottl, R.; Bors, W.; Lengfelder, E.; Saran, M.; Weser, U. Superoxide dismutase activity of low molecular weight Cu^{2+}-chelates studied by pulse radiolysis. *FEBS Lett.* **47**:72-75; 1974.

2. Halliwell, B. The superoxide dismutase activity of iron complexes. *FEBS Lett.* **56**:34-38; 1975.

3. Younes, M.; Weser, U. Inhibition of nitrobluetetrazolium reduction by cuprein (superoxide dismutase), $Cu(tyr)_2$, and $Cu(lys)_2$. *FEBS Lett.* **61**:209-212; 1976.

4. Younes, M.; Lengfelder, E.; Zienau, S.; Weser, U. Pulse radiolytically generated superoxide and Cu(II)-salicylates. *Biochem. Biophys. Res. Comm.* **81**:576-580; 1978.

5. Robertson, P.Jr.; Fridovich, I. Does copper-D-penicillamine catalyze the dismutation of $O_2^{\cdot-}$? *Arch. Biochem. Biophys.* **203**:830-831; 1980.

6. Weinstein, J.; Bielski, B.H.J. Reaction of superoxide radicals with copper(II)-histidine complexes. *J. Am. Chem. Soc.* **102**:4916-4919; 1980.

7. Archibald, F.S.; Fridovich, I. The scavenging of superoxide radical by manganous complexes: *In vitro.*; *Arch. Biochem. Biophys.* **214**:452-463; 1982.

8. Bull, C.; Fee, J.A.; O'Neil, P.; Fielden, E.M. Iron-ethylenediaminetetraacetic acid (EDTA)-catalyzed superoxide dismutation revisited: An explanation of why the dismutase activity of Fe-EDTA cannot be detected in the cytochrome *c*/xanthine oxidase assay system. *Arch. Biochem. Biophys.* **215**:551-555; 1982.

9. Bull, C.; McClune, G.J.; Fee, J.A. The mechanism of Fe-EDTA catalyzed superoxide dismutation. *J. Am. Chem. Soc.* **105**:5290-5300; 1983.

10. Roberts, N.A.; Robinson, P.A. Copper chelates of antirheumatic and anti-inflammatory agents: Their superoxide dismutase-like activity and stability. *Brit. J. Rheumatol.* **24**:128-136; 1985.

11. Darr, D.; Zarilla, K.A.; Fridovich, I. A mimic of superoxide dismutase activity based upon desferrioxamine B and manganese(IV). *Arch. Biochem. Biophys.* **258**:351-355; 1987.

12. Tachon, P. Cytotoxicity of the hypoxanthine-xanthine oxidase system on V79 cells: Comparison of the effects of superoxide dismutase and CuDIPS. *Free Rad. Res. Comms.* **7**:367-374; 1989.

13. Miesel, R.; Weser, U. Anticarcinogenic reactivity of copper-dishiffbases with superoxide dismutase-like activity. *Free Rad. Res. Comms.* **11**:39-51; 1990.

14. Darr, D.J.; Yanni, S; Pinnel, S.R. Protection of Chinese hamster ovary cells from paraquat-mediated cytotoxicity by a low molecular weight mimic of superoxide dismutase (DF-Mn). *Free Rad. Biol. Med.* **4**:357-363; 1988.

15. Rabinowitch, H.D.; Rosen, G.M.; Fridovich, I. A mimic of superoxide dismutase activity protects *Chlorella sorokiniana* against the toxicity of sulfite. *Free Rad. Biol. Med.* **6**:45-48; 1989.

16. Beyer, W.F.Jr.; Fridovich, I. Characterization of a superoxide dismutase mimic prepared from desferrioxamine and MnO_2. *Arch. Biochem. Biophys.* **271**:149-156; 1989.

17. Bandy, B.; Davison, A.J. Interactions between metals, ligands, and oxygen in the autoxidation of 6-hydroxydopamine: Mechanisms by which metal chelation enhances inhibition by superoxide dismutase. *Arch. Biochem. Biophys.* **259**:305-315; 1987.

18. Cooper, S.R.; McArdle, J.V.; Raymond, K.N. Siderophore electrochemistry: Relation to intracellular iron release mechanism. *Proc. Natl. Acad. Sci. USA* **75**:3551-3554; 1978.

19. Borg, D.C.; Schaich, K.M. Prooxidant action of desferrioxamine: Fenton-like production of hydroxyl radicals by reduced ferrioxamine. *J. Free Rad. Biol. Med.* **2**:237-243; 1986.

20. Halliwell, B. Manganese ions, oxidation reactions and the superoxide radical. *Biochem. J.* **5**:113-118; 1984.

LEGENDS TO FIGURES

Figure 1. *Effect of the green Mn-desferrioxamine complex (MnDF) on cytochrome c reduction by xanthine/xanthine oxidase.* The xanthine oxidase/cytochrome *c* reduction assay for superoxide dismutase activity was conducted under usual conditions except for omission of EDTA (as described previously[11]). Reactions were initiated by addition of 0.12 mg/ml xanthine oxidase to 50 mM potassium phosphate buffer (pH 7.4, 25°C) containing 50 μM xanthine and 10 μM cytochrome *c*. Reduction of cytochrome *c* was monitored at 550 nm. The MnDF preparation or desferrioxamine were added at 100 μM.

Figure 2. *Effects of the green Mn-desferrioxamine complex (MnDF) on autoxidation of 6-hydroxydopamine.* Autoxidation of 6-hydroxydopamine (200 μM) was followed at 490 nm in air-saturated 50 mM phosphate buffer, pH 7.4, 25°C. MnDF was added at 80 μM or 200 μM. Desferrioxamine was at 80 μM or 0.5 mM.

Figure 1. Effect of MnDF complex on reduction of cytochrome *c* by xanthine/xanthine oxidase

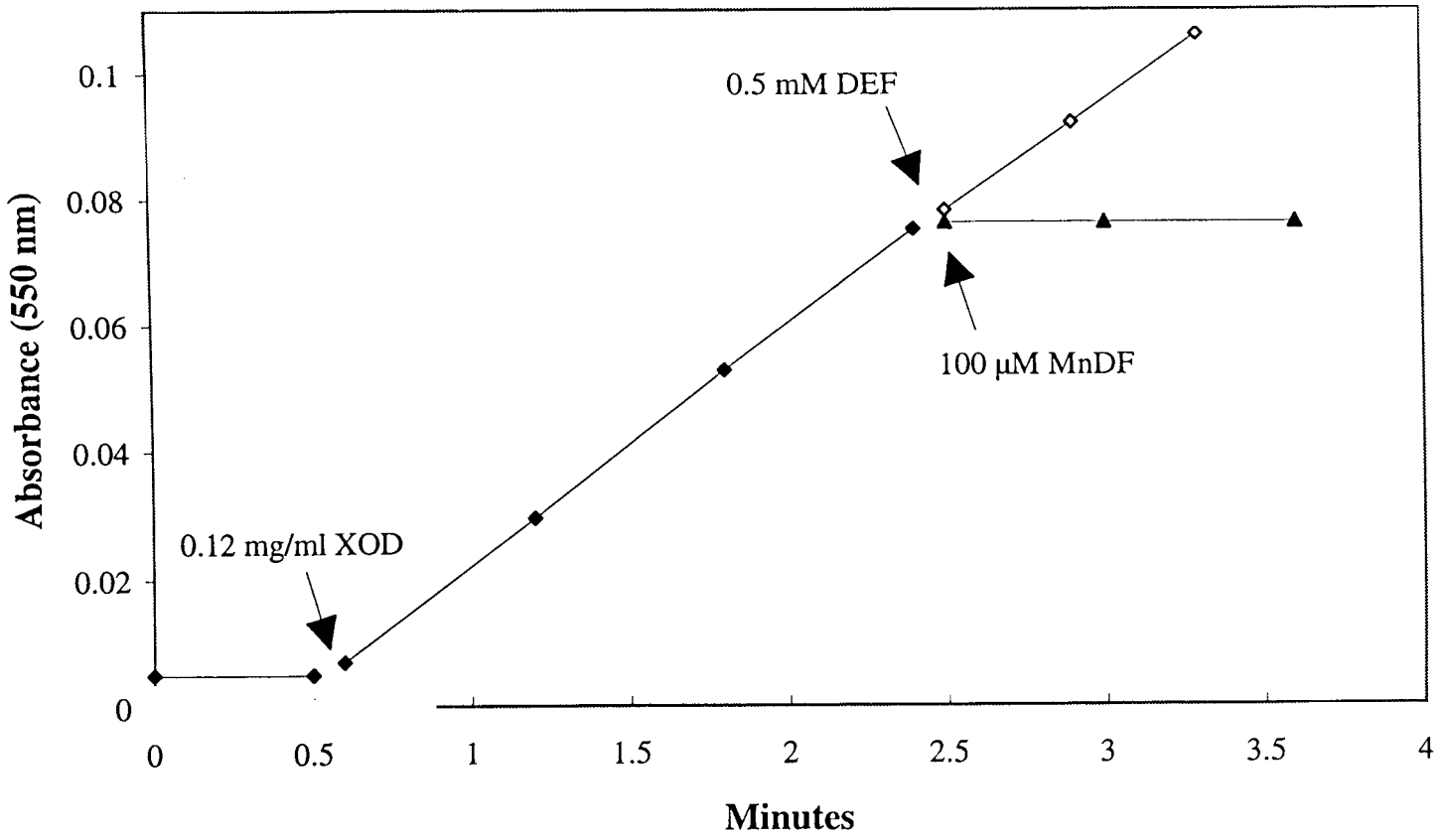
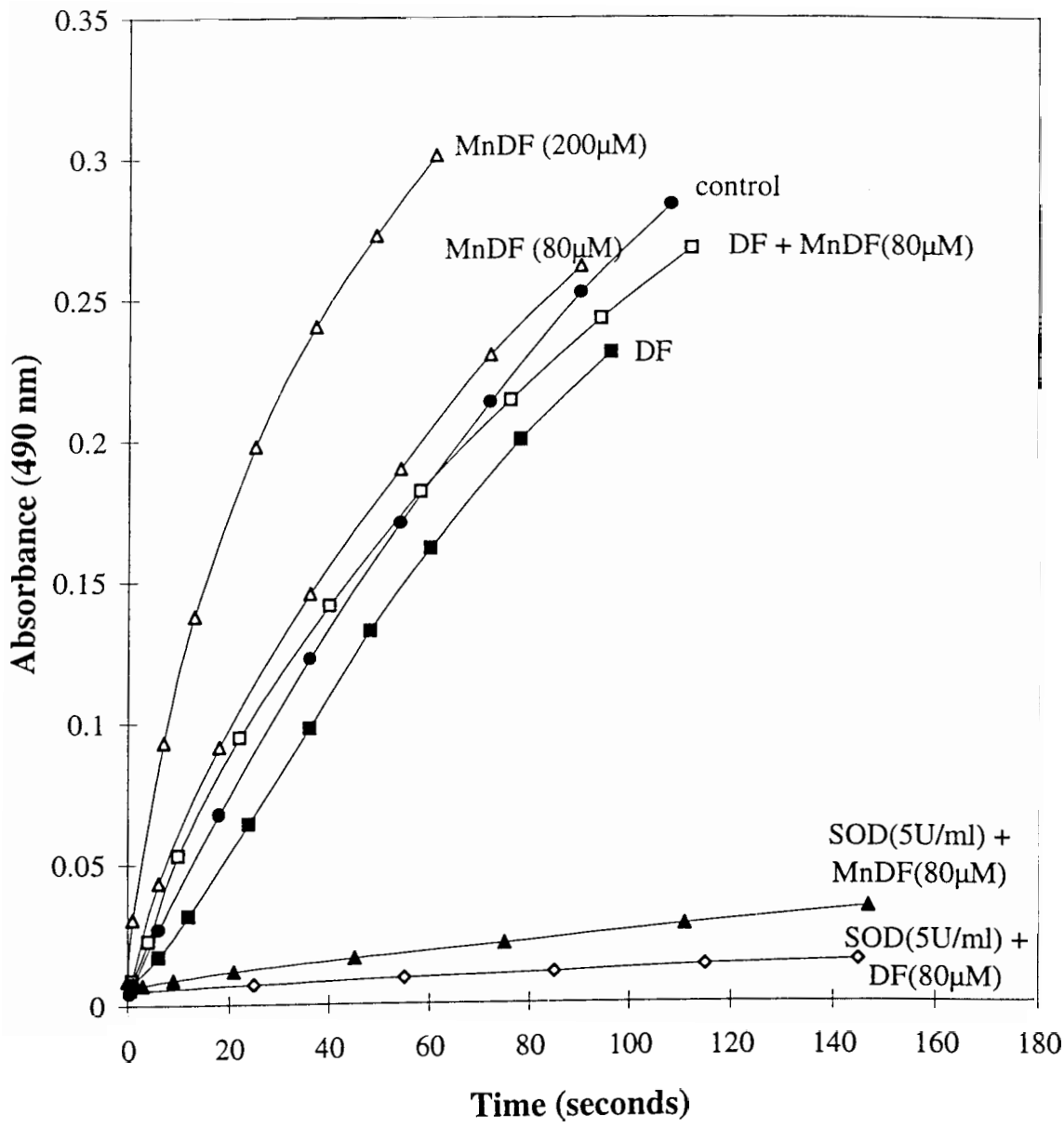


Figure 2. Effects of MnDF Complex on Autoxidation of 6-Hydroxydopamine



Chapter 7.
Inhibition by Superoxide Dismutase and Metal Chelators in the Autoxidation of Ascorbate

by

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ABSTRACT

Unlike some autoxidations, no synergism between metal chelators and superoxide dismutase was observable in the autoxidation of ascorbate due to strong (over 98%) inhibition by chelators alone. Superoxide dismutase alone inhibited ascorbate autoxidation by up to 60%. However, heat-denatured superoxide dismutase inhibited more strongly (up to 85%) than native enzyme, indicating a greater affinity of the unfolded protein for trace metals. Albumin inhibited more strongly than denatured superoxide dismutase, emphasizing its importance in blood as a metal-binding antioxidant. In autoxidation of 6-hydroxydopamine, albumin or heat-denatured superoxide dismutase, like metal chelators, inhibited synergistically with active superoxide dismutase. H_2O_2-inactivated superoxide dismutase however did not inhibit synergistically with active superoxide dismutase, indicating H_2O_2-induced loss of metal binding sites. In summary, we found no evidence for participation of $O_2^{\cdot -}$ in ascorbate autoxidation, and observed differing metal-binding affinities of different inactivated preparations of superoxide dismutase.

INTRODUCTION

Does $O_2^{\cdot-}$ participate in ascorbate autoxidation?

Contradictory reports on the participation of $O_2^{\cdot-}$ in ascorbate autoxidation raises the question of whether coordination of trace metals, like in 6-hydroxydopamine autoxidation[1], sensitizes ascorbate autoxidation to inhibition by superoxide dismutase. Ascorbate reacts efficiently with $O_2^{\cdot-}$ ($k=2.7 \times 10^5\ M^{-1}\ sec^{-1}$ at pH 7.4),[2-4] and the resulting ascorbyl radical decays mostly by disproportionation[5]. However, the extent to which autoxidation of ascorbate produces $O_2^{\cdot-}$, and proceeds as an $O_2^{\cdot-}$-propagated chain, has been a matter of debate.

In an early study, superoxide dismutase strongly inhibited ascorbate autoxidation when observed in phosphate buffer at pH 8.8.[6] A subsequent study however, showed that albumin or superoxide dismutase apoenzyme inhibited as strongly as superoxide dismutase under these conditions,[7] implying inhibition by metal binding. Moreover, no $O_2^{\cdot-}$ generation during ascorbate autoxidation was evident using epinephrine or nitro blue tetrazolium as probes.[7] At pH 10.2 (Na_2CO_3 buffer), where addition of 33 μM Fe(III):EDTA or Fe(II):EDTA stimulated autoxidation of 50 μM ascorbate (~3 fold), superoxide dismutase had little or no inhibitory effect.* Superoxide dismutase (but not albumin) did abolish $Fe^{2+}_{(aquo)}$-stimulated oxidation of ascorbate, but this effect was attributed to removal of $O_2^{\cdot-}$ produced from the rapid autoxidation of Fe^{2+} (33 μM) at alkaline pH.[7] With $Cu^{2+}_{(aquo)}$-stimulated ascorbate autoxidation (pH 10.2), superoxide dismutase, albumin, and superoxide dismutase apoenzyme inhibited equally.

Then, while confirming a strong inhibitory effect of superoxide dismutase apoenzyme on ascorbate autoxidation in untreated phosphate buffer (pH 7.4), Scarpa et al[9] observed 50%

* The lack of a similar effect of SOD with Fe(II):EDTA was considered due to the ability of Fe(II):EDTA to catalytically remove $O_2^{\cdot-}$.[8] ie. Any $O_2^{\cdot-}$ produced from autoxidation of Fe(II):EDTA is reduced efficiently to H_2O_2 by remaining Fe(II):EDTA, while Fe(III):EDTA oxidizes ascorbate to regenerate Fe(II):EDTA.

inhibition by 1.0 μM (~100 U/ml) active superoxide dismutase of the slow autoxidation in chelex treated buffer. In the presence of 0.8-5.0 μM metal-complex catalysts (Fe(III):EDTA, Fe(II):EDTA, Fe(III):ADP, Cu(II):histidine$_2$), 0.1 μM or more superoxide dismutase inhibited 50%, while albumin or superoxide dismutase apoenzyme were without effect. Generation of $O_2^{\cdot-}$ was also detected using an NMR method for measuring reduction of active site Cu^{2+} in superoxide dismutase. Metal-catalyzed autoxidation in the absence of complexing ligands (allowing inner sphere electron transfers from ascorbate to oxygen) was not tested for participation of $O_2^{\cdot-}$.

Studying $Cu^{2+}_{(aquo)}$-catalysed autoxidation of ascorbate in phosphate buffer (pH 7.4), Lovstad[10] found equal or greater inhibitory effects of albumin or azide-inhibited superoxide dismutase compared to active superoxide dismutase (I_{50}=2.2 μM). The presence of nitroblue tetrazolium to scavenge $O_2^{\cdot-}$ also had no effect on the rate of Cu^{2+}-catalysed ascorbate autoxidation. The ability of various proteins, amino acids, or chelators to inhibit Cu^{2+}-catalysed autoxidation was proportional to their affinity for cuprous or cupric ions relative to ascorbate.

Most recently, in Tris buffer with usual trace metals, Mordente et al[11] found equal inhibition by albumin and superoxide dismutase in the absence of a chelator. In the presence of desferrioxamine however, superoxide dismutase inhibited the residual ascorbate autoxidation by 50%, while albumin did not inhibit.*

These previous studies differed substantially in pH, metal concentration, and metal complex studied. Whether $O_2^{\cdot-}$ is produced and participates in ascorbate autoxidation may depend on any of several factors:

i) pH, influencing the level of ascorbate dianion (pK 11.3) and hydrolysis of metal ions,

* Paradoxically, while desferrioxamine alone slowed oxidation of 0.1 mM ascorbate, desferrioxamine accelerated oxygen consumption by 5 mM or more ascorbate, and superoxide dismutase inhibited this acceleration by 56%. This effect was attributed to the formation of a desferrioxamine anion radical which autoxidises to produce $O_2^{\cdot-}$.

ii) the background level of trace metals (relative to added ligands or metals),
iii) the level of added metals relative to ascorbate (ie. need to redox cycle),
iv) the affinity and concentration of competing ligands relative to ascorbate, and
v) the nature of the metal (eg. Cu^{2+} versus Fe^{3+}).

From previous studies we might summarize that participation of $O_2^{\cdot-}$ was detected at extremely low levels of catalytic metals (chelex treated buffer), and in the presence of low (relative to ascorbate) levels of complexed metals.[9,11] Participation of $O_2^{\cdot-}$ was not evident in the presence of significant trace metals and/or added metals (aquo),[7,10] or in the presence of a substantial (relative to ascorbate) level of complexed metal (Fe(III):EDTA, Fe(II):EDTA)[7]. Accordingly, ascorbate autoxidation, like autoxidation of 6-hydroxydopamine, may proceed by concerted inner sphere electron transfers (without release of $O_2^{\cdot-}$) when it can form a ternary ascorbate:metal:oxygen complex (as described by Martell)[12], but may proceed by a single electron, $O_2^{\cdot-}$-propagated pathway when catalytic metals (at low concentrations) are complexed.

Previous studies have not tested the relative ability of different ligands to sensitize trace metal catalysed ascorbate autoxidation to inhibition by superoxide dismutase. We thus compared the effects of several ligands (including proteins) in the presence and absence of superoxide dismutase.

Ascorbate autoxidation as a measure of metal affinities of proteins

The strong dependence of the rate of ascorbate autoxidation on the level of catalytic metals makes it useful for testing levels of adventitious metals in buffer solutions.[13] Conversely, this dependence makes ascorbate autoxidation suitable for assessing relative levels and affinities of competing ligands. Ascorbate has a relatively weak affinity for metal cations (Ka's of $10^{1.4}$-$10^{3.6}$ for deprotonated complexes) compared to other ene-diols (Ka's of 10^{5}-10^{10}).[12] This weak affinity makes ascorbate autoxidation sensitive to small changes in competing ligands. Some inhibitory effects of superoxide dismutase in biochemical or physiological

studies may be due, at least in part to binding of metals by apoprotein. Thus, we compared the inhibitory effects of native superoxide dismutase, H_2O_2-inactivated superoxide dismutase, and heat-denatured superoxide dismutase in ascorbate autoxidation.

MATERIALS AND METHODS

Ascorbic acid and Na_2CO_3 were from Sigma. $FeCl_3$ was from Fisher. Superoxide dismutase (bovine liver) was from DDI Pharmaceuticals (Mountainview, CA). Other materials were as described previously. Solutions were made with deionized, distilled water. Buffer solutions were not purified further, leaving trace metals at normal levels.[13] Fe(III):EDTA was prepared as a stock solution of equimolar EDTA plus $FeCl_3$. Ascorbate and 6-hydroxydopamine stock solutions were prepared in nitrogen-saturated water and sealed under a slight positive pressure of nitrogen.

Heat denatured superoxide dismutase was prepared by immersing the end of a test tube (covered with a marble to limit evaporation) containing 5000 U/ml superoxide dismutase in boiling water for 20 minutes. The resulting preparation had less than 1/20 th the activity of native superoxide dismutase as measured in the xanthine oxidase/cytochrome *c* reduction assay[14]. H_2O_2-inactivated superoxide dismutase[15,16] was prepared by exposing 1.4 mg/ml superoxide dismutase to 10 mM H_2O_2 for 90 minutes in 0.1 M $NaHCO_3$ buffer (pH 9.5, 25°C) and dialyzing against phosphate buffer (50 mM, pH 7.4) containing 50 U/ml catalase. Protein concentrations of superoxide dismutase preparations were determined by the method of Lowry et al[17].

Ascorbate autoxidation was conducted in 50 mM air-saturated phosphate buffer (pH 7.0 and pH 8.8) or carbonate (pH 10.2) buffers at 25°C. Ligands (0.2 mM), proteins (as indicated), or Fe(III):EDTA (5 μM) were included as indicated, and the reaction initiated by addition of ascorbate to give an initial concentration of 100 μM. Initial rates of ascorbate oxidation were determined from loss of absorbance at 265 nm (E_{265}=15000 M^{-1} cm^{-1}).

6-Hydroxydopamine autoxidation was conducted in air-saturated phosphate buffer (pH 7.4, 25°C), with ligands or proteins added as indicated. Reactions were initiated on addition of 6-hydroxydopamine by gas-tight syringe to give an initial concentration of 200 μM. Formation of *p*-quinone oxidation product was followed spectrophotometrically at 490 nm.

RESULTS AND DISCUSSION

Superoxide dismutase inhibits ascorbate autoxidation primarily by coordination of trace metals

Superoxide dismutase inhibited ascorbate autoxidation by up to 45% at pH 7.0, or 60% at pH 8.8 (Figure 1). Heat-denatured superoxide dismutase however, with less than 1/20 th residual superoxide dismutase activity, inhibited more strongly at all concentrations, up to a maximum of 80-85%. These results suggest that denaturation of superoxide dismutase opens up additional high affinity sites for coordination of trace metals. In 6-hydroxydopamine autoxidation, denatured superoxide dismutase, like metal chelators (including albumin and other proteins),[17] inhibited autoxidation synergistically with active superoxide dismutase (Figure 2). Unlike heat-denatured superoxide dismutase however, H_2O_2-inactivated superoxide dismutase provided little synergism with active superoxide dismutase in inhibiting autoxidation of 6-hydroxydopamine (Figure 2). This lessened inhibitory effect of H_2O_2-inactivated superoxide dismutase suggests a decreased affinity for trace metals. H_2O_2 presumably destroys sites on the protein which bind metals through site specific Fenton reactions. These results with different preparations of inactivated superoxide dismutase show the need for caution in choosing and interpreting controls for effects of superoxide dismutase activity. The slight residual activity of heat-denatured superoxide dismutase (or dialyzed apoprotein) may be significant under sensitive circumstances (eg. 6-hydroxydopamine autoxidation in the presence of desferrioxamine)[1]. A good control therefore might be albumin, which possesses metal binding properties but no superoxide dismutase activity.

Metal participation dominates $O_2^{\cdot-}$ in determining rates of ascorbate autoxidation

Autoxidation of ascorbate in phosphate buffer at pH 7.0 proceeded with a first order rate constant of 6.2×10^{-4} sec^{-1}. This rate agrees reasonably with values of 3.96×10^{-4} sec^{-1} for

ascorbate autoxidation at pH 7.0 (calculated from data of Buettner[18]) and 5.87×10^{-4} sec^{-1} for ascorbate monoanion (which predominates 99.9% at pH 7.0)[19]. These rates are much faster than those in chelex-treated buffers (2×10^{-6} - 6×10^{-7} sec^{-1})[9,13], indicating greater presence of metal ions. Concentrations of trace Fe^{3+} and Cu^{2+} in 50 mM phosphate buffer have been estimated at 0.3 - 0.7 μM and ~0.13 μM.[13] Calculation of k_{cat}'s in ascorbate autoxidation at pH 7.0 for Fe(III) (10 M^{-1} sec^{-1}) and Cu(II) (880 M^{-1} sec^{-1}) (including hydrolysis species present)[13] suggest that copper may have the greater influence on ascorbate autoxidation in untreated phosphate buffer. The properties of any competing ligands will obviously influence the reaction mechanism, and the extent to which different metals participate.

At pH 7.0, strong inhibition and slow autoxidation in the presence of metal chelators, including albumin or denatured superoxide dismutase, precluded observation of synergistic interactions between metal chelators and superoxide dismutase (Figure 3). Addition of 5 μM Fe(III):EDTA, a catalyst with which participation of $O_2^{\cdot-}$ had previously been observed (in chelex-treated buffer),[9] produced just a slight increase in ascorbate autoxidation (Figure 3).* While superoxide dismutase inhibited ascorbate autoxidation in the presence of Fe(III):EDTA by 15-38%, denatured superoxide dismutase was equally or more inhibitory (Figure 3). Binding of catalytic metals was apparently the primary effect of superoxide dismutase in this situation.

We then examined ascorbate autoxidation at increased pH, to increase the rate of autoxidation such that any synergistic inhibition by metal chelators and superoxide dismutase (but not denatured superoxide dismutase) might be observed. At pH 8.8, addition of 200 μM EDTA still inhibited autoxidation too strongly to observe any synergistic effect on inhibition by superoxide dismutase (Figure 4). Desferrioxamine, histidine, or albumin similarly inhibited 98-99.9 %. The rate in the presence of desferrioxamine (0.10 μM/min),

* Since EDTA abolishes catalytic effects of Cu^{2+} on ascorbate autoxidation, but increases catalysis by Fe^{3+},[18] any exchange of EDTA-bound Fe^{3+} with adventitious Cu^{2+} presumably decreased catalysis by Cu^{2+} and increased the proportion of catalysis by Fe^{2+} and Fe(III):EDTA.

was still just 24% of that where further inhibition by superoxide dismutase was observed (in Tris buffer, pH 7.4, 37°C).[11] Phytate produced little if any inhibition, but had no effect (or even decreased) inhibition by superoxide dismutase. At this pH, addition of Fe(III):EDTA inhibited ascorbate autoxidation, perhaps due to exchange coordination of catalytic Cu^{2+}. Addition of superoxide dismutase provided some further inhibition, but so did denatured superoxide dismutase.

At pH 10.2, addition of EDTA produced a slower, but readily observable rate of autoxidation (Figure 5).* Desferrioxamine, histidine, or albumin similarly produced slower but observable rates of autoxidation. In each case however, addition of superoxide dismutase provided no further inhibition.

Unlike in 6-hydroxydopamine autoxidation, these studies revealed no evidence for participation of $O_2^{\cdot-}$ in the trace metal catalysed autoxidation of ascorbate using the ligands studied. These results agree with the reported lack of inhibitory effects of superoxide dismutase in trace metal-catalysed or Cu^{2+}-catalysed autoxidation of ascorbate.[7,10] With EDTA, our results tend to support evidence against[7] and contrast evidence for[9] involvement of $O_2^{\cdot-}$ in Fe(III):EDTA catalysed ascorbate autoxidation. With desferrioxamine, we were not able to confirm evidence for an involvement of $O_2^{\cdot-}$,[11] under the conditions studied.

Scarpa et al[9] suggest that the lack of inhibition observed by Halliwell and Foyer[7] was due to insufficient superoxide dismutase. However, ascorbate reacts with $O_2^{\cdot-}$ at a rate of 2.7×10^5 - 9.3×10^4 M^{-1} sec^{-1} in the pH range 7.4 - 10.2,[3,4] while superoxide dismutase reacts with $O_2^{\cdot-}$ at 1.9×10^9 M^{-1} sec^{-1}.[20] Scarpa et al[9] required 0.1 μM superoxide dismutase to inhibit (50%) the rate of Fe(III):EDTA catalysed autoxidation of 120 μM ascorbate. Halliwell and Foyer[7] used 0.17 μM superoxide dismutase (assuming 2500 U/mg for Sigma superoxide

* The rate observed here in the presence of EDTA was comparable to that observed previously[7]. The greater rate in the absence of ligands was thus presumably due to a slightly greater level of trace copper in the buffer solutions used here.

dismutase, and a molecular weight of 32,000), which should compete effectively with the 50 μM ascorbate used.

The much higher concentration of Fe(III):EDTA relative to ascorbate in the studies of Halliwell and Foyer[7] (33 μM Fe(III):EDTA/50 μM ascorbate) compared to Scarpa et al[9] (4.7 μM Fe(III):EDTA/120 μM ascorbate) may obscure any role of $O_2^{\cdot -}$ by the limited redox cycling required of the catalyst. However, we observed no effect of superoxide dismutase in the presence of EDTA and ambient (trace) levels of Fe(III).

Differences in previous observations of inhibition by superoxide dismutase in the presence of Fe(III):EDTA[7,9], might be explicable by differences in pH. At pH 10.2, where inhibition by superoxide dismutase was not evident,[7] the ascorbate dianion (pKa 11.3; present at 7.36%) contributes more substantially to the autoxidation, and may react differently than ascorbate monoanion. For example, while the ascorbate monoanion apparently forms mixed-ligand chelates (AH^-:M^{n+}:EDTA) which exclude molecular oxygen,[12] the dianion might displace the competing ligand further to form di-ascorbate complexes accessible to oxygen (allowing concerted two-electron reduction of oxygen). Also, the rate of reaction of ascorbate with $HO_2^{\cdot}/O_2^{\cdot -}$ decreases with an increase in pH from 7 to 11.[4] However, the lack of specific inhibition by superoxide dismutase at pH 7.0 in the presence of Fe(III):EDTA in the current study argues against this explanation.

CONCLUSIONS

Rates of "spontaneous" (trace metal catalysed) and Fe^{3+} or Cu^{2+} catalysed autoxidation are reportedly dependent on oxygen concentration.[12,19] Together with first order dependencies on metal and ascorbate anion concentrations, this dependence on oxygen concentration suggests involvement of a ternary ascorbate:metal:oxygen complex.[12,19] The reaction mechanism proposed is a rate-determining inner-sphere transfer of electrons (hydride shift, or successive transfer of two electrons), preceded by equilibria of complex formation.

$$M^{n+} + HA^- \Leftrightarrow M^{n+}{:}HA^-$$

$$M^{n+}{:}HA^- + O_2 \Leftrightarrow O_2{:}M^{n+}{:}HA^-$$

$$O_2{:}M^{n+}{:}HA^- + H^+ \Rightarrow M^{n+} + A + H_2O_2$$

The stronger inhibition by denatured superoxide dismutase than superoxide dismutase tends to support such an $O_2^{\cdot-}$-dependent mechanism for trace metal catalysed autoxidation.

Rates of ascorbate autoxidation catalysed by metal chelates (eg. Fe(III):EDTA or Cu(II):EDTA) are slower than with metals alone (aquo complexes), and independent of oxygen concentration.[12,21] These rates show a linear dependence on metal:chelate concentration, and a decrease with increased stability of the chelate. Together with a lack of dependence on oxygen, these observations suggest a slow rate-determining electron transfer within a mixed ligand complex, followed by rapid reoxidation of the reduced chelate by molecular oxygen.[12,21]

$$M^{n+}{:}L + HA^- \Leftrightarrow HA^-{:}M^{n+}{:}L$$

$$HA^-{:}M^{n+}{:}L \Rightarrow M^{(n-1)+}{:}L + A^{\cdot-} + H^+$$

$$M^{(n-1)+}{:}L + O_2 \Rightarrow M^{n+}{:}L + O_2^{\cdot-}$$

The reoxidized chelate can then participate in further oxidation of ascorbate. The $O_2^{\cdot-}$ produced might also participate in further oxidation of ascorbate, but we were unable to find evidence for such a role under the conditions studied.

REFERENCES

1. Bandy, B.; Davison, A.J. Interactions between metals, ligands, and oxygen in the autoxidation of 6-hydroxydopamine: Mechanisms by which metal chelation enhances inhibition by superoxide dismutase. *Arch. Bioch. Biophys.* **259:**305-315; 1987.

2. Nishikimi, M. Oxidation of ascorbic acid with superoxide anion generated by the xanthine-xanthine oxidase system. *Biochem. Biophys. Res. Comms.* **63**:463-468; 1975.

3. Nishikimi, M.; Yagi, K. Oxidations of ascorbic acid and α-tocopherol by superoxide. In: Osamu, H., Asada, K. eds. *Biochemical and Medical Aspects of Active Oxygen.* Baltimore: University Park Press; 1977:79-87.

4. Cabelli, D.E.; Bielski, B.H.J. Kinetics and mechanism for the oxidation of ascorbic acid/ascorbate by HO_2/O_2^- radicals. A pulse radiolysis and stopped-flow photolysis study. *J. Phys. Chem.* **87**:1809-1812; 1983.

5. Bielski, B.H.J.; Richter, H.W.; Chan, P.C. Some properties of the ascorbate free radical. *Ann. N.Y. Acad. Sci.* **258**:231-237; 1975.

6. Puget, K.; Michelson, A.M. Iron containing superoxide dismutases from luminous-bacteria. *Biochimie* **56**:1255-1267; 1974.

7. Halliwell, B.; Foyer, C.H. Ascorbic acid, metal ions and the superoxide radical. *Biochem. J.* **155**:697-700; 1976.

8. Halliwell, B. The superoxide dismutase activity of iron complexes. *FEBS Lett.* **56**:34-38; 1975.

9. Scarpa, M.; Stevanato, R.; Viglino, P.; Rigo, A. Superoxide ion as active intermediate in the autoxidation of ascorbate by molecular oxygen. *J. Biol. Chem.* **258**:6695-6697; 1983.

10. Lovstad, R.A. Copper catalyzed oxidation of ascorbate (vitamin C). Inhibitory effect of catalase, superoxide dismutase, serum proteins (ceruloplasmin, albumin, apotransferrin) and amino acids. *Int. J. Biochem.* **19**:309-313; 1987.

11. Mordente, A.; Meucci, E.; Miggiano, G.A.D.; Martorana, G.E. Prooxidant action of desferrioxamine: Enhancement of alkaline phosphatase inactivation by interaction with ascorbate system. *Arch. Biochem. Biophys.* **277**:234-240; 1990.

12. Martell, A.E. Chelates of ascorbic acid. Formation and catalytic properties. In:. Seib, P.A.; Tolbert, B.M.; eds. *Ascorbic Acid: Chemistry, Metabolism and Uses*. Washington D.C.: American Chemical Society; 1982:153-178.

13. Buettner, G.R. In the absence of catalytic metals ascorbate does not autoxidize at pH 7: ascorbate as a test for catalytic metals. *J. Biochem. Biophys. Methods* **16**:27-40; 1988.

14. McCord, J.M.; Fridovich, I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244**:6056-6063; 1969.

15. Blech, D.M.; Borders, C.L.Jr. Hydroperoxide anion, HO_2^-, is an affinity reagent for the inactivation of yeast Cu,Zn-superoxide dismutase. Modification of one histidine per subunit. *Arch. Biochem. Biophys.* **224**:579-586; 1983.

16. Ishii, T.; Fridovich, I. Dual effects of superoxide dismutase on the autoxidation of 1,4-naphthohydroquinone. *Free Rad. Biol. Chem.* **8**:21-24; 1990.

17. Lowry, O.H.; Rosebrough, N.H.; Farr, A.L.; Randall, R.J. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265-275; 1951.

18. Buettner, G.R. Ascorbate autoxidation in the presence of iron and copper chelates. *Free Rad. Res. Comms.* **1**:349-353; 1986.

19. Khan, M.M.T.; Martell, A.E. Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. I. Cupric and ferric ion catalyzed oxidation. *J. Am. Chem. Soc.* **89**:4176-4185; 1967.

20. Bielski, B.H.J.; Cabelli, D.E.; Arudi, R.L.; Ross, A.B. Reactivity of HO_2/O_2^- radicals in aqueous solution. *J. Phys. Chem. Ref. Data* **14**:1041-1100; 1985.

21. Khan, M.M.T.; Martell, A.E. Metal ion and metal chelate catalyzed oxidation of ascorbic acid by molecular oxygen. II. Cupric and ferric chelate catalyzed oxidation. *J. Am. Chem. Soc.* **89**:7104-7111; 1967.

Legends to Figures

Figure 1. *Dose-response of superoxide dismutase and heat-inactivated superoxide dismutase on the autoxidation of ascorbate.* Autoxidation of ascorbate (100 μM) was followed at 265 nm in air-saturated 50 mM phosphate buffer, pH 7.0, 25°C. Superoxide dismutase (SOD) and heat-denatured superoxide dismutase (dSOD) were added at the concentrations indicated, and the inhibition of ascorbate autoxidation compared.

Figure 2. *Synergistic effects of heat-denatured superoxide dismutase and H_2O_2-inactivated superoxide dismutase on inhibition of 6-hydroxydopamine autoxidation by superoxide dismutase.* Autoxidation of 6-hydroxydopamine (200 μM) was followed at 490 nm in air-saturated 50 mM phosphate buffer, pH 7.4, 25°C. Active superoxide dismutase (SOD) was included at 5 U/ml (1.25 μg/ml). Heat-denatured superoxide dismutase (dSOD) and H_2O_2-inactivated superoxide dismutase (H_2O_2-SOD) were included at 5.6 μg/ml. Desferrioxamine was at 0.5 mM.

Figure 3. *Effects of superoxide dismutase and heat-denatured superoxide dismutase on Fe(III):EDTA-catalysed autoxidation of ascorbate.* Initial rates of autoxidation of ascorbate (100 μM) in air-saturated 50 mM phosphate buffer (pH 7.0, 25°C) are plotted. Albumin was at 25 μg/ml. Fe(III):EDTA was at 5 μM. Native superoxide dismutase (SOD) and heat-denatured superoxide dismutase (dSOD) were at 5 μg/ml or 12.5 μg/ml.

Figure 4. *Effects of metal chelators and superoxide dismutase on autoxidation of ascorbate at pH 8.8.* Initial rates of autoxidation of ascorbate (100 μM) in air-saturated 50 mM phosphate buffer (pH 8.8, 25°C) are plotted. Ligands were included at 200 μM; albumin at 25 μg/ml; and superoxide dismutase at 10 U/ml (2.5 μg/ml) or 100 U/ml (25 μg/ml).

Figure 5. *Effects of metal chelators and superoxide dismutase on autoxidation of ascorbate at pH 10.2.* Initial rates of autoxidation of ascorbate (100 μM) in air-saturated 50 mM carbonate buffer (pH 10.2, 25°C) are plotted. Ligands were included at 200 μM; albumin at 25 μg/ml; and superoxide dismutase at 5 U/ml (1.25 μg/ml), 100 U/ml (25 μg/ml), and 200 U/ml (50 μg/ml).

Figure 1. Ascorbate Autoxidation: Inhibition by SOD and Denatured SOD

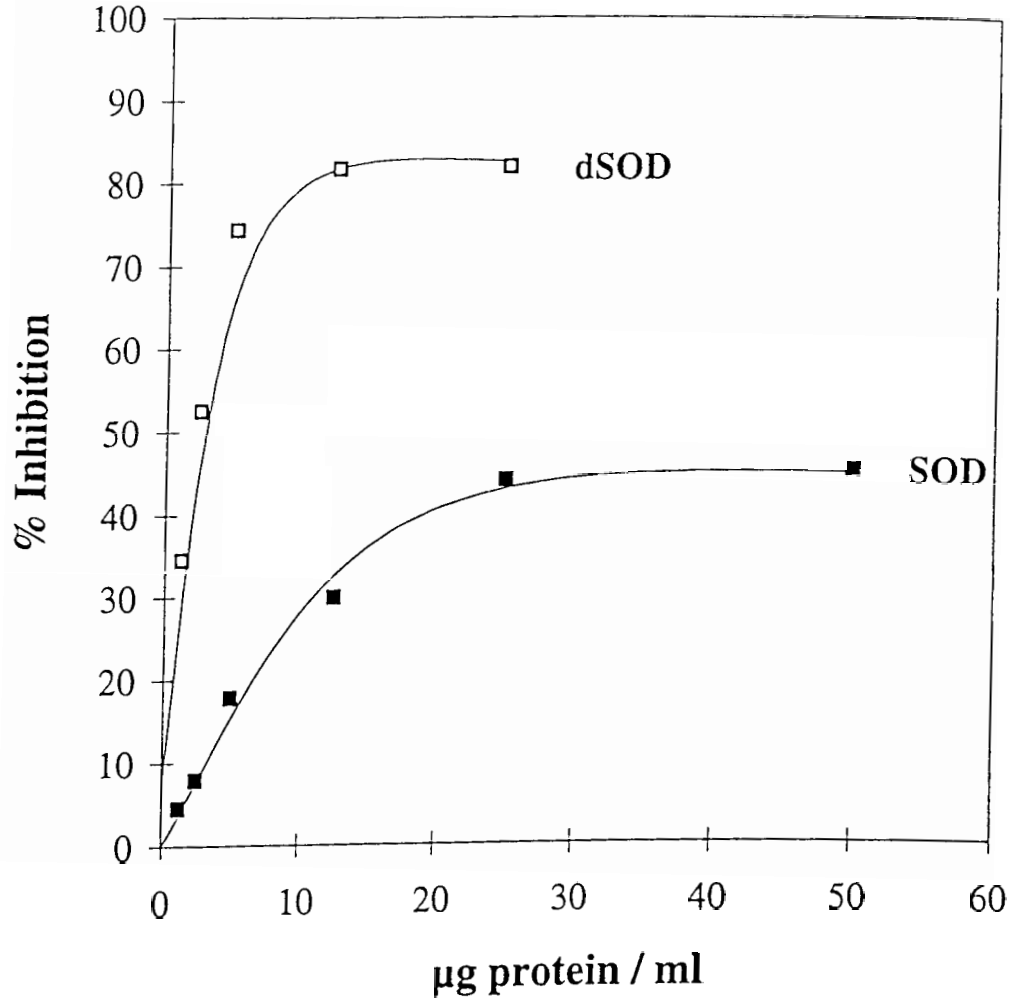

Figure 2.
6-OHDA Autoxidation: Synergism Between SOD and Heat-denatured or H_2O_2-inactivated SOD

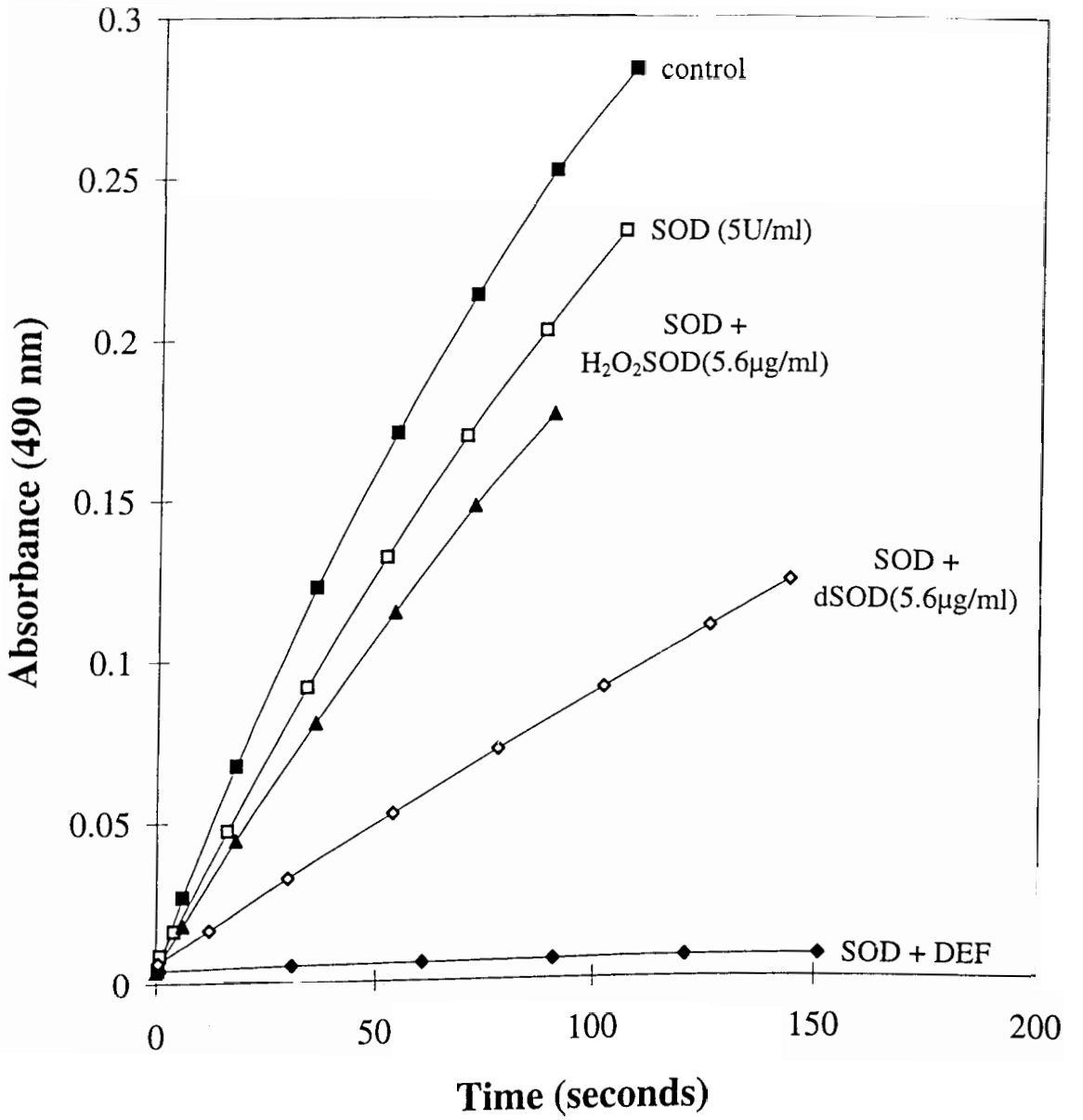

Figure 3. Ascorbate Autoxidation (pH 7.0)

Figure 4. Ascorbate Autoxidation (pH 8.8)

Figure 5. Ascorbate Autoxidation (pH 10.2)

Chapter 8.

Urate Cooperates with Ascorbate and Superoxide Dismutase to Inhibit Reduction of Oxygen by 6-Hydroxydopamine:

Synergism between metal-binding and chain-breaking antioxidant activities

by

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ABSTRACT

The plasma antioxidant, uric acid, is reportedly both a metal-binding and chain-breaking antioxidant. We sought to determine the relative metal-binding and chain-breaking activities of urate in the autoxidation of 6-hydroxydopamine. Urate alone (25-500 μM) inhibited autoxidation of 250 μM 6-hydroxydopamine by 47%. Urate and desferrioxamine inhibited autoxidation equally, and together provided no further inhibition. Together with 5 U/ml superoxide dismutase (which alone inhibited 33%), urate acted synergistically to inhibit by 96%. This superoxide dismutase-sensitizing effect was analogous to actions of metal chelators such as EDTA or desferrioxamine. Urate thus binds catalytic metals in a manner which prevents formation of a ternary 6-hydroxydopamine:metal:oxygen complex and forces outer sphere, superoxide-propagated, autoxidation. In assessing the metal-binding and chain-breaking actions of urate, a comparison with other structurally related purinols was of interest. Adenine, hypoxanthine, and xanthine had effects like those of urate, inhibiting autoxidation synergistically with superoxide dismutase. In contrast, AMP, adenosine, or inosine had no effect on autoxidation or on inhibition by superoxide dismutase. The presence of a ribose moiety on the purine ring thus prevents the metal sequestering action. The purine analogues, allopurinol and oxypurinol also inhibited 6-hydroxydopamine autoxidation and sensitized it to inhibition by superoxide dismutase. Clearly, binding of catalytic metals must not be neglected when assessing protective actions of these xanthine oxidase inhibitors.

Alone, ascorbate had no effect on 6-hydroxydopamine autoxidation, and it antagonized inhibition by superoxide dismutase. However, ascorbate cooperated with desferrioxamine or urate to inhibit autoxidation by 80%. Ascorbate evidently competes with 6-hydroxydopamine for superoxide, while urate competes with 6-hydroxydopamine for catalytic metals. Thus, in addition to chain-breaking actions, urate utilizes metal-binding, and cooperation with superoxide dismutase and ascorbate in providing antioxidant activity. *In vivo*, urate, ascorbate, and extracellular superoxide dismutase may cooperate as antioxidants in extracellular fluids.

INTRODUCTION

Urate has both chain-breaking and metal-binding antioxidant activities

Uric acid, the end-product of purine metabolism in humans, is an important antioxidant in biological fluids.[1-3] Urate inhibits lipid peroxidation,[4,5] protects erythrocytes from oxidant damage,[1] inhibits oxidative degradation of hyaluronic acid,[6] protects against DNA damage and mutagenicity by prooxidants,[7,8] and protects sensitive enzymes such as α_1-antiproteinase,[9] cyclooxygenase [10-12] and angiotensin converting enzyme[3]. Present in blood plasma and knee joint synovial fluid at 200-500 μM, urate exceeds ascorbate (50-200 μM) in these fluids. Urate is also present at substantial concentrations in other extracellular fluids such as cerebrospinal fluid, lymph, and interstitial fluids.[3,13,14]

Reportedly, urate acts as an antioxidant in two ways. As a chain-breaking antioxidant, urate reacts with oxidants such as hydroxyl radical, hypochlorous acid, singlet oxygen, peroxyl radicals, ozone, and oxo-heme species.[1,3,15,16] As a metal-binding antioxidant, urate binds free iron and copper and is likely a major low molecular weight ligand for metals in extracellular fluids.[17-19]

In scavenging oxidants, uric acid is oxidized to allantoin (and secondary products). The radical product of one-electron urate oxidation can itself produce biological damage (eg. enzyme inactivation), but is readily "repaired" (ie. reduced) by ascorbate,[9,16,20] leaving the more stable ascorbyl radical. In turn, urate protects ascorbate from autoxidation by binding free iron and copper in coordination complexes which do not catalyse ascorbate oxidation.[18,21,22]

Acting as a metal-binding antioxidant, urate is not itself significantly oxidized.[22]
A monoanion at neutral pH (pKa = 5.4)[15], urate binds Fe^{3+} and Fe^{2+} with association constants of 2.4×10^5 and 1.9×10^4.[18] Binding of a second molecule of urate is cooperative, forming a 2:1 complex with Fe^{3+} with an overall stability constant of 1.1×10^{11}. Uric acid

strongly inhibits Fe^{3+}-catalysed oxidation of ascorbate (72%, 82% and 97% at 1:1, 2:1, and 10:1 urate/ascorbate ratios),[18] as well as copper-catalysed oxidations of ascorbate[21] and NAD(P)H[23]. The mild effect of urate on the reduction potential of iron (decreasing it from 0.77 V to 0.67 V) does not likely account for this strong inhibition,[18,19] suggesting that urate inhibits ascorbate autoxidation by hindering access of ascorbate to iron. The extent to which urate hinders access of reductants with a higher affinity for metals, such as catecholamines, and the extent to which urate-metal complexes participate in other autoxidations remains largely unexplored.

Metal-binding mechanisms deserve closer attention in the antioxidant activities of allopurinol and oxypurinol

Infusion of the purine analogues, allopurinol and oxypurinol, provides protection against postischaemic reperfusion injury in several tissues, including intestine, kidney, and heart.[24-28] These compounds thus provide therapeutic benefit in such situations as cardiac surgery and organ transplantation. The protection is usually attributed to inhibition of xanthine oxidase and a resulting decrease in generation of active oxygen.[24,29,30] Evidence that allopurinol and oxypurinol scavenge hydroxyl radicals and hypochlorous acid [31-33] raised the possibility of other mechanisms for protection. Treatment with allopurinol does not influence the free radical scavenging properties of plasma or lymph,[34] but allopurinol prevents release of creatine kinase from reperfused heart as effectively as desferrioxamine,[35] and allopurinol and oxypurinol inhibit metal-catalysed oxidation of ascorbate.[36] These reports suggest a need to explore the extent to which protection by allopurinol or oxypurinol results from binding free iron or copper.

Does urate prevent pro-oxidant actions of ascorbate?

Roles of ascorbate as a chain-breaking antioxidant are well documented.[13,37,38] However, pro-oxidant actions are also seen in the presence of transition metal catalysts.[39-43] Pro-

oxidant actions of ascorbate likely occur *in vivo* only under conditions which cause release of heme or of metal ions from their binding proteins.[13,43] Under such conditions, metal coordination by low molecular weight ligands such as urate may become particularly important.

In previous studies of metal-catalysed "autoxidations", ascorbate generally acts as a coreductant, accelerating consumption of oxygen and formation of H_2O_2.[44-48] For example, ascorbate re-reduces quinone oxidation products of 6-hydroxydopamine or dialuric acid (i.e. alloxan), and thus allows redox cycling.[44-46] In this way, ascorbate may potentiate the neurotoxicity or cardiotoxicity of 6-hydroxydopamine[44,45,49-51] or other catecholamines[52,53] and the hemolytic or diabetes-causing actions of dialuric acid or alloxan[44,45]. These actions of ascorbate may help preserve reductants such as catecholamines, but perhaps at the price of increased formation of active oxygen. However, relatively little is known of the influence of ascorbate on autoxidations in the presence of metal chelators which inhibit reduction of oxygen.

6-Hydroxydopamine autoxidation can provide an index of the relative contributions of metal-binding and chain-breaking antioxidant activities.

Hydroxydopamine is a neurotoxin which at low concentrations selectively destroys catecholamine-concentrating neurons through generation of active oxygen.[44,54] Intracranial injection of 6-hydroxydopamine causes degeneration of dopaminergic and noradrenergic neurons[55] and may have implications for schizophrenia[56] and age-related pathologies such as Parkinsonism and neuromelanin deposition[57,58]. Intravenous injection induces cardiotoxicity due to degeneration of sympathetic adrenergic nerve terminals.[49,51] 6-Hydroxydopamine (in combination with ascorbate) is used clinically to purge bone marrow of neuroblastoma cells.[59]

The sensitivity of 6-hydroxydopamine autoxidation to inhibition by superoxide dismutase provides a means of assessing the relative importance of chain-breaking and metal-binding antioxidant effects of uric acid. Unlike ascorbate autoxidation, which is inhibited 92% or more by EDTA, DTPA, desferrioxamine or albumin,[18,60] 6-hydroxydopamine autoxidation is inhibited less strongly (or even accelerated) by metal chelators.[61-63] In the absence of metal chelators, 6-hydroxydopamine apparently autoxidizes by sequential transfer of two electrons to oxygen within a ternary 6-hydroxydopamine:metal:oxygen complex.[61-63] Such autoxidation is relatively insensitive to inhibition by superoxide dismutase. Metal chelators which prevent reduction of oxygen within a ternary 6-hydroxydopamine:metal:oxygen complex induce one-electron, superoxide-propagated autoxidation. From the sensitivity to superoxide dismutase therefore, 6-hydroxydopamine autoxidation distinguishes ligands which force outer sphere, one-electron transfers to oxygen.

In living systems, antioxidants of various kinds act in concert. Natural selection has optimized synergistic interactions between metal-binding and chain-breaking antioxidants. Unraveling the nature and mechanisms of these interactions is of continuing interest.

In the current study we explore the extent to which urate, and other purines, bind metals to force outer sphere electron transfers from 6-hydroxydopamine to oxygen. Comparisons with other purines (Figure 1) allow us to draw structure-activity relationships relevant to the metal binding actions of urate. Because autoxidation of 6-hydroxydopamine in the presence of metal chelators is superoxide-propagated, we could simultaneously assess the ability of urate and ascorbate to scavenge superoxide in competition with 6-hydroxydopamine.

MATERIALS AND METHODS

Materials from Sigma were sodium urate, inosine, hypoxanthine, xanthine, allopurinol, adenine, oxypurinol, adenosine, AMP, 6-hydroxydopamine, sodium ascorbate, superoxide dismutase (bovine erythrocyte), and potassium phosphate buffer salts. Disodium EDTA was from Fisher Scientific Company. Desferrioxamine (Desferal methanesulfonate) was a gift from CIBA Pharmaceuticals.

All aqueous solutions were made using deionized (Corning demineralizer 3508-A), distilled water. Buffer solutions were not purified further, so contained usual levels of trace metals present in buffer salts.

6-Hydroxydopamine was dissolved fresh before use in argon-saturated water and sealed under a slight positive pressure of argon. Aliquots were removed through a rubber septum with a Hamilton gas-tight syringe (initially purged with argon to prevent introduction of air to the vessel). Stock solutions of ascorbate were also made in argon-saturated water.

Experiments were conducted in air-saturated 50mM phosphate buffer, pH 7.4, 25°C. Urate was added at physiological concentrations (250-500 μM). Other purines were tested at 500 μM. Ascorbate was at 200 μM or 500 μM. Superoxide dismutase was at 5 U/ml. Reactions were initiated on addition of a 25 μl aliquot of 6-hydroxydopamine to 2.5 ml buffer to give an initial concentration of 250 μM. After brief mixing with a cuvette plunger, formation of quinone oxidation product was monitored spectrophotometrically at 490 nm. Concentrations of quinone product were calculated using an absorptivity of 1952 M^{-1} cm^{-1}.

RESULTS AND DISCUSSION

Urate hinders electron transfer between catalytic metals and oxygen

The results confirm a metal-binding antioxidant ability of uric acid. Urate alone inhibited the initial rate of 6-hydroxydopamine autoxidation by 47% (Figure 2). This inhibition was independent of urate concentration from 25 μM to 500 μM. In the presence of 5 U/ml superoxide dismutase, 500 μM urate enhanced inhibition from 33% to 96% (Figure 2). Inhibition decreased to 80% with 25 μM urate plus 5 U/ml superoxide dismutase (not shown). This synergistic inhibition of 6-hydroxydopamine autoxidation by urate and superoxide dismutase shows that, like EDTA or desferrioxamine,[62] urate coordinates trace metals in a manner which forces outer sphere electron transfers from 6-hydroxydopamine to oxygen.

The failure of urate to inhibit further in the presence of chelators which force $O_2^{\cdot-}$-propagated autoxidation, such as EDTA or desferrioxamine (Figure 2), indicates that urate does not compete effectively with the 6-hydroxydopamine for $O_2^{\cdot-}$. This result supports previous studies in other systems showing no significant scavenging of $O_2^{\cdot-}$ by urate.[64,65] Urate reportedly inhibits $O_2^{\cdot-}$-dependent luminol chemiluminescence, but this was interpreted as scavenging of $^{\bullet}OH$ produced through Haber-Weiss reactions.[66]

Although it does not have a large influence on the reduction potential of iron, urate presumably hinders access of 6-hydroxydopamine and/or oxygen to trace metals. By decreasing the reduction potential of iron from 0.77 to 0.67 V, urate, like EDTA, might expectedly accelerate 6-hydroxydopamine autoxidation, by accelerating reoxidation of Fe(II). Although urate does mildly accelerate autoxidation of Fe^{2+},[18] it apparently hinders access of 6-hydroxydopamine to Fe^{3+} (and other trace metals) sufficiently to inhibit catalysis. In this system then, urate renders trace metals less catalytically active than does EDTA. The iron:EDTA complex reportedly has an open coordination site.[67,68] The current results

suggest that metal complexes with urate may be "closed shell", providing no direct access of 6-hydroxydopamine or oxygen to the metal.

Other purines, but not purine nucleosides or nucleotides, share the antioxidant mechanisms of urate

Other purines vary in their ability to sensitize the autoxidation of 6-hydroxydopamine to inhibition by superoxide dismutase. Like urate, adenine, hypoxanthine, and xanthine inhibited autoxidation 31-49%, and enhanced inhibition by 5 U/ml superoxide dismutase from 33% to 93-97% (Figure 3). These purines also apparently bind catalytic metals to force outer sphere electron transfers to oxygen. In contrast, the purine nucleosides, adenosine and inosine, and the purine nucleotides, AMP and ADP, neither inhibited 6-hydroxydopamine autoxidation nor sensitized the autoxidation to inhibition by superoxide dismutase.

A previous study comparing the avidity of natural substances, including purines, for bivalent metals[17] provides some rationale for the current findings. Adenine, hypoxanthine, and urate formed 1:1 complexes with bivalent metals (including Cu^{2+} and Fe^{2+}) with Ka's from $10^{2.4}$ to $10^{6.2}$. In contrast, adenosine did not detectably bind metals. While indicative perhaps, the key to an inhibitory role in the current study is likely the ability to form dimeric complexes which hinder access of reductants or oxygen to the metal.

Differences in the structures of the purines tested in this system give insights into the mechanisms of metal-binding. The presence of ribose or ribose phosphate abolished kinetically effective metal complexing activity. Two possibilities for this effect are 1) hindrance of metal coordination involving nitrogen atom 9 on the purine ring, and 2) steric hindrance of dimeric complex formation. The common donor atoms among the compounds which complexed metals are an acidic nitrogen at position 9, a hydroxyl or amino group at position 6, and nitrogens at positions 1 and 3 on the purine ring. The lone pair electrons of

the nitrogen atoms, along with a donor group at position 6, conceivably contribute to the formation of a metal "sandwich" or "cage" within a dimeric complex (ie. resembling that of ferrocene).

From the results with allopurinol and oxypurinol, a nitrogen at position 7 was not needed for the superoxide dismutase sensitizing action. Thus, the known binding of metals by a hydroxyl group peri to a tertiary heterocyclic nitrogen atom, as seen in riboflavin or 8-hydroxyquinoline,[17] was not needed for the metal complexing actions of the 6-hydroxy purines. The lack of effect of inosine, which like hypoxanthine has a 6-hydroxy group and a peri nitrogen atom, but also a ribose on the nitrogen at position 9, supports this conclusion.

Xanthine, urate and oxypurinol inhibited slightly more strongly with superoxide dismutase than the other purines (Figure 3). This stronger synergism may reflect additional hydroxyl substituents on xanthine, urate and oxypurinol which increase the resonance stabilization of positive charge relative to adenine, hypoxanthine and allopurinol. The resulting increase in electron donor strength increases the affinity for cations, and thus may contribute to stronger metal complex formation. In addition, these hydroxyl groups offer intermolecular hydrogen bonding to increase the stability of dimeric complexes.

Metal binding affinity of allopurinol and oxypurinol may augment their abilities to inhibit superoxide generation.

Allopurinol and oxypurinol inhibit superoxide generation by blocking the xanthine/xanthine oxidase reaction. However, in the current studies they also displayed metal binding antioxidant activity (Figure 3). Allopurinol and oxypurinol inhibited 6-hydroxydopamine autoxidation 47-50%, and together with 5 U/ml superoxide dismutase inhibited by 94-97%. Thus, like uric acid, these purine analogues bind catalytic metals sufficiently to force an almost exclusive outer sphere, superoxide-propagated, autoxidation mechanism.

Binding of metals by allopurinol and oxypurinol is also evident in the metal catalysed oxidations of ascorbate and of erythrocyte membrane lipids.[36] Allopurinol and oxypurinol inhibited basal and copper-catalysed autoxidation of ascorbate.[36] In slowing ascorbate autoxidation, allopurinol and oxypurinol compete effectively with EDTA for cupric ions, but not for ferric ions. In ferric ion-induced oxidation of erythrocyte membrane lipids, allopurinol and oxypurinol inhibited, but not as strongly as uric acid.[36] The current study on 6-hydroxydopamine autoxidation extends such findings with the inference that metal-binding antioxidant activity of allopurinol and oxypurinol may be particularly protective in concert with superoxide dismutase activity.

While allopurinol and oxypurinol *do* inhibit superoxide generation from endothelial cells for example,[69] care must be taken in interpreting protective effects on reperfusion injury. An appropriate control for metal binding activity would be administration of desferrioxamine, with and without allopurinol or oxypurinol.

Urate and ascorbate cooperate to inhibit 6-hydroxydopamine autoxidation

Unlike urate, ascorbate had no effect on the autoxidation of 6-hydroxydopamine, and did not cooperate with superoxide dismutase (Figure 4). Thus ascorbate does not compete with 6-hydroxydopamine for catalytic metals, consistent with its low affinity for iron or copper (Ka's $10^{1.4}$ - $10^{3.6}$)[70]. Moreover, ascorbate antagonized inhibition by superoxide dismutase, with 500 μM ascorbate decreasing inhibition by 5 U/ml superoxide dismutase from 33% to 11%. As discussed further below, a probable explanation for this antagonism is that ascorbate intercepts some $O_2^{\cdot-}$, and the resulting ascorbyl radical propagates the chain by oxidising 6-hydroxydopamine.

Previous studies show increased oxygen consumption and H_2O_2 generation from 6-hydroxydopamine in the presence of ascorbate,[44-46] with diminished formation of *p*-quinone[45]. However, the concentration of ascorbate in the previous studies (5-10 mM) was

100 fold that of 6-hydroxydopamine (48-100 μM), so *p*-quinone and/or semiquinone were likely reduced more rapidly than in the current studies. As in the current study, inhibition by superoxide dismutase was decreased by ascorbate.[45]

In the presence of desferrioxamine, ascorbate inhibited 6-hydroxydopamine autoxidation. At 500 μM, ascorbate enhanced inhibition with desferrioxamine from 51% to 82% (Figure 4). Evident from synergistic inhibition by superoxide dismutase plus desferrioxamine, autoxidation in the presence of desferrioxamine is superoxide-propagated. The relatively facile reaction of superoxide with ascorbate ($k=10^5\ M^{-1}\ s^{-1}$ compared to $k=10^2\ M^{-1}\ s^{-1}$ for reaction with desferrioxamine)[71,72] allows ascorbate to compete with 6-hydroxydopamine for superoxide. In addition, ascorbate can reduce 6-hydroxydopamine semiquinone.[46] In either case, the resulting ascorbyl radical is obviously more stable, reacting to a greater extent by disproportionation[73]. In contrast to ascorbate, urate does not react significantly with superoxide,[64,65] and did not inhibit in the presence of desferrioxamine (Figure 2).

Previous studies on effects of ascorbate on autoxidations in the presence of chelators have produced mixed results. In the presence of EDTA, ascorbate (10mM) induced a slight lag in 6-hydroxydopamine autoxidation, but then accelerated *p*-quinone formation and oxygen consumption.[47,48] In the presence of DTPA, ascorbate amplified dopamine-induced oxygen consumption when added after the autoxidation had started, but inhibited when present before addition of dopamine.[52] Thus, in the presence of significant quinone, ascorbate may increase redox cycling and oxygen consumption. However, in the presence of chelators such as DTPA or desferrioxamine, ascorbate can slow autoxidations by scavenging superoxide and semiquinone.

Consistent with metal-binding by urate and superoxide-scavenging by ascorbate, urate and ascorbate inhibited autoxidation cooperatively (Figure 5). Together, 500 μM urate and 500 μM ascorbate provided 78% inhibition, close to the inhibition provided by desferrioxamine plus ascorbate. This cooperation produced a corresponding decrease in oxygen consumption (not shown). Addition of 5 U/ml superoxide dismutase provided 12% further inhibition.

However, ascorbate detracted somewhat from inhibition by urate plus superoxide dismutase alone, accelerating the slow autoxidation 2.7 fold. This interference by ascorbate was lessened by decreasing the concentration of ascorbate to 200 μM or increasing superoxide dismutase to 20 U/ml (not shown). Interference by ascorbate is thus explicable in terms of competition of ascorbate with superoxide dismutase for superoxide. Although superoxide dismutase reacts much more rapidly with superoxide ($k=1.9 \times 10^9\ M^{-1}\ s^{-1}$)[74], its concentration in this study (48 nM) was 10^4 less than ascorbate. The ascorbyl radical resulting from reaction of ascorbate with superoxide may then provide a means of propagating limited 6-hydroxydopamine autoxidation, either by oxidizing 6-hydroxydopamine itself, or by reducing H_2O_2 to $^{\bullet}OH$. Catalase (20 U/ml) inhibited the interference by ascorbate by only 31%, so the majority of the interference in these conditions was likely due to oxidation of 6-hydroxydopamine by the ascorbyl radical.

In summary, the synergistic inhibition of 6-hydroxydopamine autoxidation by urate and superoxide dismutase or ascorbate suggest that urate inhibits metal catalysed autoxidations by steric hindrance, forcing outer sphere electron transfers. This chelation, presumably within a 2:1 urate:metal complex,[18] renders autoxidation dependent on propagation by superoxide.

Urate, ascorbate, and superoxide dismutase may cooperate as antioxidants *in vivo*.

The current findings suggest that the presence of uric acid in human biological fluids preserves not only ascorbate, but, in concert with superoxide dismutase and/or ascorbate, may help preserve other reductants from metal catalysed oxidation. Autoxidation of epinephrine, for example, is inhibited synergistically by superoxide dismutase and metal chelators.[75] Oxidation of catecholamines in plasma requires catalytic metals,[53,76] which are sometimes released from storage sites under pathological conditions.[77-82] Urate, which is released by endothelial cells,[65,83] is then available to bind any free iron or copper. In concert with extracellular-superoxide dismutase, urate would thus prevent autoxidation of

catecholamines, ascorbate, and other reductants near the vascular endothelium. In other microenvironments (eg. bulk phase plasma) or compartments inaccessible to enzymic superoxide dismutase, urate may cooperate with ascorbate to inhibit metal-catalysed oxidations.

Other examples of metal catalysed "autoxidations" inhibited cooperatively by superoxide dismutase and metal chelators include those of 1,2,4-benzenetriol[84] and of cytotoxic pyrimidines[85]. Together, urate, ascorbate, and superoxide dismutase may help limit generation of active oxygen by both endogenous and xenobiotic prooxidants.

Extracellular-superoxide dismutase and urate may be well situated to protect cell surfaces from prooxidant damage. Extracellular-superoxide dismutase, a secretory glycoprotein produced by fibroblasts and glial cells, occurs in extracellular fluids such as plasma, lymph, synovial fluid, and in interstitial fluids.[86] For example, the activity of superoxide dismutase in human plasma is approximately 0.8 U/ml, which increases to about 1.4 U/ml on heparin-induced release of extracellular-superoxide dismutase from endothelial cell surfaces.[87,88] Uric acid, unlike high molecular weight metal-binding proteins such as albumin, transferrin, or ceruloplasmin,[13,89] has ready access to extracellular fluids, including lymphatic, cerebrospinal, interstitial, synovial, intraocular, respiratory tract, and amniotic fluids.[3] Urate may be well suited therefore to cooperate with extracellular-superoxide dismutase in extracellular fluid-plasma membrane boundary layers.

The current data provide a rationale for the cooperation between urate and superoxide dismutase dramatically evident in *Drosophila melanogaster*. Mutation to genes for either xanthine dehydrogenase (preventing urate production) or CuZn superoxide dismutase produce mutants hypersensitive to oxidative stress.[90] Mutation to both genes is lethal.

This study adds another aspect to the antioxidant actions of uric acid. In addition to directly scavenging oxidants such as hydroxyl radical and to binding catalytic metals, urate cooperates with ascorbate and with superoxide dismutase to inhibit metal-catalysed

oxidations. Urate, ascorbate, and extracellular-superoxide dismutase may thus cooperate in extracellular fluids to provide synergistic antioxidant activity.

REFERENCES

1. Ames, B.N.; Cathcart, R.; Schwiers, E.; Hochstein, P. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: A hypothesis. *Proc. Natl. Acad. Sci. USA* **78**:6858-6862; 1981.

2. Cutler, R.G. Urate and ascorbate: their possible roles as antioxidants in determining longevity of mammalian species. *Arch. Gerontol. Geriatr.* **3**:321-348; 1984.

3. Becker, B. Towards the physiological function of uric acid. *Free Rad. Biol. Med.* **14**:615-631; 1993.

4. Matsushita, S.; Ibuki, F.; Aoki, A. Chemical reactivity of the nucleic acid bases. Antioxidative ability of the nucleic acids and their related substances on the oxidation of unsaturated fatty acids. *Arch. Biochem. Biophys.* **102**:446-451; 1963.

5. Smith, R.C.; Lawing, L. Antioxidant activity of uric acid and 3-*N*-ribosyluric acid with unsaturated fatty acids and erythrocyte membranes. *Arch. Biochem. Biophys.* **223**:166-172; 1983.

6. Liu, K.M.; Swann, D.; Lee, P.; Lam, K.W. Inhibition of oxidative degradation of hyaluronic acid by uric acid. *Curr. Eye Res.* **3**:1049-1053; 1984.

7. Cohen, A.M.; Aberdroth, R.E.; Hochstein, P. Inhibition of free radical-induced DNA damage by uric acid. *FEBS Let.* **174**:147-150; 1984.

8. Al-Bekairi, A.M.; Qureshi, S.; Chaudhry, M.A.; Shah, A.H. Uric acid as an inhibitor of cyclophosphamide-induced micronuclei in mice. *Mutation Res.* **262**:115-118; 1991.

9. Aruoma, O.I.; Halliwell, B. Inactivation of α_1-antiproteinase by hydroxyl radicals. The effect of uric acid. *FEBS Lett.* **244**:76-80; 1989.

10. Ogino, N.; Yamamoto, S.; Hayaishi, O.; Tokuyama, T. Isolation of an activator for prostaglandin hydroperoxidase from bovine vesicular gland cytosol and its identification as uric acid. *Biochem. Biophys. Res. Commun.* **87**:184-191; 1979.

11. Deby, C.; Deby-Dupont, G.; Noel, F.-X.; Lavergne, L. *In vitro* and *in vivo* arachidonic acid conversions into biologically active derivatives are enhanced by uric acid. *Biochem. Pharmacol.* **30**:2243-2249; 1981.

12. Arnout, J.; Kienast, J.; Deckmyn, H.; Vermylen, J. Prostacyclin stimulatory activity of reducing cofactors in human plasma filtrate. A potential role for uric acid. *Agents and Actions* **22**:360-361; 1987.

13. Halliwell, B.; Gutteridge, J.M.C. The antioxidants of human extracellular fluids. *Arch. Biochem. Biophys.* **280**:1-8; 1990.

14. Stone, T.W.; Simmonds, H.A. *Purines: Basic and clinical aspects.* Dordrecht, The Netherlands: Kluwer Academic Publishers; 1991.

15. Simic, M.J.; Jovanovic, S.V. Antioxidation mechanisms of uric acid. *J. Am. Chem. Soc.* **111**:5778-5782; 1989.

16. Kaur, H.; Halliwell, B. Action of biologically relevant oxidizing species upon uric acid. Identification of uric acid oxidation products. *Chem.-Biol. Interactions* **73**:235-247; 1990.

17. Albert, A. Quantitative studies of the avidity of naturally occurring substances for trace metals. *Biochem. J.* **54**:646-654; 1953.

18. Davies, K.J.A.; Sevanian, A.; Muakkassah-Kelly, S.F.; Hochstein, P. Uric acid-iron complexes. A new aspect of the antioxidant functions of uric acid. *Biochem. J.* **235**:747-754; 1986.

19. Sevanian, A.; Davies, K.J.A.; Hochstein, P. Serum urate as an antioxidant for ascorbic acid. *Am. J. Clin. Nutr.* **54**:1129S-1134S; 1991.

20. Maples, K.R.; Mason, R.P. Free radical metabolite of uric acid. *J. Biol. Chem.* **263**:1709-1712; 1988.

21. Lam, K.-W.; Fong, D.; Lee, A.; Liu, K.M.D. Inhibition of ascorbate oxidation by urate. *J. Inorg. Biochem.* **22**:241-248; 1984.

22. Sevanian, A.; Davies, K.J.A.; Hochstein, P. Conservation of vitamin C by uric acid in blood. *J. Free Rad. Biol. Med.* **1**:117-124; 1985.

23. Rowley, D.A.; Halliwell, B. Formation of hydroxyl radicals from NADH and NADPH in the presence of copper salts. *J. Inorg. Biochem.* **23**:103-108; 1985.

24. Granger, D.N.; Rutili, G.; McCord, J.M. Superoxide radicals in feline intestinal ischemia. *Gastroenterology* **81**:22-29; 1981.

25. Paller, M.S.; Hoidal, J.R.; Ferris, T. Oxygen free radicals in ischemic acute renal failure in rat. *J. Clin. Invest.* **74**:1156-1164; 1984.

26. McCord, J. Oxygen-derived free radicals in postischemic tissue injury. *N. Engl. J. Med.* **312**:159-163; 1985.

27. Simpson, P.J.; Mickelson, J.K.; Lucchesi, B.R. Free radical scavengers in myocardial ischemia. *Fed. Proc.* **46**:2413-2421; 1987.

28. Flaherty, J.T.; Weisfeldt, M.L. Reperfusion injury. *Free Rad. Biol. Med.* **5**:409-419; 1988.

29. Chambers, D.E.; Parks, D.A.; Patterson, G.; Roy, R.; McCord, J.; Yoshida, S.; Parmley, L.F.; Downey, J.M. Xanthine oxidase as a source of free radical damage in myocardial ischemia. *J. Mol. Cell. Cardiol.* **17**:145-152; 1985.

30. Thompson-Gorman, S.L.; Zweier, J.L. Evaluation of the role of xanthine oxidase in myocardial reperfusion injury. *J. Biol. Chem.* **265**:6656-6663; 1990.

31. Das, D.K.; Engelman, R.M.; Clement, R.; Otani, H.; Prasad, M.R.; Rao, P.S. Role of xanthine oxidase inhibitor as free radical scavenger: a novel mechanism of action of allopurinol and oxypurinol in myocardial salvage. *Biochem. Biophys. Res. Comm.* **148**:314-319; 1987.

32. Grootveld, M.; Halliwell, B.; Moorhouse, C.P. Action of uric acid, allopurinol, and oxypurinol on the myeloperoxidase-derived oxidant hypochlorous acid. *Free Rad. Res. Commun.* **4**:69-76; 1987.

33. Moorhouse, P.C.; Grootveld, M.; Halliwell, B.; Quinlan, J.G.; Gutteridge, J.M.C. Allopurinol and oxypurinol are hydroxyl radical scavengers. *FEBS Lett.* **213**:23-28; 1987.

34. Zimmerman, B.J.; Parks, D.A.; Grisham, M.B.; Granger, D.N. Allopurinol does not enhance antioxidant properties of extracellular fluid. *Am. J. Physiol.* **255**:H202-H206; 1988.

35. Myers, C.L.; Weiss, S.J.; Kirsh, M.M.; Shlafer, M. Involvement of hydrogen peroxide and hydroxyl radical in the 'oxygen paradox': reduction of creatine kinase release by catalase, allopurinol or deferroxamine, but not by superoxide dismutase. *J. Mol. Cell. Cardiol.* **17**:675-684; 1985.

36. Ko, K.M.; Godin, D.V. Inhibition of transition metal ion-catalysed ascorbate oxidation and lipid peroxidation by allopurinol and oxypurinol. *Biochem. Pharmacol.* **40**:803-809; 1990.

37. Bendich, A.; Machlin, L.J.; Scandurra, O.; Burton, G.W.; Wayner, D.D.M. The antioxidant role of vitamin C. *Adv. Free Rad. Biol. Med.* **2**:419-444; 1986.

38. Frei, B.; England, L.; Ames, B.N. Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Natl. Acad. Sci. USA* **86**:6377-6381; 1989.

39. Ottolenghi, A. Interaction of ascorbate and mitochondrial lipids. *Arch. Biochem. Biophys.* **79**:353-363; 1959.

40. Barber, A.A. Lipid peroxidation in rat tissue homogenates: interaction of iron and ascorbic acid as the normal catalytic mechanism. *Lipids* **1**:146-151; 1966.

41. Sharma, O.P.; Krishna Murti, C.R. Ascorbic acid - a naturally occurring mediator of lipid peroxide formation in rat brain. *J. Neurochem.* **27**:299-301; 1976.

42. Peterkofsky, B.; Prather, W. Cytotoxicity of ascorbate and other reducing agents towards cultured fibroblasts as a result of hydrogen peroxide formation. *J. Cell. Physiol.* **90**:61-70; 1977.

43. Sadrzadeh, S.M.H.; Eaton, J. Hemoglobin-mediated oxidant damage to the central nervous system requires endogenous ascorbate. *J. Clin. Invest.* **82**:1510-1515; 1988.

44. Heikkila, R.E.; Cohen, G. Further studies on the generation of hydrogen peroxide by 6-hydroxydopamine. Potentiation by ascorbic acid. *Molec. Pharmacol.* **8**:241-248; 1972.

45. Heikkila, R.E.; Cohen, G. Cytotoxic aspects of the interaction of ascorbic acid with alloxan and 6-hydroxydopamine. *Ann. N. Y. Acad. Sci.* **258**:221-230; 1975.

46. Borg, D.C.; Schaich, K.M.; Elmore, J.J.; Bell, J.A. Cytotoxic reactions of free radical species of oxygen. *Photochem. Photobiol.* **28**:887-907; 1978.

47. Sullivan, S.G.; Stern, A. Effects of superoxide dismutase and catalase on catalysis of 6-hydroxydopamine and 6-aminodopamine autoxidation by iron and ascorbate. *Biochem. Pharmacol.* **30**:2279-2285; 1981.

48. Sullivan, S.G.; Stern, A. Autoxidation of 6-hydroxydopamine: influence of iron and ascorbate. In: Rodgers, M.A.J.; Powers, E.L., eds. *Oxygen and Oxy-Radicals in Chemistry and Biology*. New York: Academic Press; 1981:750-752.

49. Heikkila, R.E.; Mytilineou, C.; Cote, L.; Cohen, G. Evidence for degeneration of sympathetic nerve terminals caused by the *ortho*- and *para*-quinones of 6-hydroxydopamine. *J. Neurochem.* **20**:1345-1350; 1973.

50. Jonsson, G.; Lohmander, S.; Sachs, C. 6-Hydroxydopamine induced degeneration of noradrenaline neurons in the scorbutic guinea-pig. *Biochem. Pharmacol.* **23**:2585-2593; 1974.

51. Jonsson, G.; Sachs, C. Actions of 6-hydroxydopamine quinones on catecholamine neurons. *J. Neurochem.* **25**:509-516; 1975.

52. Pileblad, E.; Slivka, A.; Bratvold, D.; Cohen, G. Studies on the autoxidation of dopamine: interaction with ascorbate. *Arch. Biochem. Biophys.* **263**:447-452; 1988.

53. Jewett, S.L; Eddy, L.J.; Hochstein, P. Is the autoxidation of catecholamines involved in ischemia-reperfusion injury? *Free Rad. Biol. Med.* **6**:185-188; 1989.

54. Heikkila, R.E.; Cohen, G. Inhibition of biogenic amine uptake by hydrogen peroxide: a mechanism for toxic effects of 6-hydroxydopamine. *Science* **172**:1257-1257-1258; 1971.

55. Uretsky, N.J.; Iversen, L.L. Effects of 6-hydroxydopamine on catecholamine containing neurons in the rat brain. *J. Neurochem.* **17**:269-278; 1970.

56. Stein, L.; Wise, C.D. Possible etiology of schizophrenia: progressive damage to the noradrenergic reward system by 6-hydroxydopamine. *Science* **171**:1032-1036; 1971.

57. Graham, D.G. Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. *Molec. Pharmacol.* **14**:633-643; 1978.

58. Graham, D.G. Catecholamine toxicity: A proposal for the molecular pathogenesis of manganese neurotoxicity and Parkinson's disease. *Neurotoxicity* **5**:83-96; 1984.

59. Bruchelt, G.; Grygar, G.; Treuner, J.; Esterbauer, H.; Niethammer, D. Cytotoxic effects of 6-hydroxydopamine, merocyanine-540 and related compounds on human neuroblastoma- and hemopoietic stem cells. *Free Rad. Res. Comms.* **7**:205-212; 1989.

60. Buettner, G.R. Ascorbate autoxidation in the presence of iron and copper chelates. *Free Rad. Res. Comms.* **6**:349-353; 1986.

61. Gee, P.; Davison, A.J. 6-Hydroxydopamine does not reduce molecular oxygen directly, but requires a co-reductant. *Arch. Bioch. Biophys.* **231**:164-168; 1984.

62. Bandy, B.; Davison, A.J. Interactions between metals, ligands, and oxygen in the autoxidation of 6-hydroxydopamine: Mechanisms by which metal chelation enhances inhibition by superoxide dismutase. *Arch. Biochem. Biophys.* **259**:305-315; 1987.

63. Gee, P.; Davison, A.J. Intermediates in the aerobic autoxidation of 6-hydroxydopamine: relative importance under different reaction conditions. *Free Rad. Biol. Med.* **6**:271-284; 1989.

64. Goshima, N.; Wadano, A.; Miura, K. 3-Hydroxykynurenine as $O_2^{\cdot-}$ scavenger in the blowfly, *Aldrichina grahami. Biochem. Biophys. Res. Comm.* **139**:666-672; 1986.

65. Becker, B.F.; Reinholz, N; Ozcelik, T.; Leipert, B.; Gerlach, E. Uric acid as radical scavenger in the heart. *Pflugers Arch.* **415**:127-135; 1989.

66. Radi, R.; Rubbo, H.; Thomson, L.; Prodanov, E. Luminol chemiluminescence using xanthine and hypoxanthine as xanthine oxidase substrates. *Free Rad. Biol. Med.* **8**:121-126; 1990.

67. Lind, M.D.; Hamor, M.J.; Hamor, T.A.; Hoard, J.L. Stereochemistry of ethylenediaminetetraaceto complexes. *Inorg. Chem.* **3**:34-43; 1964.

68. Graf, E.; Mahoney, J.; Bryant, R.; Eaton, J. Iron-catalyzed hydroxyl radical formation. Stringent requirement for free iron coordination site. *J. Biol. Chem.* **295**:3620-3624; 1984.

69. Zweier, J.L.; Kuppusamy, P.; Lutty, G.A. Measurement of endothelial cell free radical generation: evidence for a central mechanism of free radical injury in postischemic tissues. *Proc. Natl. Acad. Sci. USA* **85**:4046-4050; 1988.

70. Martell, A.E. Chelates of ascorbic acid. In: Seib P.A.; Tolbert B.M., eds. *Ascorbic Acid: Chemistry, Metabolism, and Uses.* Washington D.C.: American Chemical Society; 1982:153-178.

71. Cabelli, D.E.; Bielski, B.H.J. Kinetics and mechanism for the oxidation of ascorbic acid/ascorbate by HO_2/O_2^- radicals. A pulse radiolysis and stopped-flow photolysis study. *J. Phys. Chem.* **87**:1809-1812; 1983.

72. Halliwell, B. Use of desferrioxamine as a "probe" for iron-dependent formation of hydroxyl radicals. Evidence for a direct reaction between desferal and the superoxide radical. *Biochem. Pharmacol.* **34**:229-233; 1985.

73. Bielski, B.H.J.; Richter, H.W.; Chan, P.C. Some properties of the ascorbate free radical. *Ann. N.Y. Acad. Sci.* **258**:231-237; 1975.

74. Bielski, B.H.J.; Cabelli, D.E.; Arudi, R.L.; Ross, A.B. Reactivity of HO_2/O_2^- radicals in aqueous solution. *J. Phys. Chem. Ref. Data* **14**:1041-1100; 1985.

75. Misra, H.P.; Fridovich, I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* 247:3170-3175; 1972.

76. Miller, D.M.; Buettner, G.R.; Aust, S.D. Transition metals as catalysts of "autoxidation" reactions. *Free Rad. Biol. Med.* **8**:95-108; 1990.

77. Halliwell, B.; Gutteridge, J.M.C. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* **219**:1-14; 1984.

78. Halliwell, B.; Gutteridge, J.M.C. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.* **246**:501-514; 1986.

79. Halliwell, B. Superoxide, iron, vascular endothelium and reperfusion injury. *Free Rad. Res. Comms.* **5**:315-318; 1989.

80. Ratych, R.E.; Chuknyiska, R.S.; Bulkley, G.B. The primary localization of free radical generation after anoxia/reoxygenation in isolated endothelial cells. *Surgery* **102**:122-131; 1987.

81. Marklund, S.L. Role of toxic effects of oxygen in reperfusion damage. *J. Mol. Cell. Cardiol.* **20(suppl. II)**:23-30; 1988.

82. Reif, D.W. Ferritin as a source of iron for oxidative damage. *Free Rad. Biol. Med.* **12**:417-427; 1992.

83. Becker, B.F.; Gerlach, E. Uric acid, the major catabolite of cardiac adenine nucleotides and adenosine originates in the coronary endothelium. In: Gerlach, E.; Becker, B.F., eds. *Topics and perspectives in adenosine research.* Berlin: Springer-Verlag; 1987:209-222; 1987.

84. Bandy, B.; Moon, J.; Davison, A.J. Multiple actions of superoxide dismutase: why can it both inhibit and stimulate reduction of oxygen by hydroquinones? *Free Rad. Biol. Med.* **9**:143-148; 1990.

85. Winterbourn, C.C.; Cowden, W.B.; Sutton, H.C. Auto-oxidation of dialuric acid, divicine and isouramil. Superoxide dependent and independent mechanisms. *Biochem. Pharmacol.* **38**:611-618; 1989.

86. Marklund, S.L. Expression of extracellular superoxide dismutase by human cell lines. *Biochem. J.* **266**:213-219; 1990.

87. Marklund, S.L. Extracellular superoxide dismutase and other superoxide dismutase isoenzymes in tissues from nine mammalian species. *Biochem. J.* **222**:649-655; 1984.

88. Karlsson, K.; Marklund, S.L. Extracellular superoxide dismutase in the vascular system of mammals. *Biochem. J.* **255**:223-228; 1988.

89. Halliwell, B. Albumin - an important extracellular antioxidant? *Biochem. Pharmacol.* **37**:569-571; 1988.

90. Hilliker, A.J.; Duyf, B.; Evans, D.; Phillips, J.P. Urate-null rosy mutants of *Drosophila melanogaster* are hypersensitive to oxygen stress. *Proc. Natl. Acad. Sci.* **89**:4343-4347; 1992.

LEGENDS TO FIGURES

Figure 1. *Structures of purines and purine analogs studied.* Arrows show the metabolic pathway of urate formation from AMP.

Figure 2. *Effects of uric acid on the autoxidation of 6-hydroxydopamine.* Autoxidation of 250 μM 6-hydroxydopamine was followed at 490 nm in air-saturated 50 mM phosphate buffer, pH 7.4, 25°C. Superoxide dismutase was included at 5 U/ml; urate at 500 μM; and EDTA or desferrioxamine (DEF) at 500 μM.

Figure 3. *Synergistic actions of purines and superoxide dismutase in inhibiting the autoxidation of 6-hydroxydopamine.* Autoxidation of 6-hydroxydopamine (250 μM) was followed at 490 nm in air-saturated 50 mM phosphate buffer, pH 7.4, 25°C. Purines were at 500 μM. Superoxide dismutase was at 5 U/ml. Rates represent initial rates within 3 minutes of reaction time.

Figure 4. *Effects of ascorbic acid on the autoxidation of 6-hydroxydopamine.* Reaction conditions were as in figure 2. Ascorbate was at 500 μM.

Figure 5. *Cooperative actions of urate, ascorbate, and superoxide dismutase.* Reaction conditions were as in figure 2. Urate and ascorbate were at 500 μM, superoxide dismutase at 5 U/ml. Rates represent the initial rate within the first 30 seconds of the reaction.

Figure 1.

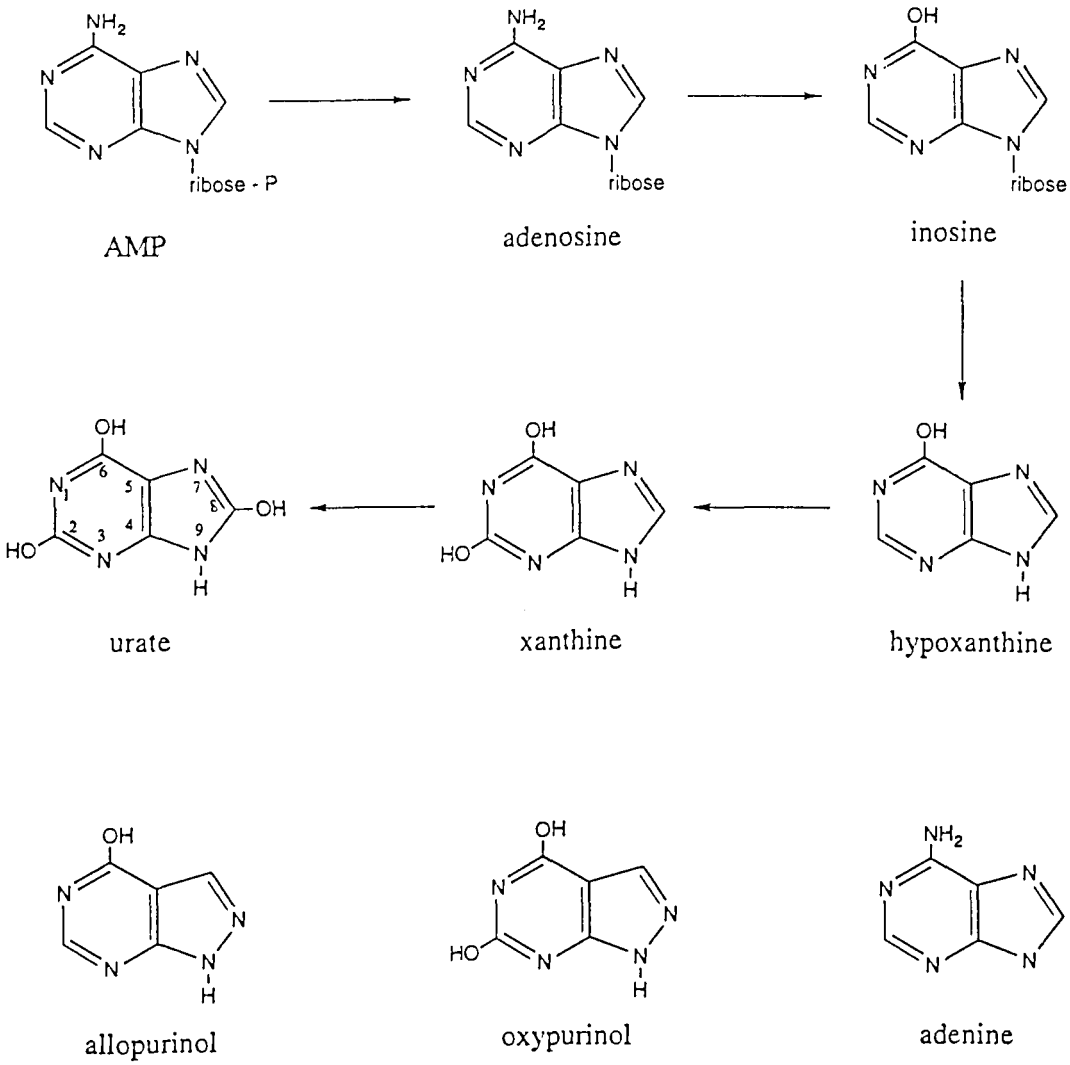
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Figure 2

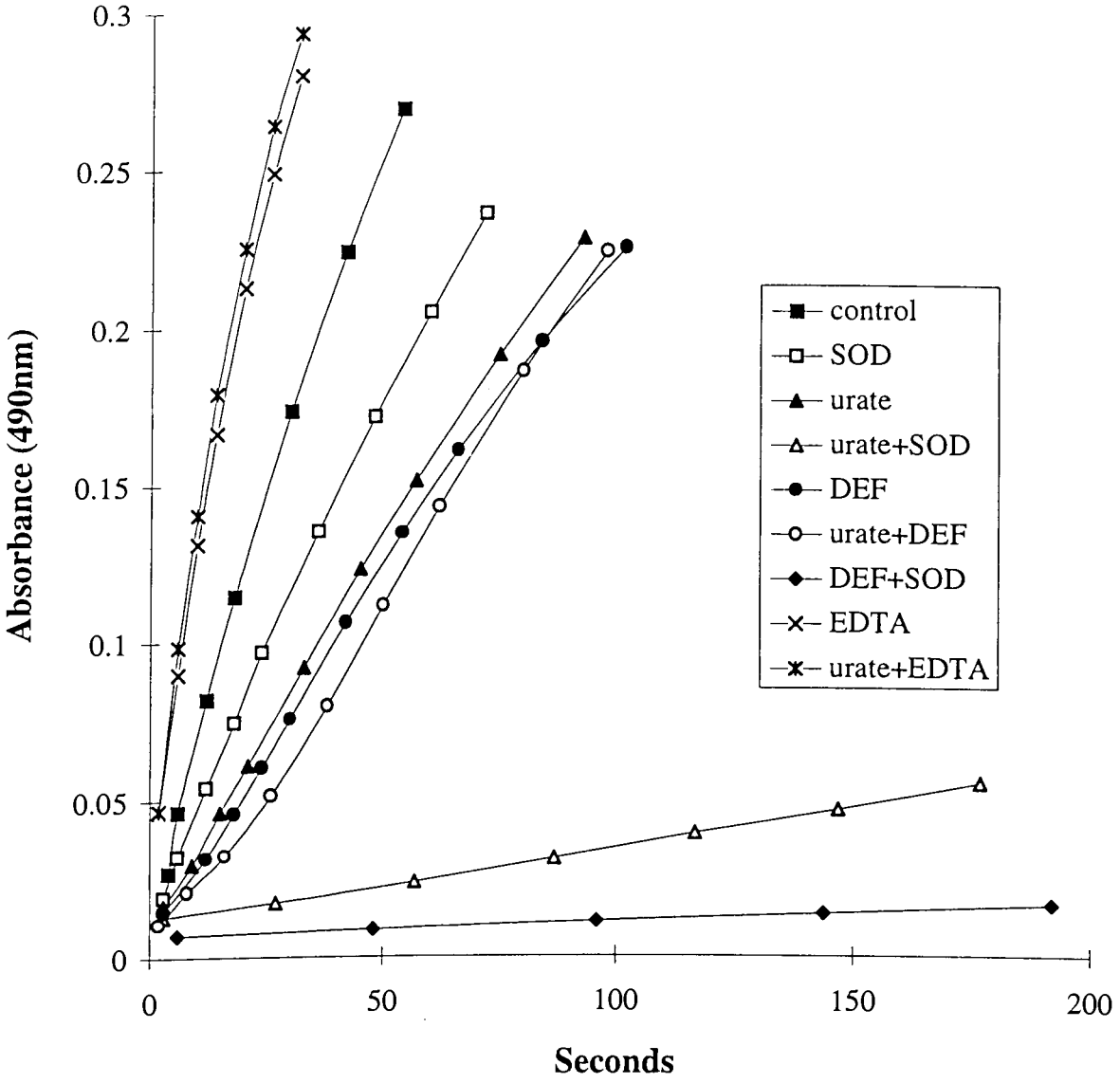
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Figure 3.

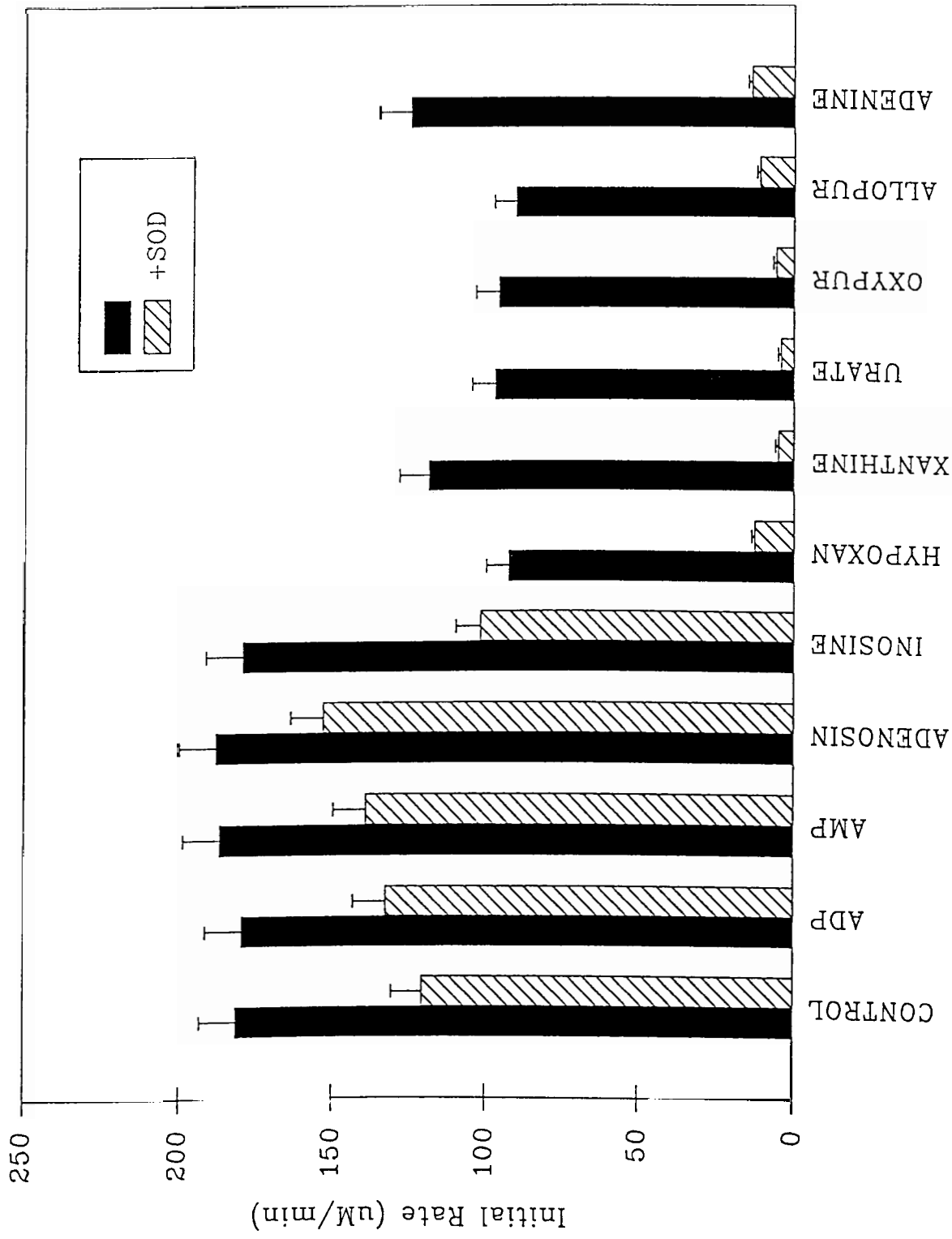
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Figure 4

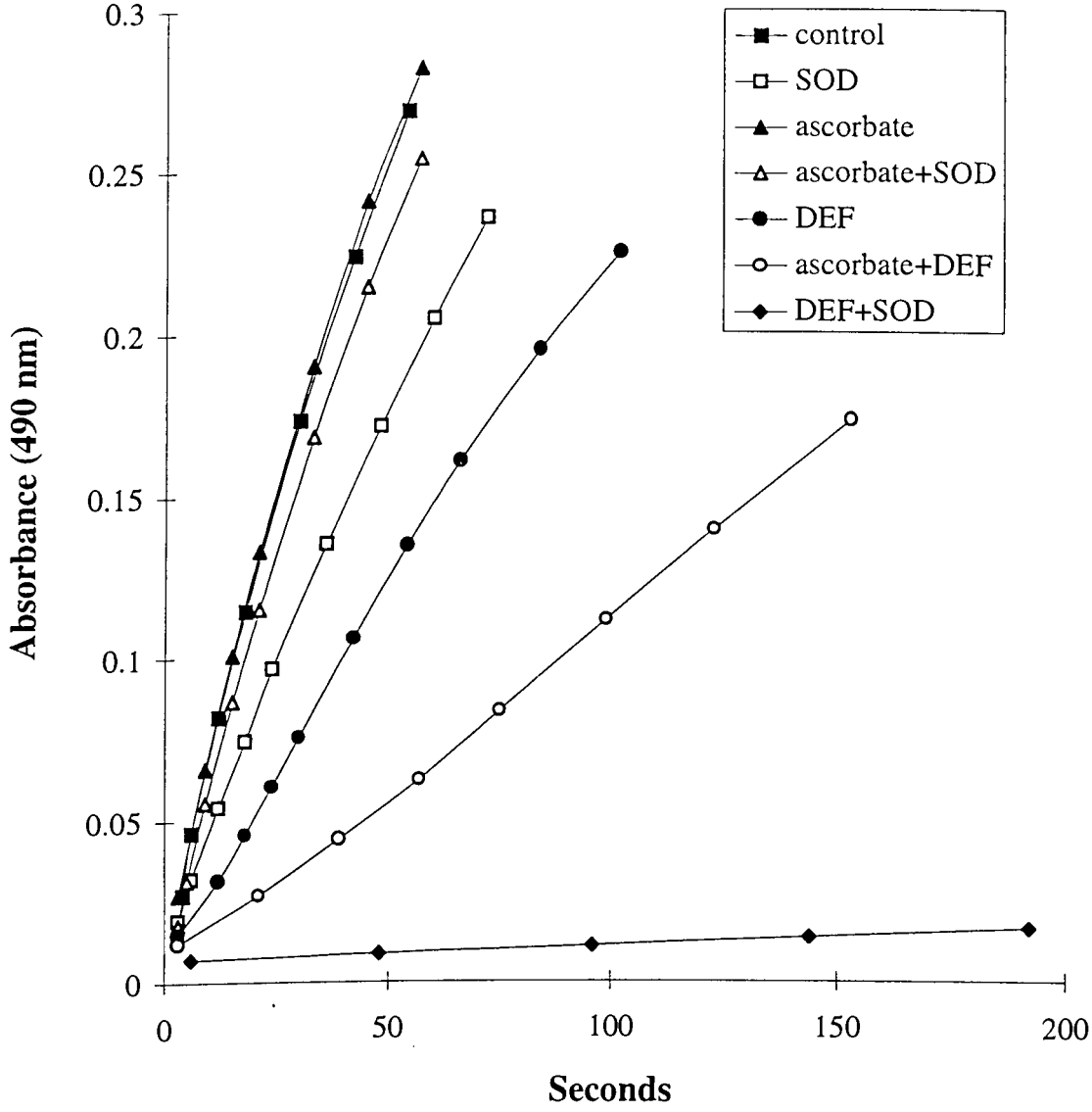
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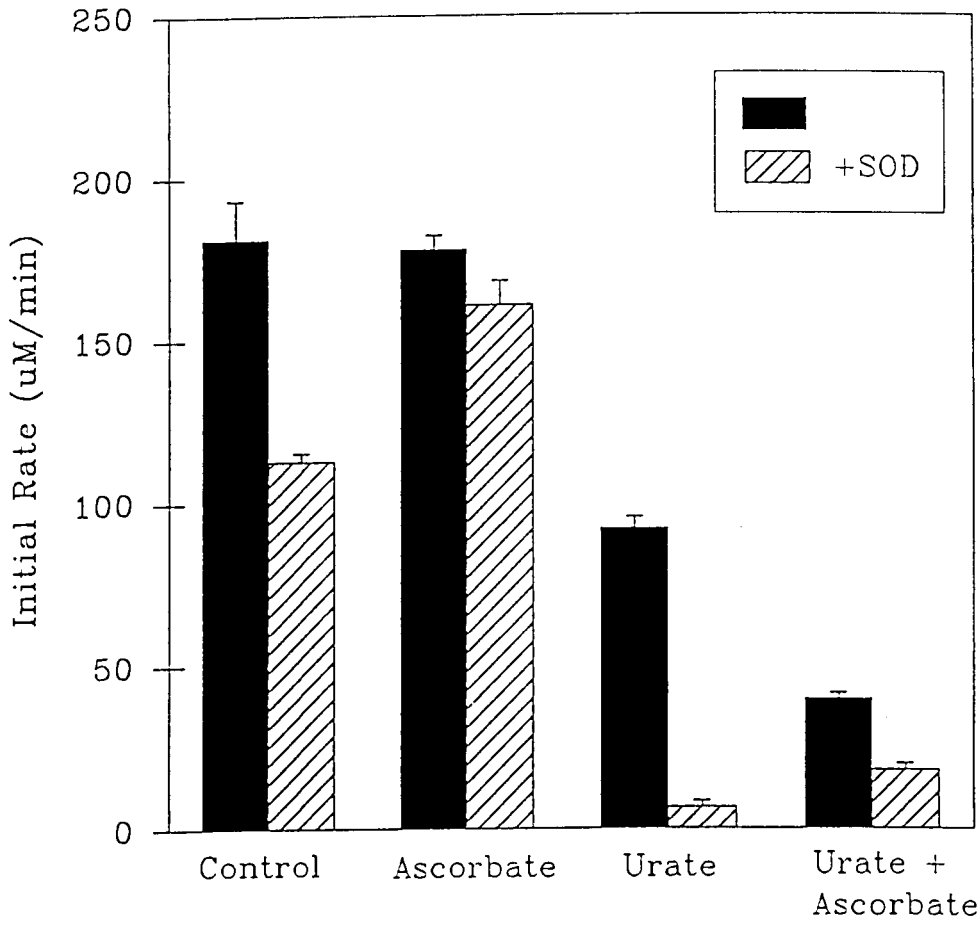
Figure 5.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Chapter 9.

Hypothesis Paper

MULTIPLE ACTIONS OF SUPEROXIDE DISMUTASE: WHY CAN IT BOTH INHIBIT AND STIMULATE REDUCTION OF OXYGEN BY HYDROQUINONES?

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Abstract—Superoxide dismutase can either inhibit or stimulate autoxidation of different hydroquinones, suggesting multiple roles for $O_2\cdot^-$. Inhibitory actions of superoxide dismutase include termination of $O_2\cdot^-$-propagated reaction chains and metal chelation by the apoprotein. Together, chelation of metals and termination of $O_2\cdot^-$-propagated chains can effectively prevent reduction of oxygen. Chain termination by superoxide dismutase can thus account for negligible accumulation of H_2O_2 without invoking a superoxide:semiquinone oxidoreductase activity for this enzyme. One *stimulatory* action of superoxide dismutase is to decrease thermodynamic limitations to reduction of oxygen. Whether superoxide dismutase inhibits or accelerates an autoxidation depends on the reduction potentials of the quinone and the availability of metal coordination for inner sphere electron transfers.

Keywords—Hydroquinone autoxidation, Superoxide dismutase, Oxygen reduction, Metal coordination, Inner sphere electron transfers, Chain propagation, Semiquinone, Free radicals

INTRODUCTION

Cadenas and coworkers have recently compiled a wealth of data on multiple effects of superoxide dismutase in hydroquinone autoxidations.[1,2] These data complement data of Winterbourn on autoxidation of pyrimidines.[3] The diverse, paradoxical, and sometimes contradictory actions of superoxide dismutase have prompted an equally wide range of explanations, including the novel suggestion that superoxide dismutase has superoxide:semiquinone oxidoreductase activity.[1] This suggestion rests on the observation that superoxide dismutase inhibits autoxidation of the hydroquinone derivatives of 2-hydroxy-*p*-benzoquinone and 1,4-naphthoquinones but prevents accumulation of H_2O_2, the product of superoxide dismutation. Arguing against this suggestion however, superoxide dismutase only inhibits autoxidation of 1,4-naphthohydroquinone in the early phase.[4] Superoxide dismutase *stimulates* the autoxidation when comproportionation of hydroquinone with quinone product becomes available to propagate the autoxidation.[4] Superoxide dismutase also does not inhibit, but accelerates oxidation of 1,2-naphthohydroquinone and 5-hydroxy-1,4-naphthohydroquinones.[1,2]

This wealth of recent data brings a need for some generalizations as to mechanisms by which superoxide dismutase can slow or accelerate autoxidations. Inhibitory actions of superoxide dismutase include termination of free radical chains and metal chelation by the apoprotein. Superoxide dismutase can also *accelerate* oxidations by at least three different mechanisms. We outline here some of the situations where superoxide dismutase might accelerate or slow hydroquinone oxidation, and offer possible explanations for some of the contrasting effects on different naphthohydroquinones.

METAL CHELATION CAN ACT SYNERGISTICALLY WITH SUPEROXIDE DISMUTASE ACTIVITY TO INHIBIT AUTOXIDATIONS

Most metal-chelating agents sensitize autoxidation of hydroquinone compounds to further inhibition by superoxide dismutase.[5,6] Metal chelators often inhibit autoxidations by decreasing the ability of the metal to

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gain or lose electrons. With a strong reductant such as 6-hydroxydopamine however, EDTA stimulates the trace metal catalyzed autoxidation[6] presumably by accelerating the (rate limiting) autoxidation of reduced iron. Desferrioxamine, a stronger chelator of ferric ions (thus decreasing the reduction potential further), slows autoxidation of 6-hydroxydopamine. Either chelator sensitizes 6-hydroxydopamine autoxidation to inhibition by superoxide dismutase.[6] By preventing formation of a ternary reductant-metal-oxygen complex, these chelators prevent sequential "inner sphere" transfer of 2 electrons and force release of $O_2\cdot^-$ and $Q\cdot^-$.[5,6] The $O_2\cdot^-$ and $Q\cdot^-$ released then serve as reaction propagators:

$$QH_2 + O_2\cdot^- \longrightarrow Q\cdot^- + H_2O_2$$

and

$$Q\cdot^- + O_2 \longrightarrow Q + O_2\cdot^-.$$

Since EDTA accelerates autoxidation of ferrous ions (and thus $O_2\cdot^-$ release) but slows reduction of ferric ions (and thus $Q\cdot^-$ formation), $O_2\cdot^-$ is the major propagator which accelerates the overall autoxidation in the presence of EDTA.

With 1,2,4-benzenetriol, both EDTA and desferrioxamine stimulated the autoxidation (although desferrioxamine caused a slight lag) (Fig. 1). Despite stimulating autoxidation of 1,2,4-benzenetriol when present alone, EDTA or desferrioxamine sensitized the autoxidation to inhibition by small, catalytic quantities of superoxide dismutase. Together, metal chelation and superoxide dismutase activity can prevent consumption of oxygen (Fig. 2). Metal binding by proteins, including superoxide dismutase apoprotein, can also play this sensitizing role[6,7] (Figs. 2 and 3). Such inhibition favors the hydroquinone form and also prevents accumulation of products, including H_2O_2, as found by Cadenas et al.[1] The concentrations of superoxide dismutase and total protein in the experiments of Cadenas et al.[1] on autoxidation of 1,2,4,-benzenetriol (present at 20 μM) were 3 μg/mL (9 U/mL) and 6.5 μg/mL (3.5 μg/mL NAD(P)H:quinone oxidoreductase stabilized with albumin). Trace metals were thus competitively bound by proteins in these experiments, and the autoxidation was sensitive to inhibition by superoxide dismutase. A superoxide:semiquinone oxidoreductase activity could prevent the autoxidation, but so could

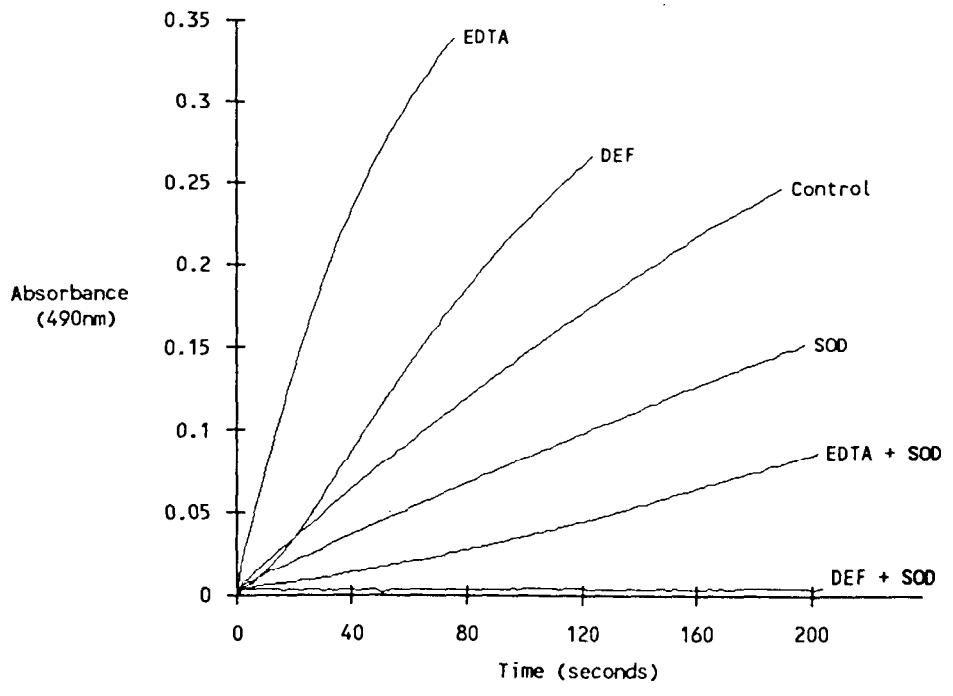

Fig. 1. 1,2,4-Benzenetriol autoxidation: Interactions between superoxide dismutase and metal chelators. Reactions were conducted in air-saturated, 50 mM phosphate buffer; pH 7.5; 25°C. Where indicated, EDTA or desferrioxamine (DEF) were present at 0.5 mM; superoxide dismutase (SOD) at 0.5 μg/mL (2 U/mL). Reactions were initiated by addition of an aliquot from an anaerobically prepared stock solution of 1,2,4-benzenetriol to give an initial concentration of 250 μM. Formation of the *p*-quinone product of oxidation was followed spectrophotometrically at 490 nm.

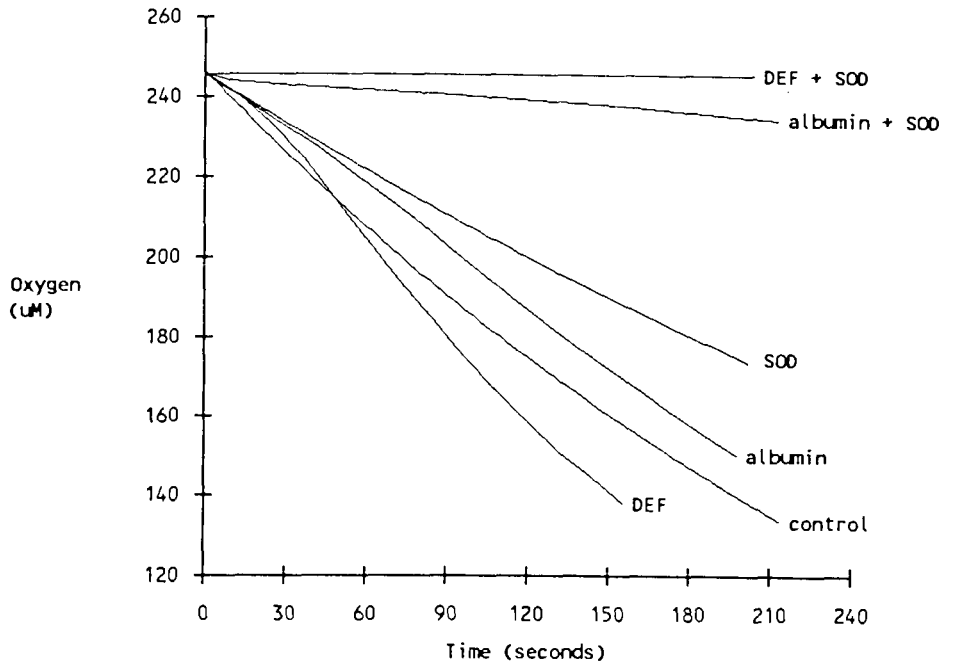

Fig. 2. 1,2,4-Benzenetriol autoxidation: Oxygen consumption in the presence of superoxide dismutase and metal chelators. Reaction conditions were as in Figure 1. Where indicated, albumin was present at 26 μg/mL. Other reagents were at the same concentrations as in Figure 1. Oxygen consumption was monitored polarographically upon addition of 1,2,4-benzenetriol.

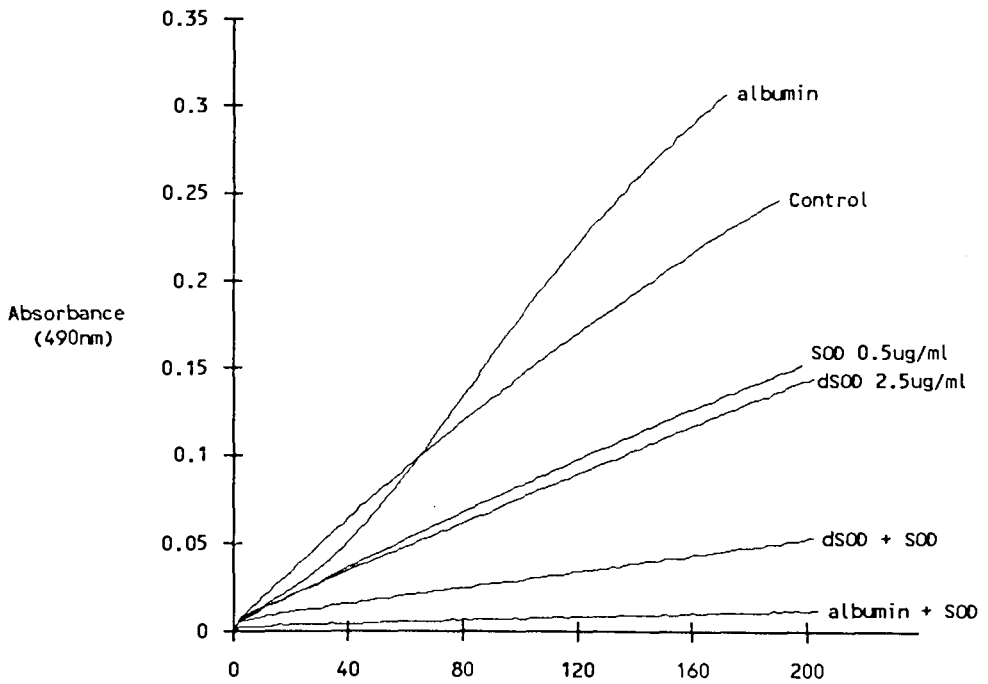

Fig. 3. 1,2,4-Benzenetriol autoxidation: Interactions between superoxide dismutase and proteins. Reactions were conducted in air-saturated, 50 mM phosphate buffer; pH 7.5; 25°C. Where indicated, superoxide dismutase (SOD) was present at 0.5 μg/mL (2 U/mL), heat-denatured (98% inactivated) superoxide dismutase (dSOD) at 2.5 μg/mL, and albumin at 26 μg/mL. Reactions were initiated by addition of an aliquot from an anaerobically prepared stock solution of 1,2,4-benzenetriol to give an initial concentration of 250 μM. Formation of the *p*-quinone product of oxidation was followed spectrophotometrically at 490 nm.

dismutation of $O_2\cdot^-$, by preventing propagation of the radical chain.

SUPEROXIDE DISMUTASE CAN INHIBIT AUTOXIDATIONS AND H_2O_2 FORMATION BY CHAIN TERMINATION

Inhibitory actions of superoxide dismutase can result from accelerated termination of free radical chains. Most if not all autoxidations are chain reactions. The main chain propagating action of superoxide in hydroquinone oxidation is:

$$QH_2 + O_2\cdot^- \longrightarrow Q\cdot^- + H_2O_2.$$

Inhibition by superoxide dismutase in autoxidations is usually considered evidence that superoxide is an intermediate in the free radical chain.[5,8] To the extent that oxidation of adrenaline is typical, superoxide has an amplification factor of up to 10.[5] In other words, one molecule of superoxide can stimulate oxidation of 10 molecules of adrenaline before its participation is ended through some chain termination reaction. On this basis, superoxide dismutase prevents the formation of 10 molecules for every half-molecule of H_2O_2 it manufactures. Thus, one explanation for the phenomena described[1,2] is that superoxide dismutase diminishes formation of H_2O_2 by facilitating chain termination. The combined effects of metal binding and chain termination can prevent oxygen consumption[9] and thus decrease H_2O_2 production to negligible levels.

METAL CHELATION BY PROXIMAL HYDROXYL GROUPS ALLOWS INNER SPHERE AUTOXIDATION

Why does superoxide dismutase not inhibit 1,2-naphthohydroquinone[1] or 5-hydroxy-1,4-naphthohydroquinone[2] oxidation? That the semiquinone is not a substrate for superoxide:semiquinone oxidoreductase was offered as one explanation for the lack of inhibition of the 1,2-naphthohydroquinone autoxidation by superoxide dismutase.[1] An alternative explanation is the difference in metal-binding capabilities of the different hydroquinones. The *o*-quinol with its adjacent phenolic hydroxyls binds metals more avidly than the (*para*) 1,4-napthohydroquinone. Thus the *o*-quinol can autoxidize by an inner sphere mechanism and is less influenced by relatively weak chelators such as proteins. The much higher concentrations of superoxide dismutase needed to inhibit autoxidation of the 2-hydroxy-1,4-napthohydroquinone (12 μg/mL, K_i = 540.8 nM)[1,2] than the 1,4-napthohydroquinone (0.3 μg/mL, K_i = 2.1 nM)[1,2] suggest more effective competition for binding of metals by the adjacent phenolic hydroxyls of 2-hydroxy-1,4-napthohydroquinone. Thus, as with autoxidation of 1,2,4-benzenetriol, sufficient protein to compete for trace metals can limit inner-sphere electron transfers and sensitize the autoxidation to inhibition by superoxide dismutase. The 5-hydroxy-1,4-naphthohydroquinone derivatives form a six-membered ring with cations (thus showing strong intramolecular hydrogen bonding).[10] These derivatives would bind metal cations avidly, and thus react primarily by concerted inner sphere transfer of electrons to oxygen.

SUPEROXIDE DISMUTASE CAN ACCELERATE AUTOXIDATIONS BY SEVERAL MECHANISMS

Why might superoxide dismutase *stimulate* autoxidation of 1,2-naphthohydroquinone[1] and 5-hydroxy-1,4-naphthohydroquinones?[2] Stimulatory effects of superoxide dismutase on autoxidations are sometimes reported.[1,2,11,12,13] In general, they occur in reactions:

a) in which superoxide is a net terminator of free radical chains

$$Q\cdot^- + O_2\cdot^- + 2H^+ \longrightarrow Q + H_2O_2,$$

b) in which hydrogen peroxide is a better oxidant than oxygen

$$\text{eg. } QH_2 + H_2O_2 \longrightarrow Q\cdot^- + \cdot OH + OH^- + 2H^+$$
$$Q\cdot^- + H_2O_2 \longrightarrow Q + \cdot OH + OH^-,$$

or c) in which semiquinone or quinone formation is limited by accumulation of superoxide

$$QH_2 + O_2 \rightleftharpoons Q\cdot^- + O_2\cdot^-$$

or

$$Q\cdot^- + O_2 \rightleftharpoons Q + O_2\cdot^-.$$

These stimulatory effects require that they are not overpowered by metal chelation and chain termination by superoxide dismutase. Stimulatory effects of superoxide dismutase are most apparent when $O_2\cdot^-$-dependent propagation becomes less significant, such as when accumulation of quinone allows comproportionation with the hydroquinone.[4] By inhibiting chain propagation by $O_2\cdot^-$ and stimulating semiquinone autoxidation,[2,14] one biologically important effect of superoxide dismutase is to limit formation of semiquinones.

SUPEROXIDE DISMUTASE CAN ACCELERATE OXIDATIONS IN WHICH EQUILIBRIA FORMING SEMIQUINONES OR QUINONES ARE LIMITED BY THERMODYNAMIC CONSIDERATIONS

Superoxide dismutase can accelerate an autoxidation by displacing the equilibria: $Q\cdot^- + O_2 \leftrightarrow Q + O_2\cdot^-$ in the direction of quinone[2,13,14] or $QH_2 + O_2 \leftrightarrow Q\cdot^- + O_2\cdot^-$ in the direction of semiquinone.[13] This effect is not seen for strong reductants with reduction potentials low enough to minimize product inhibition by $O_2\cdot^-$ (such as 6-hydroxydopamine, 1,2,4-benzenetriol or 2-hydroxy-1,4-naphthohydroquinone). 1,2-Naphthohydroquinone is a weaker reductant than 1,4-naphthohydroquinone or 2-hydroxy-1,4-naphthohydroquinone ($E^{\circ\prime}(Q/QH_2) = +143$ mV vs. $+36$ mV and -139 mV)[15] and thus is more unfavorable for one-electron reduction of oxygen, increasing product inhibition and favoring acceleration by superoxide dismutase. Similarly, 5-hydroxy-1,4-naphthohydroquinone is a weaker reductant than 1,4-naphthohydroquinone ($E_{1/2} = -140$ mV vs. -180 mV)[16] and additionally binds metals strongly to favor inner-sphere electron transfers. 1,4-Benzohydroquinone would not bind metals as strongly as 1,2,4-benzenetriol, but is a much weaker reductant ($E^{\circ\prime}(Q/QH_2) = +280$ mV versus $+106$ mV),[15] and autoxidation was stimulated by superoxide dismutase.[11]

The contrast between 1,2-naphthoquinone and 2-hydroxy-1,4-naphthoquinone resembles the contrast between the stimulation by superoxide dismutase of autoxidation of gallic acid[12] and inhibition of autoxidation of pyrogallol.[17] These compounds have identical *o*-hydroxy substitutes capable of binding metal ions. However, an electron withdrawing *p*-carboxyl substituent on gallic acid raises the reduction potential compared to pyrogallol, making it a weaker reductant. This effect slows autoxidation, and increases product inhibition.

Thus on the one hand, superoxide dismutase can stimulate 1,2-naphthohydroquinone autoxidation by decreasing product inhibition (i.e., the 2-hydroxy-1,4-napthohydroquinone is less affected by product inhibition because of the additional electron-donating hydroxy group). On the other hand, superoxide dismutase can inhibit autoxidation of 1,4-napthohydroquinone and 2-hydroxy-1,4-napthohydroquinone by metal chelation and chain termination.

REDUCTION POTENTIALS FAVOR *OXIDATION* (RATHER THAN *REDUCTION*) OF SEMIQUINONE BY SUPEROXIDE

Finally, the proposed novel enzyme activity might be questioned on thermodynamic grounds. Semiquinone:superoxide oxidoreductase activity may be more feasible than the proposed superoxide:semiquinone oxidoreductase activity. This is because enzymes have no effect on the overall free energies of the reactions they catalyze and the most stable products are the quinone and H_2O_2. On the *Principle of Microscopic Reversibility*,[18] to the extent that superoxide dismutase accelerates the transfer of an electron from $O_2\cdot^-$ to $Q\cdot^-$, it will also facilitate transfer of an electron from $Q\cdot^-$ to $O_2\cdot^-$. In a reaction mixture in which both $O_2\cdot^-$ and $Q\cdot^-$ are present, the direction of the reaction, and thus the product distribution, depend on thermodynamic considerations. Such considerations more strongly favor the formation of Q and H_2O_2 than the reverse reaction. Although reduction of the semiquinone by superoxide may be thermodynamically feasible, other things being equal, oxidation of the semiquinone by superoxide is more favorable.[a] Unless the enzyme were to act by a mechanism which favored one half-reaction over another (e.g., by specifically providing protons to one product), the thermodynamics favor formation of quinone and H_2O_2.

CONCLUSION

The diversity of actions of superoxide dismutase reflects the diversity of roles played by $O_2\cdot^-$. Superoxide propagates or terminates free radical chains, accelerates or retards oxidation of hydroquinones or semiquinones. The availability and coordination state of metals and the reduction potentials of the quinone and semiquinone all influence the roles which $O_2\cdot^-$ plays. Such diversity offers a range of explanations for paradoxical effects of superoxide dismutase which deserve exploration. In general, we conclude:

1. To the extent that reduction of oxygen occurs by sequential inner sphere electron transfers, superoxide dismutase will be without effect.
2. To the extent that reduction of oxygen by semiquinone is thermodynamically unfavorable, superoxide dismutase will accelerate hydroquinone autoxidation.
3. To the extent that reduction of oxygen by semiquinone is thermodynamically favorable, and

[a]An example is 1,4-naphthohydroquinone, the weakest reductant tested inhibitable by superoxide dismutase (therefore most thermodynamically favored for a semiquinone reductase activity). The two-electron reduction potential at pH 7 is $+36$ mV.[15,19] The one-electron reduction potentials for 1,4-naphthoquinone are also known ($E^{\circ\prime}(Q\cdot^-/Q^{2-}) = +212$ mV and $E^{\circ\prime}(Q/Q\cdot^-) = -140$ mV).[2] Thus, *reduction* of the semiquinone by superoxide ($E^{\circ\prime}(O_2/O_2\cdot^-) = -155$ mV at unit concentration) has a deltaE$^{\circ\prime}$ of $+367$ mV. However, *oxidation* of the semiquinone by superoxide ($E^{\circ\prime}(O_2\cdot^-/H_2O_2) = +865$ mV) has a deltaE$^{\circ\prime}$ of $+1005$ mV.

$O_2\cdot^-$ is the major propagating species, superoxide dismutase will inhibit hydroquinone autoxidation.

4. To the extent that comproportionation of hydroquinone and quinone is the major propagating pathway, superoxide dismutase will accelerate autoxidation.

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REFERENCES

1. Cadenas, E.; Mira, D.; Brumark, A.; Lind, C.; Segura-Aguilar, J; Ernster, L. Effect of superoxide dismutase on the autoxidation of various hydroquinones—a possible role of superoxide dismutase as a superoxide:semiquinone oxidoreductase. *Free Radical Biol. Med.* **5**:71–79; 1988.
2. Ollinger, K.; Buffington, G. D.; Ernster, L.; Cadenas, E. Effect of superoxide dismutase on the autoxidation of substituted hydro- and semi-naphthoquinones. *Chem. Biol. Interact.* **73**:53–76; 1990.
3. Winterbourn, C. C. Inhibition of the autoxidation of divicine and isouramil by the combination of superoxide dismutase and reduced glutathione. *Arch. Biochem. Biophys.* **271**:447–455; 1989.
4. Ishii, T.; Fridovich, I. Dual effects of superoxide dismutase on the autoxidation of 1,4-naphthohydroquinone. *Free Radical Biol. Med.* **8**:21–24; 1990.
5. Misra, H. P.; Fridovich, I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* **247**:3170–3175; 1972.
6. Bandy, B.; Davison, A. J. Interactions between metals, ligands and oxygen in the autoxidation of 6-hydroxydopamine: mechanisms by which metal chelation enhances inhibition by superoxide dismutase. *Arch. Biochem. Biophys.* **259**:305–315; 1987.
7. Lovstad, R. A. Copper catalyzed oxidation of ascorbate (vitamin C). Inhibitory effect of catalase, superoxide dismutase, serum proteins (ceruloplasmin, albumin, apotransferrin) and amino acids. *Int. J. Biochem.* **19**:309–313; 1987.
8. Gee, P.; Davison, A. J. Intermediates in the aerobic autoxidation of 6-hydroxydopamine: relative importance under different reaction conditions. *Free Radical Biol. Med.* **6**:271–284; 1989.
9. Gee, P.; Davison, A. J. 6-Hydroxydopamine does not reduce oxygen directly but requires a co-reductant. *Arch. Biochem. Biophys.* **231**:164–168; 1984.
10. Mukherjee, T. One-electron reduction of juglone (5-hydroxy-1,4-naphthoquinone): a pulse radiolysis study. *Radiat. Phys. Chem.* **29**:455–462; 1987.
11. Greenlee, W. F.; Sun, J. D.; Bus, J. S. A proposed mechanism of benzene toxicity: formation of reactive intermediates from polyphenol metabolites. *Toxicol. Appl. Pharmacol.* **59**:187–195; 1981.
12. Fatur, D. The effect of naturally occurring phenolics on the activity of ornithine decarboxylase in C3H 10T 1/2 cells: possible involvement of active species of oxygen. Simon Fraser University, Burnaby, B.C., Canada, 1988. 99–116. M.Sc. Thesis.
13. Ishii, T.; Iwahashi, H.; Sugata, R.; Kido, R.; Fridovich, I. Superoxide dismutases enhance the rate of autoxidation of 3-hydroxyanthranilic acid. *Arch. Biochem. Biophys.* **276**:248–250; 1990.
14. Winterbourn, C. C. Cytochrome *c* reduction by semiquinone radicals can be indirectly inhibited by superoxide dismutase. *Arch. Biochem. Biophys.* **209**:159–167; 1981.
15. Loach, P. A. Oxidation-reduction potentials. In: Fasman, G. D., ed. *Handbook of biochemistry and molecular biology*. Physical and Chemical Data. 3rd ed. vol. 1. Cleveland: Chemical Rubber Company; 1976:122–130.
16. Buffington, G. D.; Ollinger, O.; Brunmark, A.; Cadenas, E. DT-diaphorase-catalysed reduction of 1,4-naphthoquinone derivatives and glutathionyl-quinone conjugates. Effect of substituents on reaction rates. *Biochem. J.* **257**:561–571; 1989.
17. Marklund, S.; Marklund, G. Involvement of superoxide anion radical in autoxidation of pyrogallol and a convenient assay for superoxide-dismutase. *Eur. J. Biochem.* **47**:469–474; 1974.
18. Moore, W. J. *Physical chemistry*, 4th ed. Englewood Cliffs, NJ: Prentice-Hall Inc.; 1972:342.
19. Morton, R. A. Introductory account of quinones. In: Morton, R. A., ed. *Biochemistry of quinones*. London: Academic Press; 1965:1–22.

Chapter 10.

Mechanisms of Oxygen Reduction by Benzo- and Naphtho-Hydroquinones:

Participation of catalytic metals, superoxide, and comproportionation

by

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ABSTRACT

The autoxidations of selected benzenediols, benzenetriols, and naphthalenediols in phosphate buffer (pH 7.4) differ in their responses to superoxide dismutase. Autoxidation of the weakest reductants, catechol and 1,4-hydroquinone, accelerated 70% and 3-fold on addition of 10 U/ml superoxide dismutase. This effect is consistent with rate-limiting reduction of oxygen by hydroquinone and semiquinone; both reactions being thermodynamically unfavourable for these benzenediols. Conversely, superoxide dismutase (5 U/ml) inhibited autoxidation of 1,2,4-benzenetriol, pyrogallol, 2,3,4-trihydroxybenzoic acid, and gallic acid (70%, 62%, 16%, and 21%). The more favourable reduction of oxygen by benzenetriol semiquinones presumably provides enough steady state $O_2^{\cdot -}$ to propagate the autoxidation. When added at later stages of the autoxidation superoxide dismutase no longer inhibited but accelerated (6%) autoxidation of the weaker benzenetriol reductants gallic acid and 2,3,4-trihydroxybenzoic acid. Comproportionation of hydroquinone with accumulated quinone (producing semiquinone) thus apparently becomes the major propagating reaction in later stages. Superoxide remains the major propagating species for the stronger benzenetriol reductants. With 1,4-naphthalenediol and 1,2-naphthalenediol, 5 U/ml superoxide dismutase briefly inhibited initial rates of autoxidation 26% and 15%, but then accelerated autoxidation. Comproportionation apparently quickly predominates as a propagation pathway in these naphthalenediol autoxidations, consistent with more favourable quinhydrone formation.

Whether inhibiting or accelerating autoxidation alone, EDTA or desferrioxamine acted synergistically with superoxide dismutase to strongly inhibit autoxidation of the benzenetriols. Chelation of trace metals thus forced outer sphere $O_2^{\cdot -}$-propagated autoxidation. In benzenediol and naphthalenediol autoxidations, EDTA or desferrioxamine did not sensitize to inhibition by superoxide dismutase, apart from producing a slight lag in 1,2-naphthalenediol autoxidation, and had little influence on accelerating actions of superoxide dismutase. Also, EDTA or desferrioxamine alone had little influence on naphthalenediol autoxidations, together suggesting that comproportionation provides a relatively metal-independent propagation pathway.

In general, superoxide dismutase accelerates hydroquinone autoxidations when thermodynamic limitations to reduction of oxygen outweigh propagating actions of $O_2^{\cdot -}$, or when comproportionation provides a major propagating pathway. Whether superoxide dismutase inhibits or accelerates, one biologically important effect of superoxide dismutase is to limit steady state levels of semiquinone. In addition, superoxide dismutase may facilitate redox pathways in the excretion of cytotoxic quinones.

INTRODUCTION

We previously advanced a theoretical basis for explaining paradoxical actions of superoxide dismutase on hydroquinone autoxidations.[1] We now provide an experimental foundation, exploring the relative contributions of active species in the reduction of oxygen by hydroquinones. Our observations extend to selected benzenediols, benzenetriols, and naphthalenediols differing in reduction potentials and affinity for metals. The data allow us to discuss structure-activity relationships relevant to mechanisms of hydroquinone autoxidation, and to possible roles of superoxide dismutase in modulating quinone cytotoxicity.

Pathways of hydroquinone autoxidation influence the production of cytotoxic intermediates.

Quinones and polyphenolics are widely prevalent in foods, the environment, and in pharmacology,[2-8] and have long been a subject of study.[9] Phenolics sometimes act as antioxidants or anticarcinogens,[5,10-14] but they are often cytotoxic or mutagenic.[5,10,12,15,16] Catechol for example, contributes to the carcinogenicity of tobacco smoke,[17,18] while chemotherapy with antitumour quinones is dose-limited by cardiotoxicity.[19] Other cytotoxic quinones arise through endogenous metabolism of xenobiotics. For examples, phenolic metabolites of benzene (catechol, 1,4-hydroquinone, 1,2,4-benzenetriol) participate in its myelotoxicity and carcinogenicity,[20-24] while endogenous catecholamines contribute to neuromuscular pathologies such as Parkinsonism.[25]

Due to their redox characteristics, cytotoxic quinones exert toxic and mutagenic effects through cyclic reduction (by enzymic reductases) and reoxidation (by molecular oxygen), with concomitant generation of active oxygen ($O_2^{\cdot-}$, H_2O_2, $^{\bullet}OH$).[19,26-28] Cytotoxicity depends on a balance between reduction, oxidation, and conjugation. Enzymic reduction proceeds by the action of one- or two-electron transfer flavoproteins; the two-electron pathway avoiding release of semiquinone radical intermediate.[29-32] Conjugation of hydroquinones with glucuronate or sulphate, and of quinones with glutathione increases hydrophilicity and

facilitates excretion.[28] Autoxidation counters reduction and conjugation however, producing reactive oxygen and electrophilic products. The rate of reaction with oxygen depends on the properties of the hydroquinone or semiquinone, the participation of metals, and the influence of superoxide dismutase activity.[28,33,34] While many details of hydroquinone autoxidations have been uncovered, systematic studies of different hydroquinones are needed to reveal generalizations for mechanisms of autoxidation. In particular, few studies have examined the relative roles of catalytic metals and $O_2^{\cdot -}$ in different hydroquinone autoxidations.

The influence of superoxide dismutase activity on hydroquinone autoxidation and cytotoxicity is enigmatic.

On the one hand, superoxide dismutase slows autoxidation of some quinoid compounds, including catecholamines[35,36], pyrogallol[37], 1,2,4-benzenetriol[38], 2-hydroxy-1,4-naphthohydroquinone[39], and certain flavonols[40]. In these autoxidations, superoxide dismutase removes $O_2^{\cdot -}$ and prevents it from acting as a chain propagator. Catalytic metals such as iron and copper, if available, accelerate these autoxidations and diminish inhibition by superoxide dismutase.[40,41] However, metals are generally bound or sequestered in cells, so superoxide dismutase should help protect against cytotoxicity of these compounds.

On the other hand, superoxide dismutase accelerates autoxidation of semiquinones[38,42-44], and of some hydroquinones, including 1,4-benzohydroquinone[20], 1,2-naphthohydroquinone[45], and 3-hydroxyanthranilic acid[48]. Superoxide dismutase accelerates these autoxidations by preventing back reactions of $O_2^{\cdot -}$. In this way, superoxide dismutase kinetically offsets thermodynamic adversity to the reduction of oxygen. In the case of semiquinones, enhanced reaction with oxygen may help diminish cytotoxicity.[42-44] In other cases, accelerated autoxidation by superoxide dismutase can enhance cytotoxicity.[49]

In some cases, such as with 1,4-naphthohydroquinone[50] or cytotoxic pyrimidines[51], superoxide dismutase initially suppresses, and later accelerates autoxidation. Superoxide dismutase inhibits initially by removing $O_2^{\cdot -}$ as a propagator, and then accelerates when comproportionation of the hydroquinone with accumulated quinone oxidation product

serves to propagate the autoxidation. In this situation, glutathione as a reducing agent may prolong protection by preventing accumulation of oxidized product.[51-53] However, roles of superoxide dismutase need further characterization.

MATERIALS AND METHODS

Hydroquinones were obtained from Sigma (catechol, 1,4-benzohydroquinone, resorcinol, pyrogallol, gallic acid, phlorglucinol, 6-hydroxydopamine), Fluka (2,3,4-trihydroxybenzoate, 1,4-naphthohydroquinone) and Aldrich (1,2,4-benzenetriol, 1,2-naphthohydroquinone). Potassium phosphate buffer salts, Tris buffer, and albumin (bovine serum) were from Sigma. Disodium EDTA and $FeCl_3$ were from Fisher Scientific (Ottawa). $FeSO_4$ was from BDH Pharmaceuticals (Toronto). Catalase, from Boehringer Mannheim Canada Ltd. (Laval), was confirmed to be free of any contaminating superoxide dismutase activity. Desferrioxamine was a gift from CIBA. Superoxide dismutase (bovine erythrocyte) was from Sigma and from DDI Pharmaceuticals (Mountainview, CA).

All aqueous solutions were made using deionized (Corning demineralizer 3508-A), distilled water. Buffer solutions were not purified further, so contained usual levels of trace metals present in buffer salts.

Hydroquinone solutions were prepared fresh before use in argon-saturated water (benzohydroquinones) or ethanol (naphthohydroquinones) and sealed under a slight positive pressure of argon. Aliquots were removed through a rubber septum with a Hamilton gas-tight syringe (initially purged with argon to prevent introduction of air to the hydroquinone vessel).

Absorption spectra of hydroquinones and partially oxidized samples revealed appropriate wavelengths for monitoring autoxidation. Autoxidation of catechol, which was previously undetected[20], was observable at 320 nm on a high sensitivity setting. Autoxidations of resorcinol (1,3-benzenediol) and phlorglucinol (1,3,5-benzenetriol) were not readily observable at neutral pH.

Experiments were conducted in air-saturated 50 mM phosphate buffer, pH 7.4, 25°C. Reactions were initiated on addition by gas-tight syringe of a 20-50 µl aliquot from an

argon-saturated preparation of hydroquinone. Formation of quinone products was monitored spectrophotometrically. Oxygen consumption was followed polarographically with a YSI Model 53 oxygen monitor and stirrer bath assembly (Yellow Springs Instruments, Ohio). An initial concentration of approximately 246 μM oxygen was assumed for air-saturated 50 mM phosphate buffer at the altitude of our laboratory. Superoxide dismutase and metal chelators (EDTA or desferrioxamine) were added either initially, or during the course of a reaction. Albumin was used as a superoxide dismutase-inactive protein control.

Concentrations of quinone oxidation product were calculated using molar absorptivities estimated from the difference in absorbance of 100 μM hydroquinone before (in argon-saturated buffer) and after oxidation to completion. For the benzohydroquinones (except catechol), the end point oxidation was conducted at pH 9.0 in Tris buffer to accelerate reaction with oxygen and minimize bleaching reactions observed on accumulation of quinone products at neutral pH. For the slower autoxidations, $CuSO_4$ (10 μM) was added to further accelerate reaction with oxygen. End point oxidation of catechol was determined in 0.01 M NaOH. Absorptivities obtained are shown in Table I.

RESULTS

Kinetics of autoxidation

The rates of oxygen consumption during hydroquinone autoxidation correlated approximately with the strength of the reductant (Figure 1, Table I). Gallic acid was unlike the other benzohydroquinones in displaying a slight lag period before the maximal rate of autoxidation. The naphthohydroquinones also differed in their kinetics of autoxidation, with 1,4-naphthohydroquinone displaying a significant lag period (Figure 2a). Maximal rates of autoxidation were all first order with respect to hydroquinone concentration, except that 1,4-naphthohydroquinone deviated at concentrations less than 120 μM (Figure 2b).

Addition of catalase, regenerating one-half mole of oxygen per H_2O_2 generated, approximately halved the maximal rates of oxygen consumption (Table II), revealing H_2O_2 as a major product of autoxidation. At the completion of oxygen consumption following autoxidation of 1,2,4-benzenetriol or 6-hydroxydopamine, addition of catalase regenerated slightly less than half of the oxygen consumed (Figure 3). Also, consumption of oxygen was slightly (10-15%) less than stoichiometric, revealing some reduction of H_2O_2. Nevertheless, H_2O_2 was clearly the major oxygen reduction product.

Effects of superoxide dismutase and metal chelators

Benzenediols

Superoxide dismutase accelerated autoxidation of the weakest reductants catechol (76%) and 1,4-hydroquinone (3.7 fold) (Figure 4). Reduction of O_2 to $O_2^{\cdot -}$ ($E^{o'}$=-0.155 V at 1 M O_2) is thermodynamically unfavourable for both the hydroquinone or semiquinone of these benzenediols (Table I). Superoxide dismutase thus lessens thermodynamic limitations to reduction of oxygen by removing product, and shifts the equilibria toward reduction of oxygen.

$QH_2 + O_2 \Leftrightarrow Q^{\cdot-} + O_2^{\cdot-}$

$Q^{\cdot-} + O_2 \Leftrightarrow Q + O_2^{\cdot-}$

Metal chelators had mild but contrasting effects on benzenediol autoxidations. Both EDTA and desferrioxamine inhibited autoxidation of 1,4-benzohydroquinone by 25%. Catechol, unlike 1,4-benzohydroquinone, binds trace metals avidly.[56] EDTA had little effect on autoxidation of catechol, while desferrioxamine *stimulated* 3-fold. Superoxide dismutase also accelerated autoxidation in the presence of chelators, except in the desferrioxamine-stimulated autoxidation of catechol.

Benzenetriols

Superoxide dismutase decreased the initial rate of autoxidation of each of the benzenetriols (Figures 5 and 6). The degree of inhibition by superoxide dismutase depended on the strength of the reductant. 5 Units/ml superoxide dismutase inhibited the initial rate of autoxidation of 1,2,4-benzenetriol 70%, pyrogallol 62%, and 2,3,4-trihydroxybenzoate 16%, and the maximal rate of autoxidation of gallate 21%.

When added at later stages, superoxide dismutase had contrasting effects on autoxidation of the different benzenetriols (Figures 5 and 6 insets). Superoxide dismutase continued to inhibit autoxidation of the stronger reductants, 1,2,4-benzenetriol and pyrogallol, but mildly accelerated the later autoxidation of gallate and 2,3,4-trihydroxybenzoate. With the weaker reductants, the main pathway of autoxidation thus apparently changed from one propagated by $O_2^{\cdot-}$ to one propagated by $O_2^{\cdot-}$-independent mechanisms.

The effects of metal chelators on benzenetriol autoxidations differed notably (Figures 5 and 6). EDTA or desferrioxamine both inhibited autoxidations of pyrogallol (27% and 30%) and 2,3,4-trihydroxybenzoate (32% and 41%). Chelation of trace metals thus slowed these autoxidations. With 1,2,4-benzenetriol, EDTA and desferrioxamine accelerated autoxidation (4.5 fold and 1.9 fold). Desferrioxamine differed from EDTA in producing a slight lag

before accelerating autoxidation. With gallic acid, EDTA had relatively little effect, producing a slight (but consistent) inhibition (8%). In contrast, desferrioxamine accelerated gallic acid autoxidation 45%.

Accelerating effects of desferrioxamine on gallate and 1,2,4-benzenetriol were also evident with $FeCl_3$ added as catalyst (Figure 7). With gallate, addition of $FeCl_3$ removed much of the lag period in the presence (or absence) of desferrioxamine. With 1,2,4-benzenetriol, the lag period in the presence of desferrioxamine was still evident with added $FeCl_3$.

Regardless of whether EDTA or desferrioxamine accelerated or inhibited autoxidation, they enhanced inhibition by superoxide dismutase in autoxidation of each of the benzenetriols (Figures 5 and 6). Chelation of trace metals thus forces outer sphere $O_2^{\bullet -}$-propagated pathways to predominate these autoxidations.

When 1,2,4-benzenetriol was allowed to autoxidize to completion, absorbance at 490 nm due to accumulation of quinone products reached a plateau and then began to decline (Figure 8). Concomitantly, new peaks appeared at 266 nm and 350 nm. As with the initial autoxidation, desferrioxamine accelerated the loss of absorbance at 490 nm. Catalase did not prevent the bleaching, discounting H_2O_2 as the responsible species. Addition of superoxide dismutase however, mildly accelerated the loss of absorbance. This accelerated bleaching was also unaffected by catalase, so was not due to increased production of H_2O_2. The accelerating effect of superoxide dismutase thus suggests involvement of residual oxygen. This bleaching response needs further characterization, but might represent oxidative polymerization[57] of 2-hydroxy-*p*-quinone product.

Naphthalenediols

Superoxide dismutase briefly slowed, and then accelerated autoxidation of both 1,4-naphthohydroquinone and 1,2-naphthohydroquinone (Figure 9). As observed previously for 1,4-naphthohydroquinone,[50] acceleration by superoxide dismutase was enhanced by the

presence of oxidized hydroquinone (Figure 10). The increased concentration of quinone accelerated autoxidation, and eliminated any latent period or initial inhibition by superoxide dismutase. Thus, comproportionation of hydroquinone with accumulated quinone becomes increasingly available as a propagation pathway as the autoxidations progress. In contrast to the benzohydroquinones, EDTA had no effect on autoxidation of the naphthohydroquinones, or on the influences of superoxide dismutase (not shown). Desferrioxamine also had no effects on 1,4-naphthohydroquinone autoxidation, but inhibited 1,2-naphthohydroquinone autoxidation mildly (28%) and enhanced the initial inhibition by superoxide dismutase from 19% to 57% (Figure 11). Desferrioxamine did not prevent the accelerating action of superoxide dismutase on 1,2-naphthohydroquinone autoxidation, but decreased the maximal rate 39% (Figure 11, Table II).

DISCUSSION

Effects of superoxide dismutase on benzohydroquinone autoxidations depend largely on thermodynamic properties

The influence of superoxide dismutase on autoxidation of the benzohydroquinones depended on the thermodynamic properties of the quinone. Superoxide dismutase accelerated autoxidation of the weak reductants; catechol and 1,4-benzohydroquinone. With these benzenediols, reduction of oxygen to $O_2^{\cdot -}$ is unfavourable for both hydroquinone (+15.8 and +14.2 kcal $mole^{-1}$) and semiquinone (+8.4 and +5.4 kcal $mole^{-1}$) (Table I). By removing product ($O_2^{\cdot -}$), superoxide dismutase shifts the equilibria for reduction of oxygen, avoiding the thermodynamic limitations to reduction of oxygen and accelerating autoxidation.

$$QH_2 + O_2 \Leftrightarrow Q^{\cdot -} + O_2^{\cdot -} + 2H^+$$

$$Q^{\cdot -} + O_2 \Leftrightarrow Q + O_2^{\cdot -}$$

In contrast, with the stronger reductants, 1,2,4-benzenetriol and pyrogallol, superoxide dismutase inhibited autoxidation. With these benzenetriols, reduction of oxygen by semiquinone is more favourable. After initial (metal catalyzed) formation of semiquinone, reduction of oxygen provides sufficient steady state $O_2^{\cdot -}$ to propagate the autoxidation. The presence of superoxide dismutase accelerates semiquinone autoxidation, but prevents $O_2^{\cdot -}$ from oxidizing hydroquinone to propagate the chain.

$$Q^{\cdot -} + O_2 \Leftrightarrow Q + O_2^{\cdot -}$$

$$QH_2 + O_2^{\cdot -} \Rightarrow Q^{\cdot -} + H_2O_2$$

Superoxide dismutase first inhibited and later accelerated autoxidation of the moderately strong reductants 2,3,4-trihydroxybenzoic acid and gallic acid. An electron withdrawing carboxyl group on these benzenetriols increases the reduction potentials compared to pyrogallol. Superoxide thus propagates the autoxidation initially, until $O_2^{\cdot -}$-independent propagation mechanisms become available at later stages. Accumulated H_2O_2 does not

contribute significantly to propagation as catalase failed to inhibit autoxidation (not shown) or eliminate the later acceleration by superoxide dismutase (Figure 5 inset). Comproportionation of hydroquinone with accumulated quinone, producing semiquinone, thus becomes increasingly available as a propagation mechanism. Superoxide dismutase then accelerates net autoxidation by accelerating thermodynamically unfavourable autoxidation of semiquinone.

$$QH_2 + Q \Leftrightarrow 2Q^{\cdot -}$$

$$Q^{\cdot -} + O_2 \Leftrightarrow Q + O_2^{\cdot -}$$

Effects of superoxide dismutase on naphthohydroquinone autoxidations do not follow the thermodynamic dependence observed for benzohydroquinones

As reductants, the naphthalenediols are equal to or stronger than 1,2,4-benzenetriol or pyrogallol (Table I), and autoxidize more rapidly (Figure 1). In contrast to its effects on autoxidations of 1,2,4-benzenetriol or pyrogallol however, superoxide dismutase induces a brief latent period but then accelerates autoxidation of these naphthohydroquinones. Thus $O_2^{\cdot -}$ propagates initially, but comproportionation quickly takes over. Stronger van der Waals interactions between naphthalene derivatives (compared with benzene derivatives) favour dimer ("quinhydrone") formation and presumably increase the rate of comproportionation as a propagating pathway. The large planar π electron orbitals facilitate formation of a charge transfer complex[58] and thus accelerate semiquinone formation. On this basis, superoxide dismutase accelerates net hydroquinone autoxidation by accelerating autoxidation of naphthosemiquinone[38,45] formed through comproportionation.

$$QH_2 + Q \Leftrightarrow [HQ{:}QH]$$

$$[HQ{:}QH] \Leftrightarrow 2Q^{\cdot -} + 2H^+$$

$$Q^{\cdot -} + O_2 \Leftrightarrow Q + O_2^{\cdot -}$$

Quinhydrones equilibrate with free semiquinone[59] leaving open the question whether quinhydrone, semiquinone, or both act as reductant of oxygen.

Other naphthohydroquinone autoxidations reportedly accelerated by superoxide dismutase include those of 5-hydroxy derivatives and glutathione conjugates of 1,2- or 1,4-naphthohydroquinones.[38,45] Superoxide dismutase inhibits initial autoxidation of 2-hydroxy-1,4-naphthohydroquinone,[38,45] but because of the coupled enzymic reduction system that was used it is unknown whether superoxide dismutase might accelerate the subsequent autoxidation when quinone can accumulate.

Effects of metal coordination on kinetics of autoxidation

The hydroquinones studied differ in their affinity for trace metals, and thus in their capacity to reduce oxygen within a ternary reductant:metal:oxygen complex. Catechol, having vicinal hydroxyl groups, binds Fe^{3+} and Cu^{2+} avidly (Table III). The stabilities of dimeric or trimeric complexes of catechol with Fe^{3+} or Cu^{2+} exceed the stability of coordination complexes of EDTA or desferrioxamine. Other hydroquinones with vicinal hydroxyl groups presumably also coordinate metals with affinities dependent on their pKa's, and compete with EDTA and desferrioxamine for trace metals. Conversely, 1,4-benzohydroquinone and 1,4-naphthohydroquinone have little ability to coordinate trace metals.

The effects of EDTA or desferrioxamine on autoxidation of the different hydroquinones did not reveal anticipated systematic patterns, but instead produced intriguing anomalies. Inhibition of autoxidations of 1,4-benzohydroquinone, 2,3,4-trihydroxybenzoate, and pyrogallol by EDTA and desferrioxamine likely reflects impaired reduction of trace metals. With the stronger reductant 1,2,4-benzenetriol, the accelerated autoxidation induced by EDTA or desferrioxamine may reflect acceleration of otherwise rate-limiting reoxidation of catalytic metals. However, desferrioxamine (but not EDTA) accelerates autoxidation of the weak reductants catechol and gallic acid. Explanations are not readily apparent. Moreover, EDTA and desferrioxamine inhibit autoxidation of 1,4-benzohydroquinone and 2,3,4-trihydroxybenzoate but not of catechol or gallate. Catechol may be able to compete better for metals with EDTA and desferrioxamine than 1,4-benzohydroquinone. The

differences between responses of 2,3,4-trihydroxybenzoate and gallate may in some way be related to a salicylate-like group on 2,3,4-trihydroxybenzoate, which binds metals with affinities close to those of catecholate groups.[56]

The effects of EDTA and desferrioxamine on the naphthalenediol autoxidations were somewhat more explicable. The mild (28%) inhibition of 1,2-naphthohydroquinone, but not 1,4-naphthohydroquinone autoxidation by desferrioxamine is consistent with a greater affinity of 1,2-naphthohydroquinone for catalytic metals. This difference in affinity for metals may also explain the differences in kinetics of autoxidation (Figures 9 and 11) and the more rapid autoxidation and oxygen consumption of the weaker reductant 1,2-naphthohydroquinone (Table I, Table II). More efficient catalysis by trace metals in 1,2-naphthohydroquinone autoxidation avoids any lag for accumulation of propagating intermediates. Thus, at 40 μM, where low availability of quinone limits comproportionation, autoxidation and oxygen consumption were faster for 1,2-naphthohydroquinone than for 1,4-naphthohydroquinone. At higher initial concentrations (250 μM), more quinone was generated to propagate autoxidation, and the rates of oxygen consumption correlated with reduction potentials.

Influence of metal chelators on effects of superoxide dismutase

The influence of EDTA and desferrioxamine on the effects of superoxide dismutase provided useful mechanistic information. In previous studies on autoxidations of 6-hydroxydopamine[41], epinephrine[35], or sulphite[60], EDTA or desferrioxamine sensitized the autoxidation to inhibition by superoxide dismutase. By preventing inner sphere (two-electron) transfers to oxygen, these chelators apparently force outer sphere $O_2^{\cdot-}$-propagated autoxidation. In the current studies, EDTA or desferrioxamine sensitized the autoxidations of the benzenetriols (1,2,4-benzenetriol, pyrogallol, 2,3,4-trihydroxybenzoate, and to a lesser extent gallic acid) to inhibition by superoxide dismutase. This synergistic inhibition occurred whether the metal chelator alone inhibited (2,3,4-trihydroxybenzoate, pyrogallol)

or accelerated (1,2,4-benzenetriol) autoxidation, showing the variable effectiveness of $O_2^{\cdot-}$ -propagation. The enhanced inhibition was most dramatic with the strongest benzenetriol reductant (1,2,4-benzenetriol), and decreasingly evident with weaker reductants. With a strong reductant such as 1,2,4-benzenetriol, slowed reduction of trace metals in the presence of EDTA or desferrioxamine apparently does not limit autoxidation. Accelerated autoxidation of reduced metals in the presence of EDTA and desferrioxamine[61-63] accelerates $O_2^{\cdot-}$ release, propagating 1,2,4-benzenetriol autoxidation, and sensitizing it to inhibition by superoxide dismutase. With weaker reductants such as pyrogallol or 2,3,4-trihydroxybenzoate, the presence of EDTA or desferrioxamine also increases propagation by $O_2^{\cdot-}$, but to a lesser extent.

Removal of one pathway for propagating an autoxidation increases the contribution of alternate pathways, as observed in autoxidation of 6-hydroxydopamine.[64] Thus, chelation of trace metals, preventing inner sphere electron transfers, augments propagation by $O_2^{\cdot-}$. In the presence of a chelator plus superoxide dismutase, remaining autoxidation proceeds largely by metal-independent, $O_2^{\cdot-}$-independent pathways. H_2O_2 or comproportionation remain as possible propagators.

In those autoxidations accelerated by superoxide dismutase (catechol, 1,4-benzohydroquinone, 1,2-naphthohydroquinone, 1,4-naphthohydroquinone), metal chelators did not induce inhibition by superoxide dismutase (apart from producing a lag in 1,2-naphthohydroquinone autoxidation), and had little or no effect on the acceleration by superoxide dismutase. The lag in autoxidation of 1,2-naphthohydroquinone produced by desferrioxamine plus superoxide dismutase was soon overcome as quinone accumulated. These results suggest that comproportionation, and perhaps autoxidation of semiquinone, are largely metal-independent (or at least that comproportionation is metal-independent and rate limiting).

The different benzohydroquinones display similar rates of autoxidation in the presence of superoxide dismutase plus a chelator (Table II), suggesting similar rates of

comproportionation. Autoxidation of the naphthalenediols in the presence of superoxide dismutase plus a chelator is two orders of magnitude faster, presumably reflecting more facile quinhydrone formation.

Latent periods reflect time for accumulation of propagating species

In some cases the latent period reflected time for accumulation of sufficient quinone product for comproportionation to serve as a propagating pathway. For example, a latent period in the autoxidation of a first aliquot of 1,4-naphthohydroquinone was absent on addition of a second aliquot to the cuvette, where accumulated quinone product was available. H_2O_2 was not the accumulated propagator, as catalase had no effect on rates of autoxidation, and as superoxide dismutase accelerated autoxidation in the presence of already accumulated H_2O_2.

The lack of a latent period in autoxidation of 1,2-naphthohydroquinone presumably reflects coordination of trace metals by the hydroquinone, allowing formation of a ternary reductant:metal:oxygen complex. The inhibition by desferrioxamine, and more pronounced latent period in the presence of superoxide dismutase plus desferrioxamine is consistent with this suggestion. That superoxide dismutase, or superoxide dismutase plus desferrioxamine induced a latent period in autoxidations of 1,4- and 1,2-naphthohydroquinone, suggests that $O_2^{\bullet -}$ serves as an initial propagator until sufficient quinone accumulates for comproportionation to overtake the contribution of $O_2^{\bullet -}$.

In previous studies where superoxide dismutase plus a chelator induce a latent period before accelerating to maximal rates of autoxidation, such as with dialuric acid and other pyrimidines[51] or with 6-hydroxydopamine[40], addition of oxidation product eliminates the latent period. In the autoxidation of pyrimidines, decreasing the initial concentration of pyrimidine increased the lag period, as more time was required for accumulation of oxidation products. In the present studies, the deviation of 1,4-naphthohydroquinone

autoxidation from first order kinetics at low concentrations may reflect limiting quinone accumulation for maximal comproportionation.

Some latent periods are removed by addition of metal, or induced by addition of a chelator. For example, addition of Fe^{3+} removed the latent period in the slow autoxidation of gallic acid. Superoxide dismutase still produced mild inhibition in the presence of Fe^{3+} (not shown), so greater steady state levels of $O_2^{\cdot-}$ (and $Q^{\cdot-}$) rather than accumulation of quinone were likely responsible for the absence of a latent period. Desferrioxamine accelerated catalysis by Fe^{3+}, and superoxide dismutase again produced mild inhibition. At later stages of autoxidation, comproportionation gained in importance, as superoxide dismutase mildly accelerated autoxidation. Together, these results suggest a balance between $O_2^{\cdot-}$ and comproportionation as propagators in the autoxidation of gallic acid, with inner-sphere two-electron transfer to oxygen contributing relatively little.

Desferrioxamine produced a brief latent period before accelerating autoxidation of 1,2,4-benzenetriol. Further addition of superoxide dismutase produced strong inhibition, so the latent period was for maximal steady state generation of superoxide.

When does superoxide dismutase inhibit or accelerate autoxidations?

These and previous studies provide a foundation for revealing when superoxide dismutase inhibits or accelerates autoxidations. We previously offered a theoretical framework to explain contrasting effects of superoxide dismutase in hydroquinone autoxidations.[1] The current results give experimental support to each of the proposals.

1. Theorem: *"To the extent that reduction of oxygen occurs by sequential inner sphere electron transfers, superoxide dismutase will be without effect."* **Experimental support:** Autoxidations involving sequential inner sphere electron transfers are most evident with the benzenetriols (herein) and catecholamines[35,41], where metal chelators sensitize the

autoxidation to inhibition by superoxide dismutase.* In general, compounds where metal chelators sensitize autoxidation to inhibition by superoxide dismutase are hydrophilic, and contain vicinal hydroxyl or other groups capable of coordinating trace metals. Examples include benzenetriols[1,20,37], catecholamines[35,41], and hydroxylated pyrimidines[65,66]. An extreme example is the autoxidation of ascorbic acid, which is highly dependent on trace metals[67,68], and sensitive to inhibition by superoxide dismutase activity only in the presence of added Fe:EDTA[69].

2. Theorem: *"To the extent that reduction of oxygen by semiquinone is thermodynamically unfavourable, superoxide dismutase will accelerate hydroquinone autoxidation."*

Experimental support: Autoxidations of 1,4-benzohydroquinone and catechol provide examples where autoxidation of the semiquinone, as well as of the hydroquinone, is strongly unfavourable. Net autoxidation is thus driven by O_2/H_2O_2 ($E^{o'}$=+0.281 V). The equilibria for reduction of oxygen by hydroquinone and semiquinone both lie far to the left, and will be displaced forward by superoxide dismutase. Superoxide dismutase thus accelerates autoxidation of any semiquinone which forms either through oxidation of hydroquinone by trace metals or through comproportionation. In these cases, sequential inner sphere electron transfers to oxygen do not seem to be a major pathway for oxidation, since superoxide dismutase accelerated autoxidation and since metal chelators had only mild effects. Comproportionation of hydroquinone with accumulating quinone is thus likely the main propagating pathway. Another example where single electron transfers to oxygen are both strongly unfavourable may be autoxidation of the amino phenol, 3-hydroxyanthranilic acid, which is very slow, and is accelerated by superoxide dismutase.[46-48] Other examples of weak reductants whose autoxidations accelerate with superoxide dismutase include glutathione conjugates of 2-methyl- and 2,5-dimethyl-1,4-benzohydroquinone.[70]

*Caution is needed interpreting the effects of superoxide dismutase on such autoxidations, since at greater than catalytic concentrations, coordination of trace metals by superoxide dismutase apoprotein can sensitize the autoxidation to inhibition by superoxide dismutase activity.[41] Conversely, studies done only in the presence of a metal chelator may preclude an inner sphere pathway.

3. Theorem: *"To the extent that reduction of oxygen by semiquinone is thermodynamically favourable, and $O_2^{\cdot-}$ is the major propagating species, superoxide dismutase will inhibit hydroquinone autoxidation."* **Experimental support:** Autoxidations of 1,2,4-benzenetriol and 6-hydroxydopamine represent examples where reduction of oxygen by semiquinone is relatively favourable. Reduction of oxygen by semiquinone thus provides steady-state $O_2^{\cdot-}$ to help propagate the autoxidation. While superoxide dismutase accelerates autoxidation of semiquinone, removal of $O_2^{\cdot-}$ slows rate limiting oxidation of hydroquinone to semiquinone. In these cases, propagation by $O_2^{\cdot-}$ only becomes fully prevalent when sequential inner sphere electron transfers are prevented by metal chelators.

4. Theorem: *"To the extent that comproportionation of hydroquinone and quinone is the major propagating pathway, superoxide dismutase will accelerate autoxidation."* **Experimental support:** Autoxidations of 1,2- and 1,4-naphthohydroquinone serve as examples where comproportionation is the major propagating pathway. While the relatively favourable reduction of oxygen by semiquinone provides $O_2^{\cdot-}$ to propagate the autoxidation initially, comproportionation of hydroquinone with accumulating quinone quickly predominates. Superoxide dismutase may thus inhibit briefly, but then accelerates autoxidation when autoxidation of semiquinone becomes rate limiting. With reductants such as gallic acid or 2,3,4-trihydroxybenzoate, comproportionation is slower, and other autoxidation pathways dominate until sufficient quinone accumulates to provide competitive steady state levels of semiquinone.

Whether superoxide dismutase inhibits, accelerates, or has no effect on autoxidation depends on an interplay between available propagating pathways. This interplay obviously depends on the physico-chemical characteristics of the quinone, and the composition of the reaction medium (eg. presence of metals, ionic strength). At any one time, more than one reaction pathway may be operating. Also, a pathway may become more or less pronounced as a reaction proceeds or as conditions change.

One action of superoxide dismutase in vivo *may be to limit steady state levels of semiquinone*

Observations that superoxide dismutase accelerates autoxidation of semiquinones[38] and suppresses reactions of semiquinones with biological constituents such as hemoproteins[43,44] led to the important suggestion that one protective action of superoxide dismutase may be to help remove semiquinones.[43,44] In addition, by preventing $O_2^{\cdot-}$ from propagating autoxidation of hydroquinones, superoxide dismutase can inhibit *formation* of semiquinones. Together these actions of superoxide dismutase may help suppress biologically damaging reactions of semiquinones such as release of iron from ferritin[71-73] or site specific reduction of H_2O_2 to $^{\bullet}OH$[74-76].

In biological systems, sequestration and coordination of metals minimize metal-catalyzed inner-sphere autoxidation and thus force $O_2^{\cdot-}$-propagated or comproportionation-propagated autoxidation pathways. In cases where $O_2^{\cdot-}$ acts as propagator of hydroquinone autoxidation, superoxide dismutase acts in concert with glutathione[52,53] and DT-diaphorase[77] to keep the quinone in a fully reduced state, and facilitate excretion. In cases where comproportionation is the major propagating pathway, superoxide dismutase acts counter to DT-diaphorase and glutathione, accelerating autoxidation. With weak reductants such as 1,4-benzohydroquinone, autoxidation is slow, so even with superoxide dismutase present, reductases likely maintain the quinone in a sufficiently reduced steady state to allow sulphation or glucuronidation.[78] With stronger reductants such as 1,2-naphthohydroquinone, superoxide dismutase may increase redox cycling more dangerously. However, since less hydrophilic quinones such as 1,2- and 1,4-naphthohydroquinone are apparently more susceptible to comproportionation, increased quinone formation by superoxide dismutase may facilitate nucleophilic addition of glutathione and help trap the quinone in the aqueous phase. Conjugation with glutathione reportedly does not decrease redox cycling[70,79-81], but it facilitates excretion. Superoxide dismutase may thus help poise quinone reduction/oxidation for efficient excretion, while minimizing levels of semiquinone (and $O_2^{\cdot-}$).

CONCLUSIONS

Hydroquinone autoxidation may proceed by one or more pathways depending on the properties of the hydroquinone and the reaction conditions. The predominant pathway may change as a reaction proceeds or as conditions change, and removal of one pathway may increase the contribution of competing pathways. Once initiated, presumably through oxidation of hydroquinone by trace metal, metal:oxygen complex, or very slowly by oxygen alone, the autoxidation can be maintained by any of several chain propagators. In autoxidation of hydroquinones in air-saturated aqueous solution, the major propagators include superoxide, and comproportionation of hydroquinone with quinone. In addition, if trace metals can be coordinated by hydroquinone, autoxidation may proceed by sequential inner-sphere electron transfers to oxygen. H_2O_2 is the major reduction product under the conditions studied, and peroxidatic oxidation does not contribute significantly to the oxidation rate. Only at low oxygen, or in the presence of superoxide dismutase plus a metal chelator, might H_2O_2 contribute significantly. The extent to which any pathway predominates depends on the properties of the hydroquinone, and the presence of chain-breaking and/or metal-binding antioxidants.

The influence of superoxide dismutase on hydroquinone autoxidation depends on the quinone reduction potentials, coordination of metals, and the contribution of comproportionation. Superoxide dismutase accelerates autoxidation of weak benzenediol reductants by helping overcome thermodynamic limitations to reduction of oxygen by both hydroquinone and semiquinone. Superoxide dismutase inhibits autoxidation of stronger benzenetriol reductants (pyrogallol, 1,2,4-benzenetriol) where more favourable reduction of oxygen by semiquinone provides $O_2^{\cdot-}$ to propagate the autoxidation. With moderately strong benzenetriol reductants (gallic acid, 2,3,4-trihydroxybenzoic acid), superoxide dismutase inhibits initially, but accelerates when accumulation of oxidized quinone becomes available to propagate the autoxidation. The hydrophilic, strongly metal binding character of benzenetriols allows sequential inner sphere reductions of oxygen, and metal chelators sensitize these autoxidations to inhibition by superoxide dismutase. With naphthalenediols,

greater ease of quinhydrone formation favours comproportionation, accelerating autoxidation and inducing an accelerating action of superoxide dismutase. While these studies yielded some useful insights into mechanisms of hydroquinone autoxidations, they need to be extended to other quinones to reveal further generalities.

REFERENCES

1. Bandy, B.; Moon, J.; Davison, A.J. Multiple actions of superoxide dismutase: Why can it both inhibit and stimulate reduction of oxygen by hydroquinones? *Free Rad. Biol. Med.* **9**:143-148; 1990.

2. Thomson, R.H. *Naturally Occurring Quinones.* London and New York; Academic Press; 1971.

3. Thomson, R.H. *Naturally Occurring Quinones. III. Recent Advances.* London: Chapman & Hall; 1987.

4. Mager, J.; Chevion, M.; Glaser, G. Favism. In: Liener, L.I. ed. *Toxic Constituents of Plant Foodstuffs.* New York: Academic Press; 1980:265-294.

5. Ames, B.N. Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. *Science* **221**:1256-1264; 1983.

6. Fox, M.A.; Olive, S. Photooxidation of anthracene on atmospheric particulate matter. *Science* **205**:582-583; 1979.

7. Scheutzle, D. Sampling of vehicle emissions for chemical analysis in biological testing. *Environ. Health Perspect.* **47**:65-80; 1983.

8. Powis, G. Metabolism and reactions of quinoid anticancer agents. *Pharmac. Ther.* **35**:57-162; 1987.

9. Willson, R.L. Quinones, semiquinone free radicals and one-electron transfer reactions: a walk in the literature from Peru to S.O.D. *Free Rad. Res. Comms.* **8**:201-217; 1990.

10. Brown, J.P. A review of the genetic effects of naturally occurring flavonoids, anthraquinones and related compounds. *Mutation Research* **75**:243-277; 1980.

11. Larson, R.A. The antioxidants of higher plants. *Phytochemistry* **27**:969-978; 1988

12. Laughton, M.J.; Halliwell, B.; Evans, P.; Hoult, J.R.S. Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin. Effects on lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA. *Biochem. Pharmacol.* **38**:2859-2865; 1989.

13. Josephy, P.D.; Lord, H.L.; Sniekus, V.A. Inhibition of benzo[a]pyrene dihydrodiol epoxide mutagenicity by synthetic analogues of ellagic acid. *Mutation Res.* **242**:143-149; 1990.

14. Zhou, Y.-C.; Zheng, R.-L. Phenolic compounds and an analog as superoxide anion scavengers and antioxidants. *Biochem. Pharmacol.* **42**:1177-1179; 1991.

15. Stich, H.F.; Rosin, M.P.; Wu, C.H.; Powrie, W.D. The action of transition metals on the genotoxicity of simple phenols. *Cancer Lett.* **14**:251-260; 1981.

16. Chesis, P.L.; Levin, D.E.; Smith, M.T.; Ernster, L.; Ames, B.N. Mutagenicity of quinones: pathways of metabolic activation and detoxification. *Proc. Natl. Acad. Sci. USA* **81**:1696-1700; 1984.

17. Hecht, S.; Carmella, S.; Mori, H.; Hoffman, D. A study of tobacco carcinogenesis. Role of catechol as a major cocarcinogen in the weakly acidic fraction of smoke condensate. *J. Natl. Cancer Inst.* **66**:163-169; 1981.

18. Pryor, W.A.; Hales, B.J.; Premovic, P.I.; Church, D.F. The radicals in cigarette tar: their nature and suggested physiological implications. *Science* **220**:425-427; 1982.

19. Powis, G. Free radical formation by antitumor quinones. *Free Rad. Biol. Med.* **6**:63-101; 1989.

20. Greenlee, W.F.; Sun, J.D.; Bus, J.S. A proposed mechanism of benzene toxicity: formation of reactive intermediates from polyphenol metabolites. *Toxicol. Appl. Pharmacol.* **59**:187-195; 1981.

21. Lewis, J.G.; Steward, W.; Adams, D.O. Role of oxygen radicals in induction of DNA damage by metabolites of benzene. *Cancer Res.* **48**:4762-4765; 1988.

22. Glatt, H.; Padykula, R.; Berchtold, G.A.; Ludewig, G.; Platt, K.L.; Klein, J.; Oesch, F. Multiple activation pathways of benzene leading to products with varying genotoxic characteristics. *Environmental Health Perspectives* **82**:81-89; 1989.

23. Smith, M.T.; Yager, J.W.; Steinmetz, K.L.; Eastmond, D.A. Peroxidase-dependent metabolism of benzene's phenolic metabolites and its potential role in benzene toxicity and carcinogenicity. *Environmental Health Perspectives* **82**:23-29; 1989.

24. Kawanishi, S.; Inoue, S.; Kawanishi, M. Human DNA damage induced by 1,2,4-benzenetriol, a benzene metabolite. *Cancer Research* **49**:164-168; 1989.

25. Graham, D.G. Catecholamine toxicity: a proposal for the molecular pathogenesis of manganese neurotoxicity and Parkinson's disease. *Neurotoxicity* **5**:83-96; 1984.

26. Smith, M.; Evans, C.G.; Thor, H.; Orrenius, S. Quinone-induced oxidative injury to cells and tissues. In: Sies, H., ed. *Oxidative Stress.* London: Academic Press; 1985:91-113.

27. Nohl, H.; Jordan, W.; Youngman, R.J. Quinones in biology: functions in electron transfer and oxygen activation. *Adv. Free Rad. Biol. Med.* **2**:211-279; 1986.

28. Brunmark, A.; Cadenas, E. Redox and addition chemistry of quinoid compounds and its biological implications. *Free Rad. Biol. Med.* **7**:435-477; 1989.

29. Yamazaki, I. One-electron and two-electron transfer mechanisms in enzymic oxidation-reduction reactions. *Adv. Biophys.* **2**:33-76; 1971.

30. Powis, G.; Appel, P.L. Relationship of the single-electron reduction potential of quinones to their reduction by flavoproteins. *Biochem. Pharmacol.* **29**:2567-2572; 1980.

31. Lind, C.; Hochstein, P.; Ernster, L. DT-diaphorase as a quinone reductase: a cellular control device against semiquinone and superoxide radical formation. *Arch. Biochem. Biophys.* **216**:178-188; 1982.

32. Wermuth, B.; Platts, K.L.; Seidel, A.; Oesch, F. Carbonyl reductase provides the enzymatic basis of quinone detoxification in man. *Biochem. Pharmacol.* **35**:1277-1282; 1986.

33. Wardman, P. Bioreductive activation of quinones: redox properties and thiol reactivity. *Free Rad. Res. Comms.* **8**:219-229; 1990.

34. Miller, D.M.; Buettner, G.R.; Aust, S.D. Transition metals as catalysts of "autoxidation" reactions. *Free Rad. Biol. Med.* **8**:95-108; 1990.

35. Misra, H.P.; Fridovich, I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* 247:3170-3175; 1972.

36. Heikkila, R.E.; Cohen, G. 6-hydroxydopamine: evidence for superoxide radical as an oxidative intermediate. *Science* **181**:456-457; 1973.

37. Marklund, S.; Marklund, G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.* **47**:469-474; 1974.

38. Ollinger, K.; Buffington, G.D.; Ernster, L.; Cadenas, E. Effect of superoxide dismutase on the autoxidation of substituted hydro- and semi-naphthoquinones. *Chem.-Biol. Interact.* **73**:53-76; 1990.

39. Canada, A.T.; Giannella, E.; Nguyen, T.D.; Mason, R.P. The production of reactive oxygen species by dietary flavonols. *Free Rad. Biol. Med.* **9**:441-449; 1990.

40. Sullivan, S.G.; Stern, A. Effects of superoxide dismutase and catalase on catalysis of 6-hydroxydopamine and 6-aminodopamine autoxidation by iron and ascorbate. *Biochemical Pharmacol.* **30**:2279-2285; 1981.

41. Bandy, B.; Davison, A.J. Interactions between metals, ligands, and oxygen in the autoxidation of 6-hydroxydopamine: mechanisms by which metal chelation enhances inhibition by superoxide dismutase. *Arch. Bioch. Biophys.* **259:**305-315; 1987.

42. Winterbourn, C.C.; French, J.K.; Claridge, R.F.C. Superoxide dismutase as an inhibitor of reactions of semiquinone radicals. *FEBS Lett.* **94**:269-272; 1978.

43. Winterbourn, C.C. Cytochrome *c* reduction by semiquinone radicals can be indirectly inhibited by superoxide dismutase. *Arch. Biochem. Biophys.* **209**:159-167; 1981.

44. Sutton, H.C.; Sangster, D.F. Reactivity of semiquinone radicals and its relation to the biochemical role of superoxide. *J. Chem. Soc. Faraday Trans. 1* **78**:695-711; 1982.

45. Cadenas, E.; Mira, D.; Brunmark, A.; Lind, C.; Segura-Aguilar, J.; Ernster, L. Effect of superoxide dismutase on the autoxidation of various quinones - a possible role of superoxide dismutase as a superoxide:semiquinone oxidoreductase. *Free Rad. Biol. Med.* **5**:71-79; 1988.

46. Dykens, J.A.; Sullivan, S.G.; Stern, A. Oxidative reactivity of the tryptophan metabolites 3-hydroxyanthranilate, cinnabarinate, quinolinate, and picolinate. *Biochem. Pharmacol.* **36**:211-217; 1987.

47. Iwahashi, H.; Ishii, T.; Sugata, R.; Kido, R. Superoxide dismutase enhances the formation of hydroxyl radicals in the reaction of 3-hydroxyanthranilic acid with molecular oxygen. *Biochem. J.* **251**:893-898; 1988.

48. Ishii, T.; Iwahashi, H.; Sugata, R.; Kido, R.; Fridovich, I. Superoxide dismutases enhance the rate of autoxidation of 3-hydroxyanthranilic acid. *Arch. Biochem. Biophys.* **276**:248-250; 1990.

49. Ishii, T.; Iwahashi, H.; Sugata, R.; Kido, R. Superoxide dismutase enhances the toxicity of 3-hydroxyanthranilic acid to bacteria. *Free Rad. Res. Comms.* **14**:187-194; 1991.

50. Ishii, T.; Fridovich, I. Dual effects of superoxide dismutase on the autoxidation of 1,4-naphthohydroquinone. *Free Rad. Biol. Chem.* **8**:21-24; 1990.

51. Winterbourn, C.C.; Cowden, W.B.; Sutton, H.C. Auto-oxidation of dialuric acid, divicine and isouramil. Superoxide dependent and independent mechanisms. *Biochem. Pharmacol.* **38**:611-618; 1989.

52. Winterbourn, C.C. Inhibition of autoxidation of divicine and isouramil by the combination of superoxide dismutase and reduced glutathione. *Arch. Biochem. Biophys.* **271**:447-455; 1989.

53. Winterbourn, C.C.; Munday, R. Concerted action of reduced glutathione and superoxide dismutase in preventing redox cycling of dihydroxypyrimidines and their role in antioxidant stress. *Free Rad. Res. Comms.* **8**:287-293; 1990.

54. Wardman, P. Reduction potentials of one-electron couples involving free radicals in aqueous solution. *J. Phys. Chem. Ref. Data* **18**:1637-1755; 1989

55. Clarke, W.M. *Oxidation Reduction Potentials of Organic Systems.*, The Williams and Wilkins Company, Baltimore, 1960.

56. Martell, A.E.; Smith, R.M. *Critical Stability Constants*, Vol. 2: Other Organic Ligands, Plenum, New York, 1977.

57. Musso, H. Phenol coupling. In: Taylor, W.I., Battersby, A.R., eds., *Oxidative Coupling of Phenols.*; New York: Marcel Dekker Inc.; 1967:1-94.

58. Foster, R.; Forman, M.I. Quinone complexes. In: Patai, S. ed. *The Chemistry of the Quinoid Compounds.* London: John Wiley & Sons; 1974:257-333.

59. Michaelis, L.; Fletcher, E.S. The equilibrium of the semiquinone of phenanthrene-3-sulfonate with its dimeric compound. *J. Am. Chem. Soc.* **59**:2460-2467; 1937.

60. McCord, J.M.; Fridovich, I. The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen. *J. Biol. Chem.* **244**:6056-6063; 1969.

61. Kaden, T.; Fallab, S. Chelate catalysed autoxidation of iron(II) complexes. In: Kirschner, S., ed. *Advances in the Chemistry of Coordination Compounds.* New York: MacMillan Co.; 1961:654-663.

62. Harris, D.C.; Aisen, P. Facilitation of Fe(II) autoxidation by Fe(III) complexing agents. *Biochim. Biophys. Acta* **329**:156-158; 1973.

63. Zuberbuhler, A.D. Interaction of Cu(I) complexes with dioxygen. In: Sigel, H., ed. *Metal Ions in Biological Systems.* 5:325; 1976.

64. Gee, P.; Davison, A.J. Intermediates in the aerobic autoxidation of 6-hydroxydopamine: relative importance under different reaction conditions. *Free Rad. Biol. Med.* **6**:271-284; 1989.

65. Cohen, G.; Heikkila, R.E. The generation of hydrogen peroxide, superoxide radical and hydroxyl radical by 6-hydroxydopamine, dialuric acid and related cytotoxic agents. *J. Biol. Chem.* **249**:2447-2452; 1974.

66. Winterbourn, C.C.; Benatti, U.; De Flora, A. Contribution of superoxide, hydrogen peroxide, and transition metal ions to auto-oxidation of the favism-inducing pyrimidine aglycone, divicine, and its reactions with haemoglobin. *Biochem. Pharmacol.* **35**:2009-2015; 1986.

67. Buettner, G.R. Ascorbate autoxidation in the presence of iron and copper chelates. *Free Rad. Res. Comms.* **1**:349-353; 1986.

68. Buettner, G.R. In the absence of catalytic metals ascorbate does not autoxidize at pH 7: ascorbate as a test for catalytic metals. *J. Biochem. Biophys. Methods* **16**:27-40; 1988.

69. Scarpa, M.; Stevanato, R.; Viglino, P.; Rigo, A. Superoxide ion as active intermediate in the autoxidation of ascorbate by molecular oxygen. *J. Biol. Chem.* **258**:6695-6697; 1983.

70. Brunmark, A.; Cadenas, E. Reductive addition of glutathione to *p*-benzoquinone, 2-hydroxy-*p*-benzoquinone, and *p*-benzoquinone epoxides. Effect of the hydroxy- and glutathionyl substituents on *p*-benzohydroquinone autoxidation. *Chem.-Biol. Interact.* **68**:273-298; 1988.

71. Boyer, R.F.; Clark, H.M.; Laroche, A.P. Reduction and release of ferritin iron by plant phenolics. *J. Inorg. Biochem.* **32**:171-181; 1988.

72. Monteiro, H.P.; Vile, G.F.; Winterbourn, C.C. Release of iron from ferritin by semiquinone, anthracycline, bipyridyl, and nitroaromatic radicals. *Free Rad. Biol. Med.* **6**:587-591; 1989.

73. Reif, D.W. Ferritin as a source of iron for oxidative damage. *Free Rad. Biol. Med.* **12**:417-427; 1992.

74. Winterbourn, C.C. Evidence for the production of hydroxyl radicals from the adriamycin semiquinone and H_2O_2. *FEBS Lett.* **136**:89-94; 1981.

75. Nohl, H.; Jordan, W. OH^--generation by adriamycin semiquinone and H2O2: an explanation for the cardiotoxicity of anthracycline antibiotics. *Biochem. Biophys. Res. Comm.* 114:197-205; 1983.

76. Nohl, H.; Jordan, W. The involvement of biological quinones in the formation of hydroxyl radicals via the Haber-Weiss reaction. *Bioorg. Chem.* **15**:374-382; 1987.

77. Lind, C.; Vadi, H.; Ernster, L. Metabolism of benzo[a]pyrene-3,6-quinone and 3-hydroxy-benzo[a]pyrene in liver microsomes from 3-methyl-cholanthrene-treated rats. A possible role of DT-diaphorase in the formation of glucuronyl conjugates. *Arch. Biochem. Biophys.* **190**:97-108; 1978.

78. Lind, C. Relationship between the rate of reduction of benzo[a]pyrene 3,6-quinone and the formation of benzo[a]pyrene 3,6-quinol glucuronides in rat liver microsomes. *Biochem. Pharmacol.* **34**:895-897; 1985.

79. Buffington, G.D.; Ollinger, K.; Brunmark, A.; Cadenas, E. DT-diaphorase-catalysed reduction of 1,4-naphthoquinone derivatives and glutathionyl-quinone conjugates. *Biochem. J.* **257**:561-571; 1989.

80. Llopis, J.; Ernster, L.; Cadenas, E. Effect of glutathione on the redox transitions of naphthohydroquinone derivatives formed during DT-diaphorase catalysis. *Free Rad. Res. Comms.* **8**:271-285; 1990.

81. Jarabak, J. Polycyclic aromatic hydrocarbon quinone-mediated oxidation reduction cycling catalyzed by a human placental NADPH-linked carbonyl reductase. *Arch. Biochem. Biophys.* 291:334-338; 1991.

Table I. *Structures and physical data for the hydroquinones studied.* Wavelengths at which autoxidations were monitored (λ), and molar absorptivities (ε) are shown. Midpoint reduction potentials (E_{m7}) for one- and two-electron couples are from references 54 and 55.

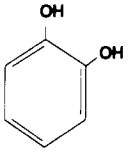
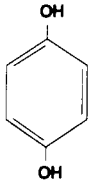
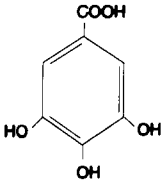
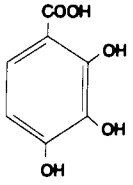
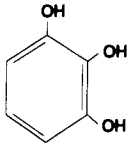
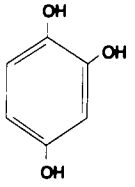
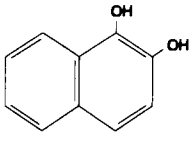
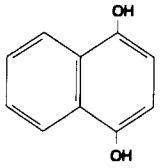
		λ (nm)	ε ($M^{-1}\,cm^{-1}$)	E_{m7} (mV)		
				Q/QH_2	$Q/Q^{\cdot-}$	$Q^{\cdot-}/QH_2$
catechol		320	7680	370	210	530
1,4-benzohydroquinone		248	15,410	280	99	459
gallic acid		450	3100	-	-	-
2,3,4-trihydroxybenzoate		405	2760	-	-	-
pyrogallol		420	3610	-	-	-
1,2,4-benzenetriol		490	2494	106	-	-
1,2-naphthohydroquinone		259	21,294	156	-89	401
1,4-naphthohydroquinone		256 266	22,411 12,646	36	-140	212

Table II. *Kinetic data in autoxidation of selected hydroquinones.* Oxygen consumption and quinone formation were measured separately at the initial hydroquinone concentrations indicated. Values represent maximal rates of oxygen consumption and quinone formation under the experimental conditions shown: catalase, 20 U/ml; superoxide dismutase (SOD), 5 U/ml; EDTA, 0.5 mM; desferrioxamine (DEF), 0.5 mM. Standard deviations from at least three determinations are shown for the control reactions. The relative standard deviations for the experimental conditions were similar to those of control reactions and were omitted for clarity.

	$[QH_2]_{initial}$ (μM)	Oxygen Consumption (μM/min)		Quinone Formation (μM/min)			
		control	catalase	control	SOD	EDTA+SOD	DEF+SOD
catechol	500	0.045 ± 0.006	0.025	0.016 ± 0.002	0.029	0.021	0.051
1,4-benzohydroquinone	250	0.131 ± 0.021	0.076	0.293 ± 0.018	1.179	1.079	0.977
gallic acid	250	0.478 ± 0.004	0.295	0.208 ± 0.018	0.160	0.144	0.116
2,3,4-trihydroxybenzoate	250	0.935 ± 0.070	0.474	0.511 ± 0.028	0.434	0.049	0.035
pyrogallol	250	4.26 ± 0.62	2.15	2.22 ± 0.20	0.873	0.090	0.077
1,2,4-benzenetriol	250	40.9 ± 2.3	17.7	32.4 ± 1.8	11.1	1.848	0.129
1,2-naphthohydroquinone	250	217.9 ± 13.7	181.3	-	-	-	-
	40	42.4 ± 2.7	-	46.6 ± 2.5	39.4	34.5	28.6
1,4-naphthohydroquinone	250	319.8 ± 9.6	201.7	-	-	-	-
	40	29.5 ± 1.2	-	29.4 ± 1.7	38.5	33.5	36.6

Table III. Stability constants (log Ka) reflecting affinity of the ligand (L) for metal (M) (data from reference 56).

		EDTA	DEF	catechol
Fe^{2+}	(ML/M,L)	14.3	7.2	7.9
	(ML_2/M,L^2)			13.5
Fe^{3+}	(ML/M,L)	25.3	30.6	20.0
	(ML_2/M,L^2)			34.7
	(ML_3/M,L^3)			43.8
Cu^{2+}	(ML/M,L)	18.7	14.2	13.6
	(ML_2/M,L^2)			24.9

LEGENDS TO FIGURES

Fig. 1. *Relative rates of oxygen consumption in hydroquinone autoxidations.* Samples were injected by gas-tight syringe into 3 ml air-saturated 50 mM phosphate buffer (pH 7.4, 25°C) and oxygen tension monitored polarographically. Initial concentrations of hydroquinone were 250 μM except for catechol (666 μM).

Fig. 2. a) *Kinetics of 1,2- and 1,4-naphthohydroquinone autoxidation.* Oxygen consumption was followed polarographically on addition of naphthohydroquinone (125 μM) to air-saturated 50 mM phosphate buffer, pH 7.4, 25°C.
b) *Rates of naphthalenediol autoxidation as a function of initial hydroquinone concentration.* Rates of oxygen consumption were determined at different initial hydroquinone concentrations. Conditions were as in figure 2a. Rates represent initial rate for 1,2-naphthohydroquinone and maximal rate after the lag for 1,4-naphthohydroquinone.

Fig. 3. *Effects of catalase on consumption of oxygen by autoxidizing 1,2,4-benzenetriol and 6-hydroxydopamine.* Oxygen consumption was monitored polarographically on addition of 100 μM hydroquinone. Reaction conditions were as in previous figures. Catalase (20 U/ml) was present either before addition of hydroquinone, or added on cessation of oxygen consumption.

Fig. 4. *Autoxidation of benzenediols.* Autoxidations of catechol (500 μM) or 1,4-benzohydroquinone (250 μM) in air-saturated phosphate buffer (pH 7.4, 25°C) were followed spectrophotometrically. Desferrioxamine (500 μM), EDTA (500 μM), albumin (5.6 mg/ml), or superoxide dismutase (10 U/ml) were added as the reaction progressed.

Fig. 5. *Autoxidation of trihydroxybenzoates.* Reaction conditions were as in figure 4. Autoxidations of hydroquinones (250 μM) in air-saturated buffer were followed spectrophotometrically. Desferrioxamine (500 μM), EDTA (500 μM), and superoxide dismutase (5 U/ml) were present either before addition of hydroquinone, or added as the

reaction progressed. Insets show autoxidations over a longer time course with additions of superoxide dismutase (5 U/ml) at later times.

Fig. 6. *Autoxidation of benzenetriols.* Reaction conditions were as in figure 4. Autoxidations of hydroquinones (250 μM) in air-saturated buffer were followed spectrophotometrically. Desferrioxamine and EDTA were at 500 μM. Superoxide dismutase was at 5U/ml for pyrogallol and 2 U/ml for 1,2,4-benzenetriol. Insets show autoxidations over a longer time course with additions of superoxide dismutase (5 U/ml) at later times.

Fig. 7. *Accelerating influence of desferrioxamine on iron-stimulated autoxidations of gallic acid and 1,2,4-benzenetriol.* Autoxidations were initiated as in previous figures and followed spectrophotometrically. $FeCl_3$ was present at 10 μM; desferrioxamine at 500 μM.

Fig. 8. *1,2,4-Benzenetriol autoxidation: loss of 490 nm absorbance of quinone oxidation product.* Autoxidation of 1,2,4-benzenetriol was followed spectrophotometrically over an extended time in the presence or absence of desferrioxamine (500 μM). Catalase (20 U/ml) and superoxide dismutase (10 U/ml) were added as the absorbance declined.

Fig. 9. *Naphthalenediol autoxidations.* Autoxidations were initiated by addition of naphthohydroquinone (20 μM) to air-saturated 50 mM phosphate buffer (pH 7.4, 25°C), and formation of quinone product was followed spectrophotometrically. Where indicated, superoxide dismutase was at 5 U/ml.

Fig. 10. *Autoxidation of a second aliquot of 1,4-naphthohydroquinone.* A second aliquot of 1,4-naphthohydroquinone (40 μM) was added to the cuvette after autoxidation of a first aliquot (40 μM) had reached completion. Autoxidation was followed in the presence and absence of superoxide dismutase (5 U/ml).

Fig. 11. *Effects of desferrioxamine plus superoxide dismutase on naphthalenediol autoxidations.* Initial naphthohydroquinone concentrations were 40 μM. Autoxidations were

monitored at wavelengths different from those in figure 9 to minimize contribution of hydroquinone to the initial absorbance. EDTA (100 μM) had no influence on these autoxidations or on the effects of superoxide dismutase (not shown). Desferrioxamine (100 μM) however acted synergistically with superoxide dismutase to inhibit 1,2-naphthohydroquinone autoxidation but not 1,4-naphthohydroquinone autoxidation.

Figure 1

Hydroquinone Autoxidations: Relative Rates of Oxygen Consumption

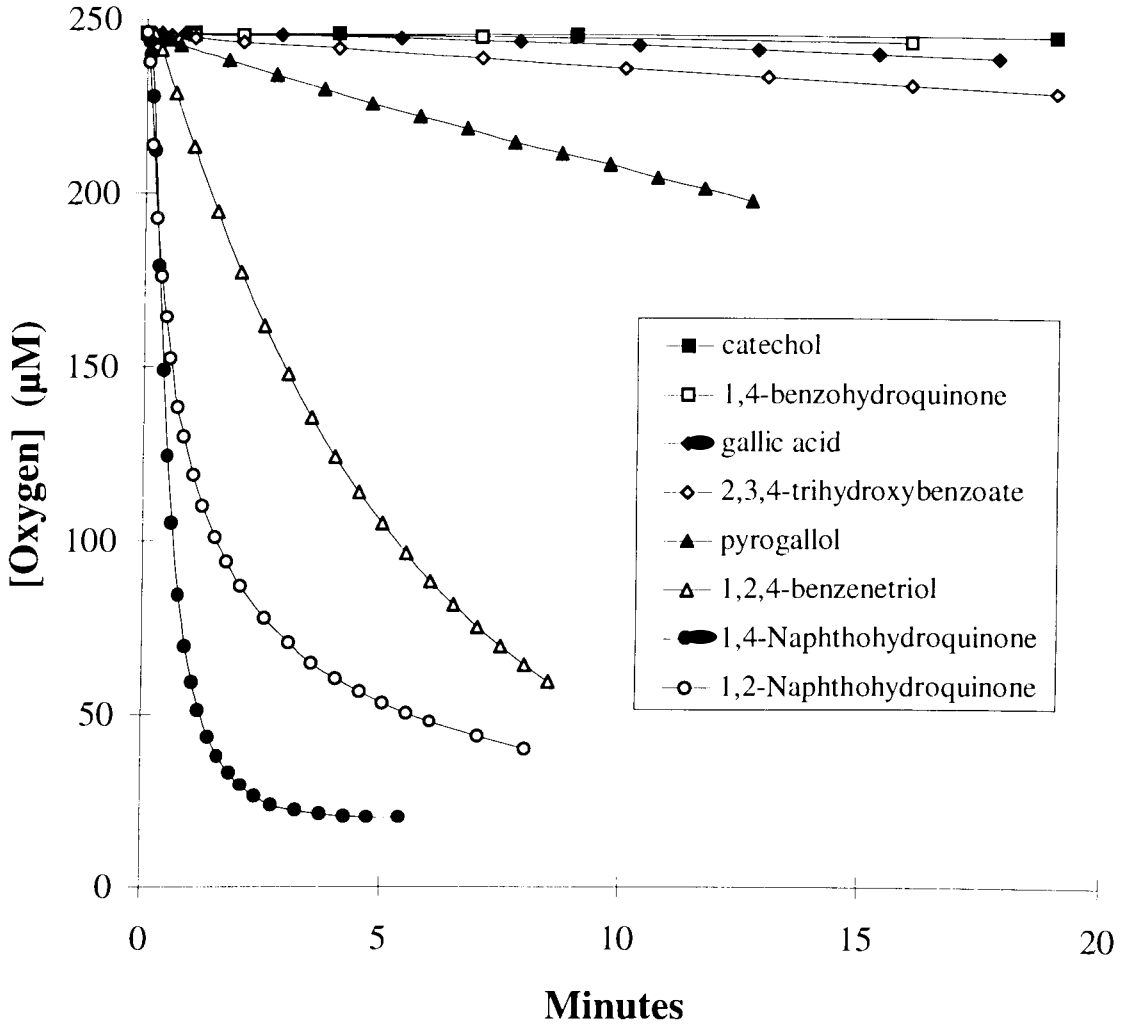


Figure 2

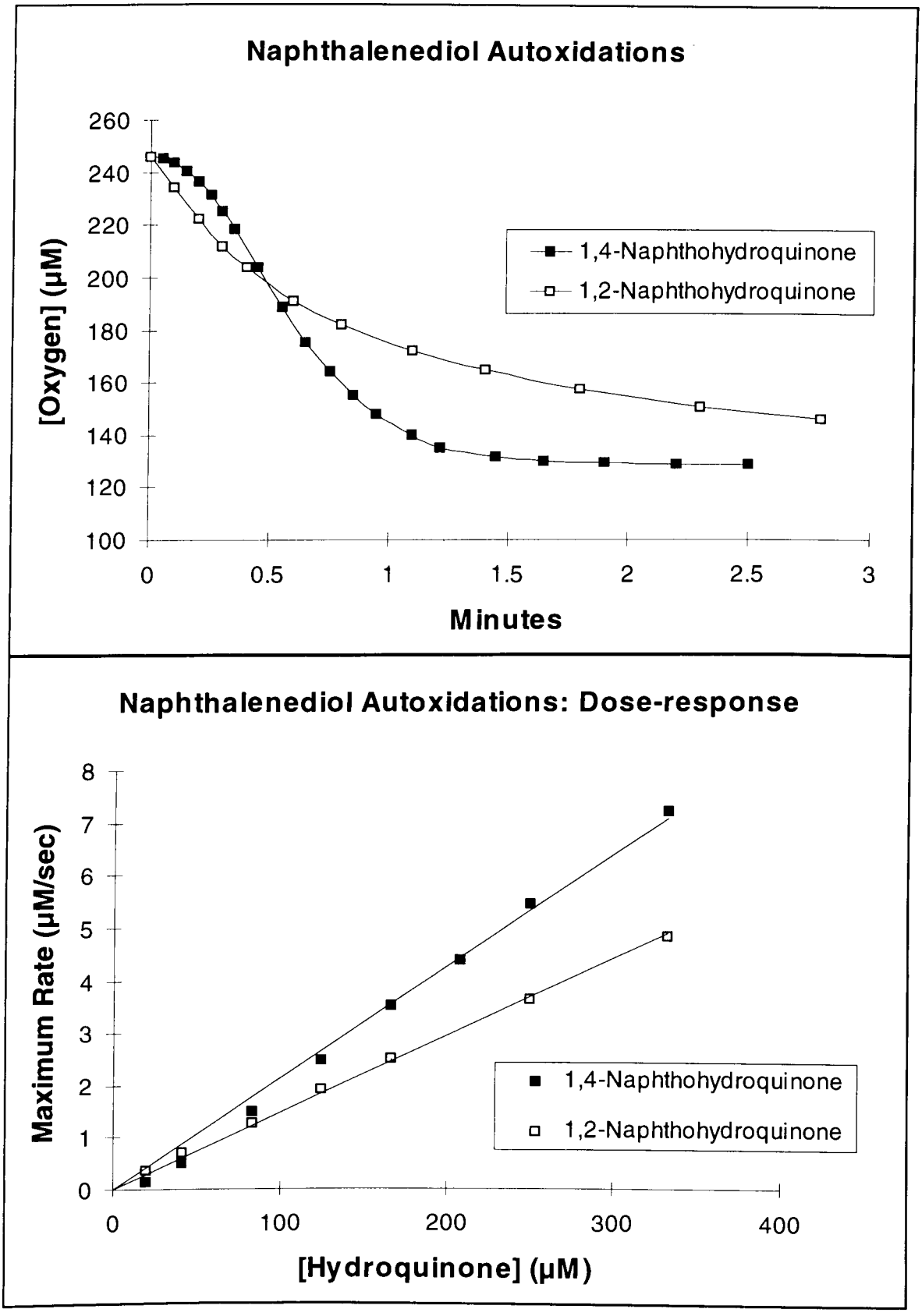

Naphthalenediol Autoxidations
[Oxygen] (μM)
260
240
220
200
180
160
140
120
100
0
0.5
1
1.5
2
2.5
3
Minutes
1,4-Naphthohydroquinone
1,2-Naphthohydroquinone
Naphthalenediol Autoxidations: Dose-response
Maximum Rate (μM/sec)
8
7
6
5
4
3
2
1
0
0
100
200
300
400
[Hydroquinone] (μM)
1,4-Naphthohydroquinone
1,2-Naphthohydroquinone

Figure 3

Oxygen Consumption by 1,2,4-Benzenetriol and 6-Hydroxydopamine: Effects of Catalase

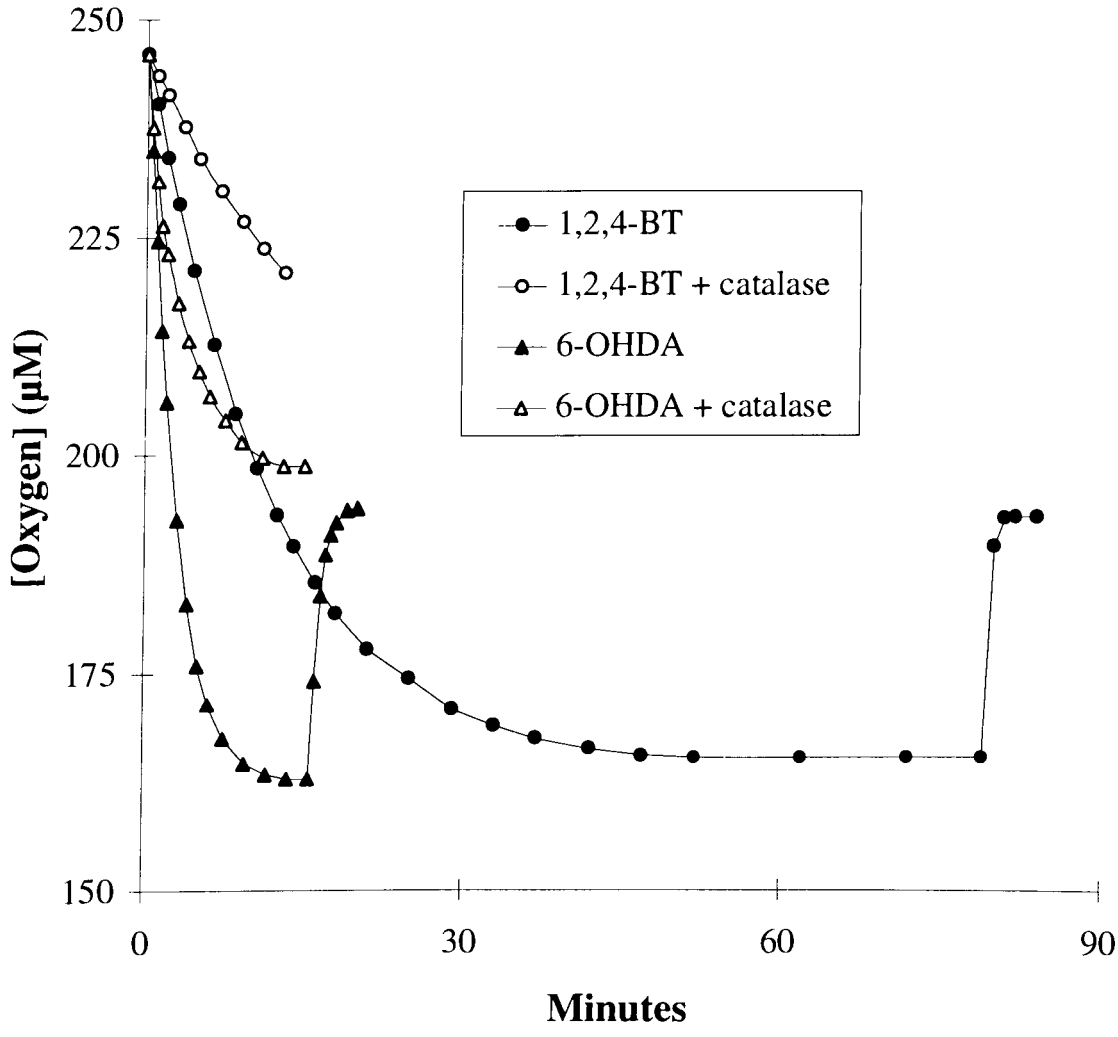


Figure 4

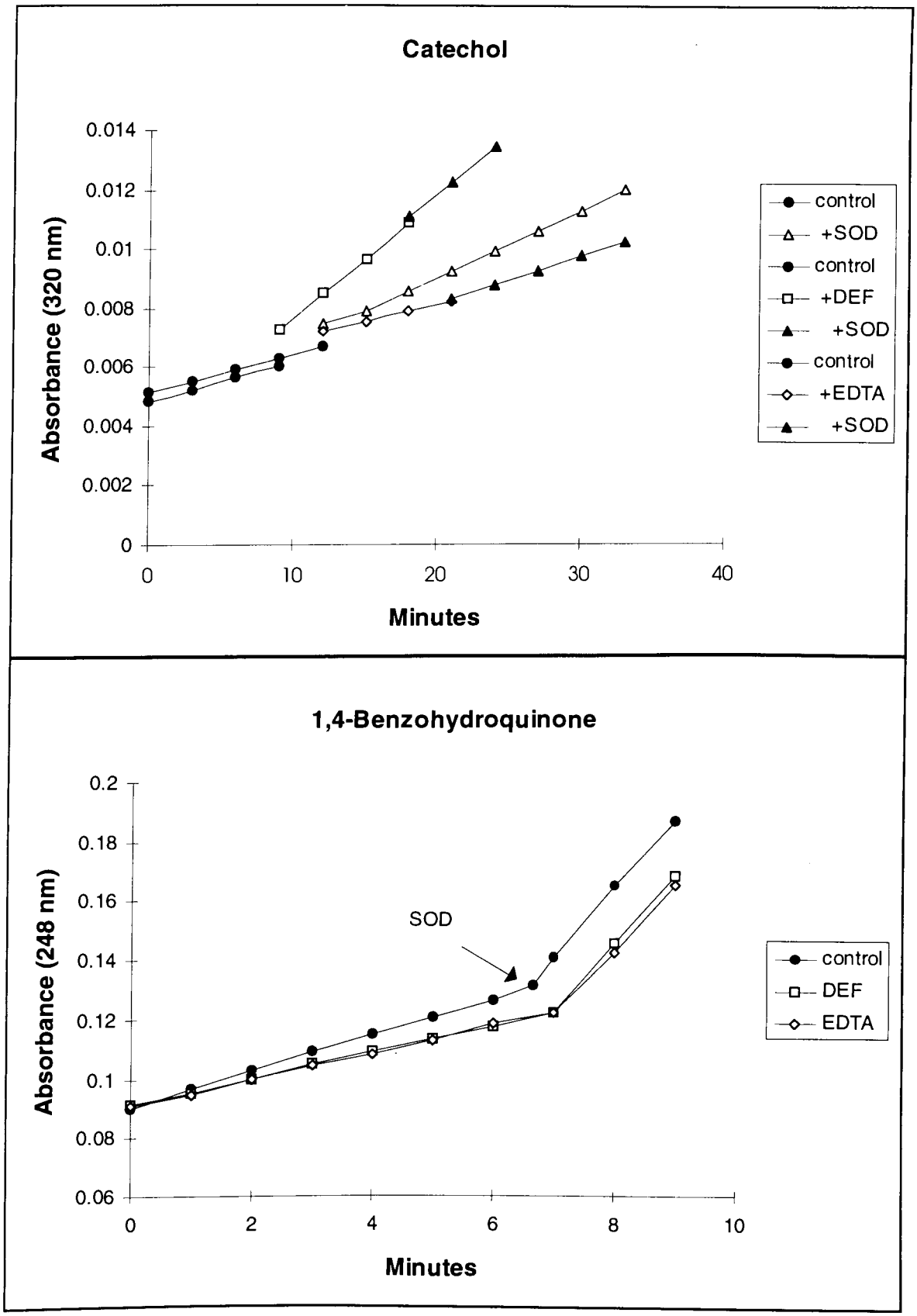

Catechol
Absorbance (320 nm)
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0
10
20
30
40
Minutes
control
+SOD
control
+DEF
+SOD
control
+EDTA
+SOD
1,4-Benzohydroquinone
Absorbance (248 nm)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
SOD
0
2
4
6
8
10
Minutes
control
DEF
EDTA

Figure 5

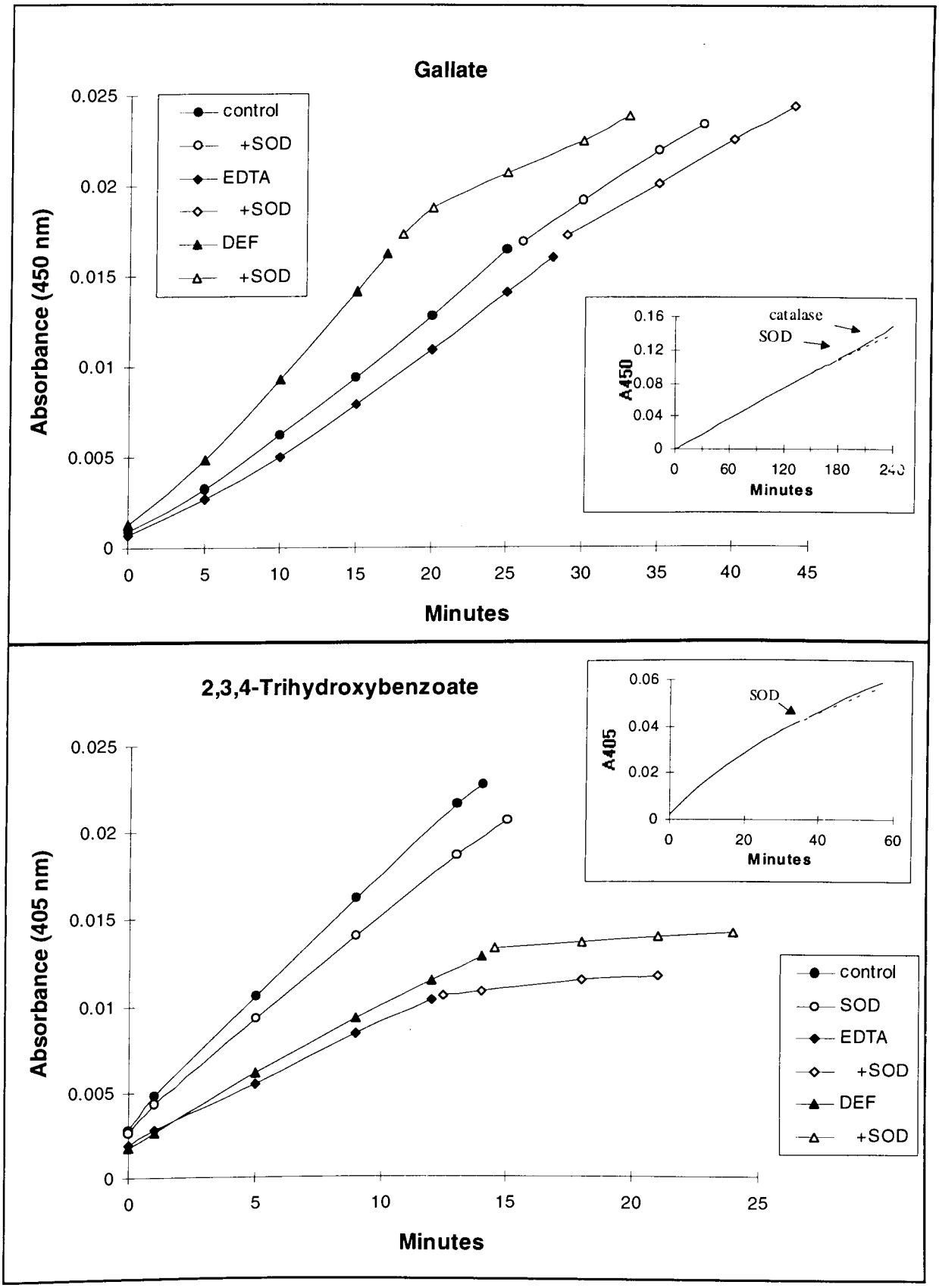

Gallate
Absorbance (450 nm)
0.025
0.02
0.015
0.01
0.005
0
0
5
10
15
20
25
30
35
40
45
Minutes
control
+SOD
EDTA
+SOD
DEF
+SOD
A450
0.16
0.12
0.08
0.04
0
0
60
120
180
Minutes
catalase
SOD
2,3,4-Trihydroxybenzoate
Absorbance (405 nm)
0.025
0.02
0.015
0.01
0.005
0
0
5
10
15
20
25
Minutes
A405
0.06
0.04
0.02
0
0
20
40
60
Minutes
SOD
control
SOD
EDTA
+SOD
DEF
+SOD

Figure 6

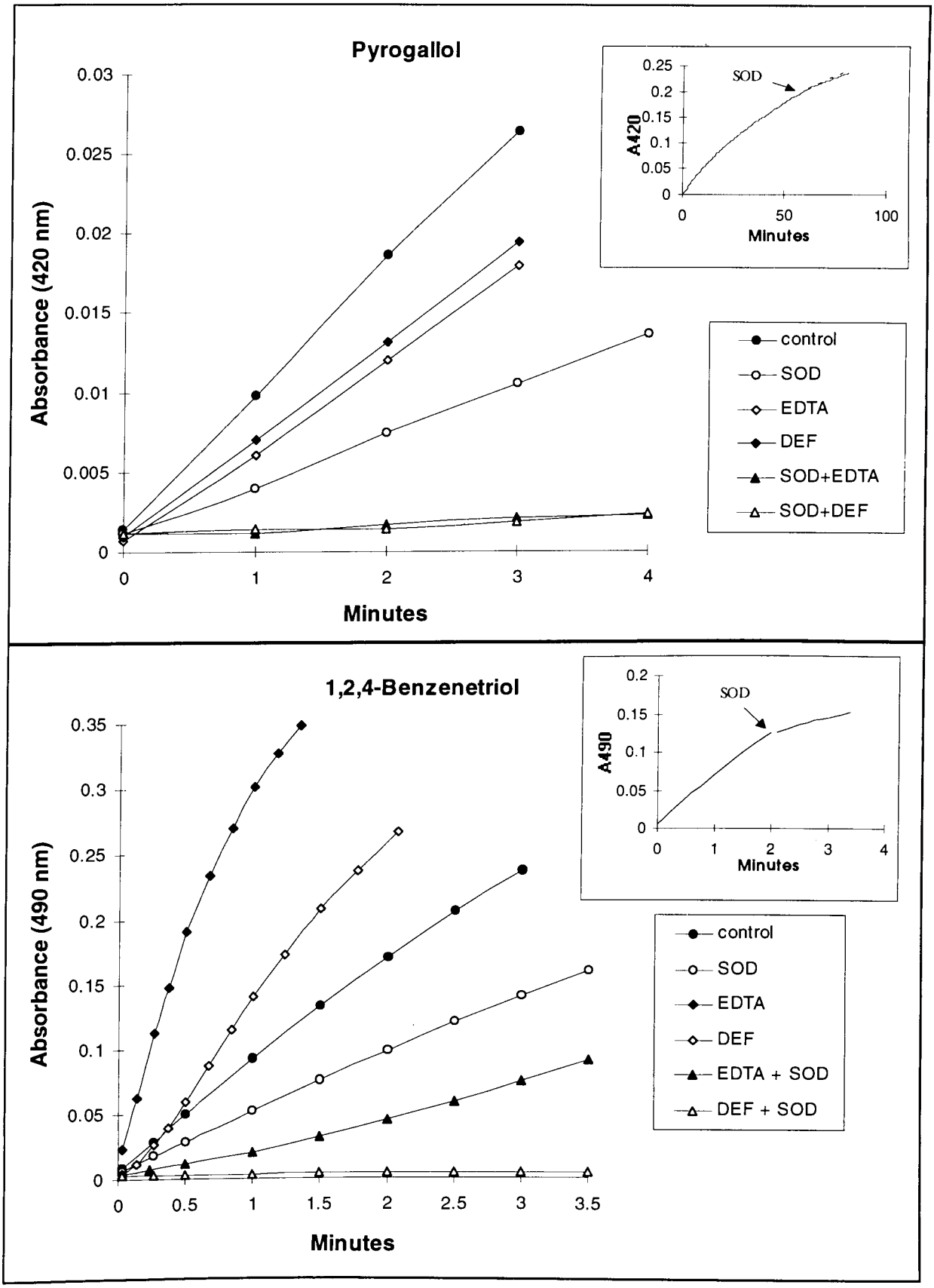

Pyrogallol
Absorbance (420 nm)
0.03
0.025
0.02
0.015
0.01
0.005
0
0
1
2
3
4
Minutes
control
SOD
EDTA
DEF
SOD+EDTA
SOD+DEF
SOD
A420
0.25
0.2
0.15
0.1
0.05
0
0
50
100
Minutes
1,2,4-Benzenetriol
Absorbance (490 nm)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.5
1
1.5
2
2.5
3
3.5
Minutes
control
SOD
EDTA
DEF
EDTA + SOD
DEF + SOD
SOD
A490
0.2
0.15
0.1
0.05
0
0
1
2
3
4
Minutes

Figure 7

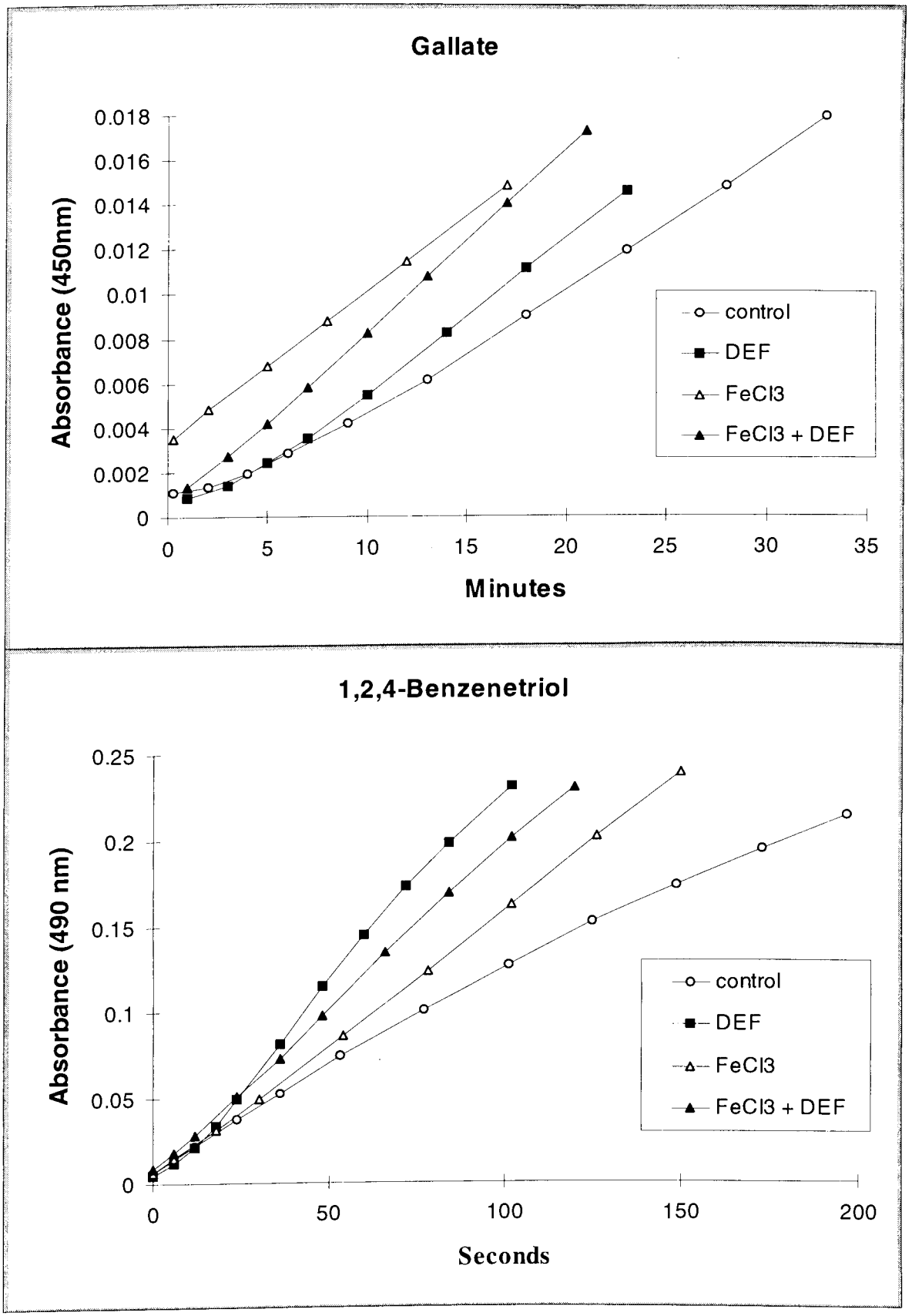

Gallate
Absorbance (450nm)
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0
5
10
15
20
25
30
35
Minutes
control
DEF
FeCl3
FeCl3 + DEF
1,2,4-Benzenetriol
Absorbance (490 nm)
0.25
0.2
0.15
0.1
0.05
0
0
50
100
150
200
Seconds
control
DEF
FeCl3
FeCl3 + DEF

Figure 8

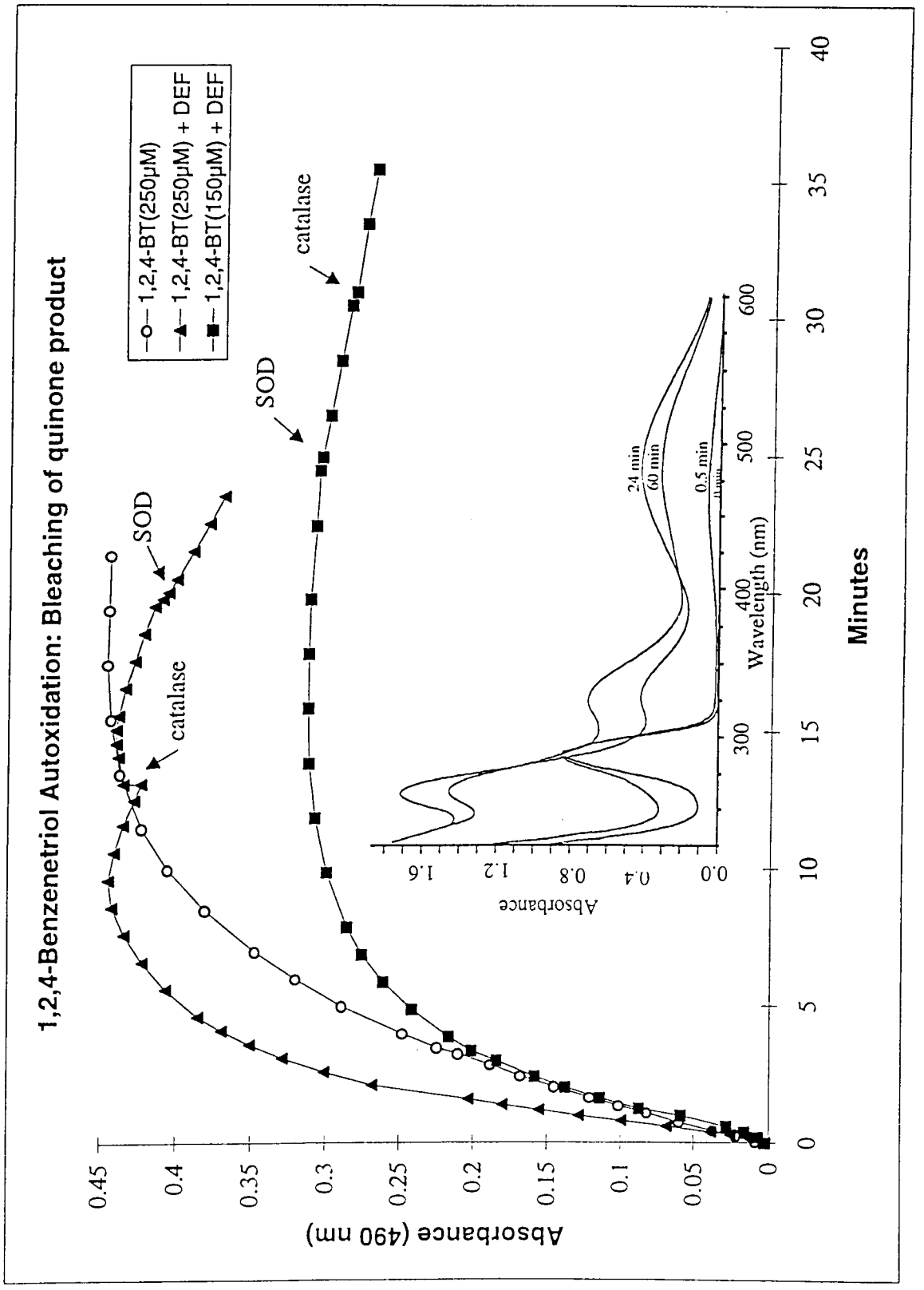

1,2,4-Benzenetriol Autoxidation: Bleaching of quinone product
1,2,4-BT(250μM)
1,2,4-BT(250μM) + DEF
1,2,4-BT(150μM) + DEF
SOD
catalase
SOD
catalase
Absorbance (490 nm)
Minutes
Absorbance
Wavelength (nm)
24 min
60 min
0.5 min

Figure 9

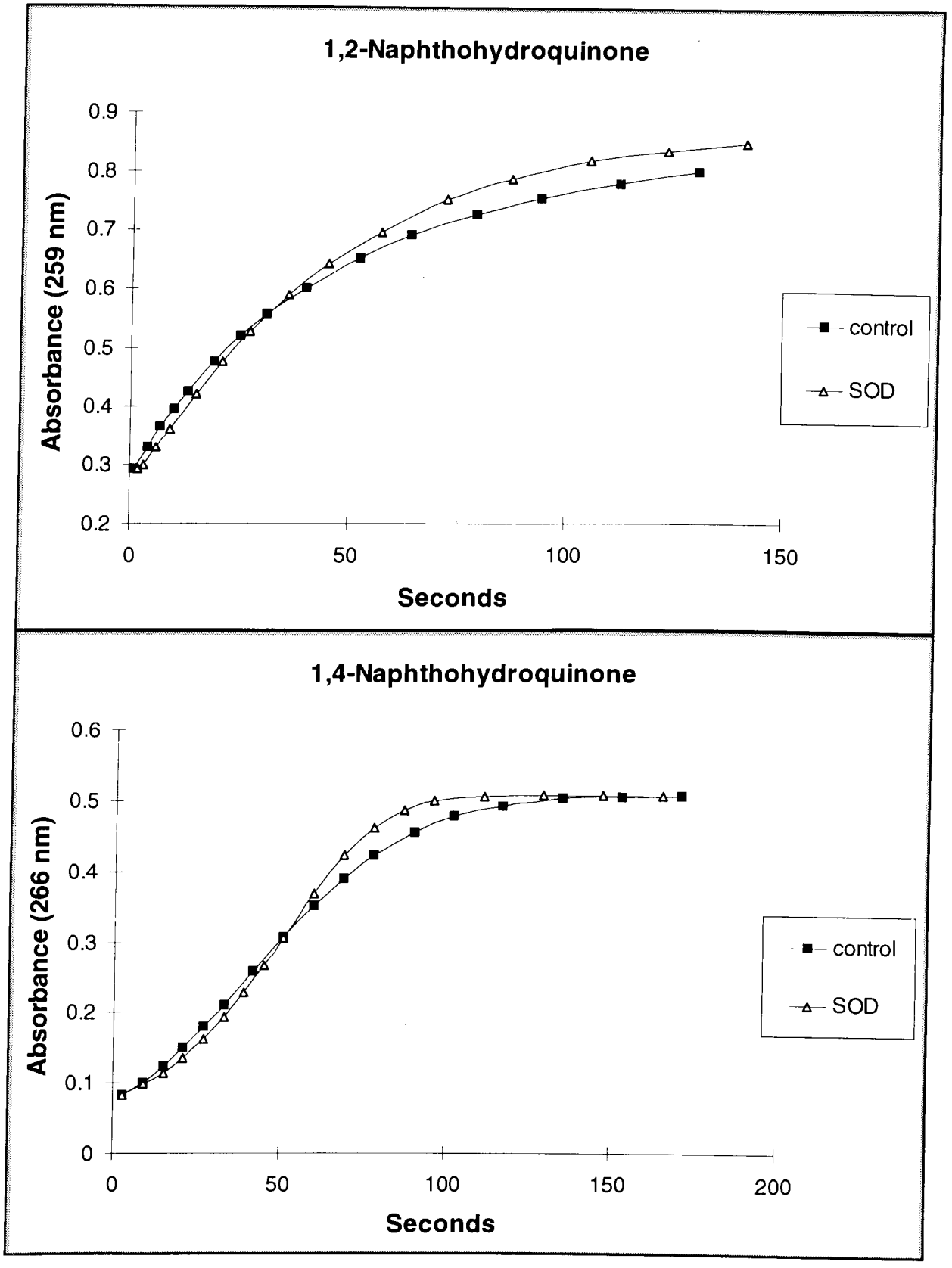

1,2-Naphthohydroquinone
Absorbance (259 nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
50
100
150
Seconds
control
SOD
1,4-Naphthohydroquinone
Absorbance (266 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
50
100
150
200
Seconds
control
SOD

Figure 10

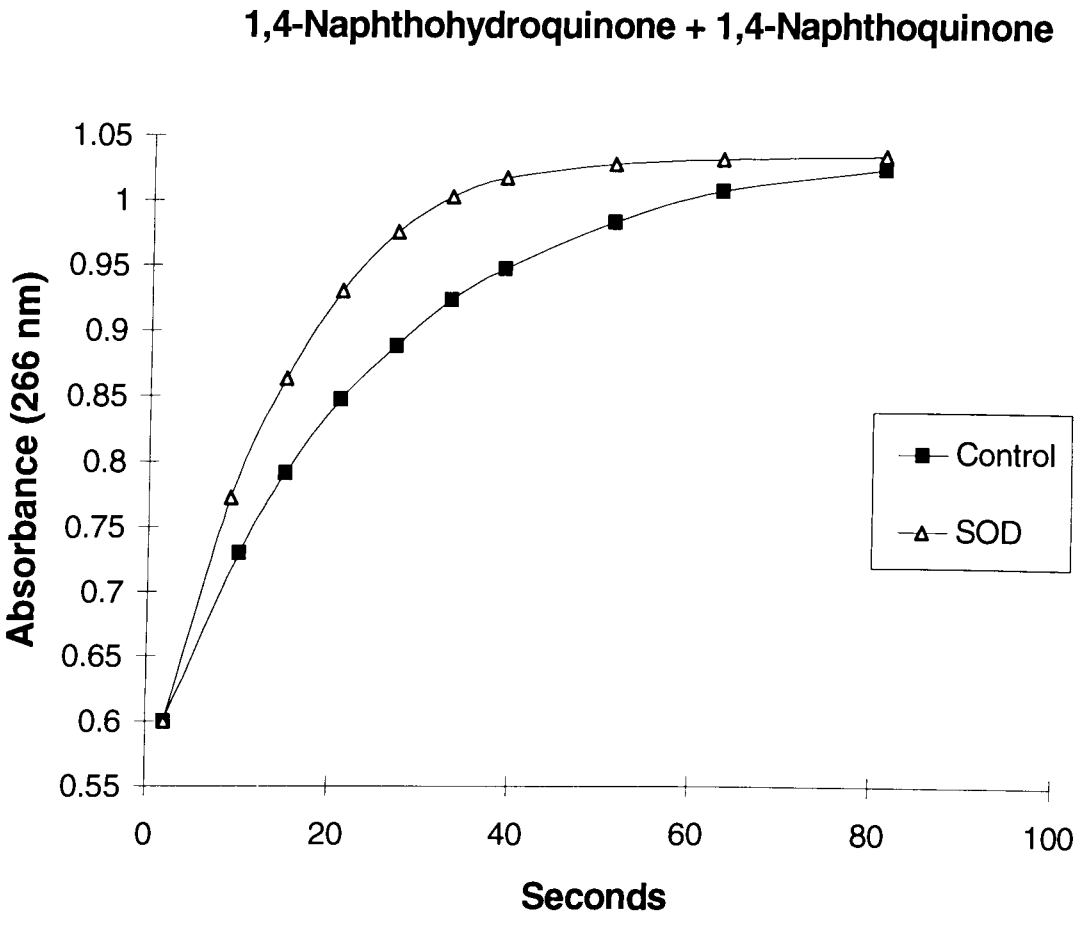
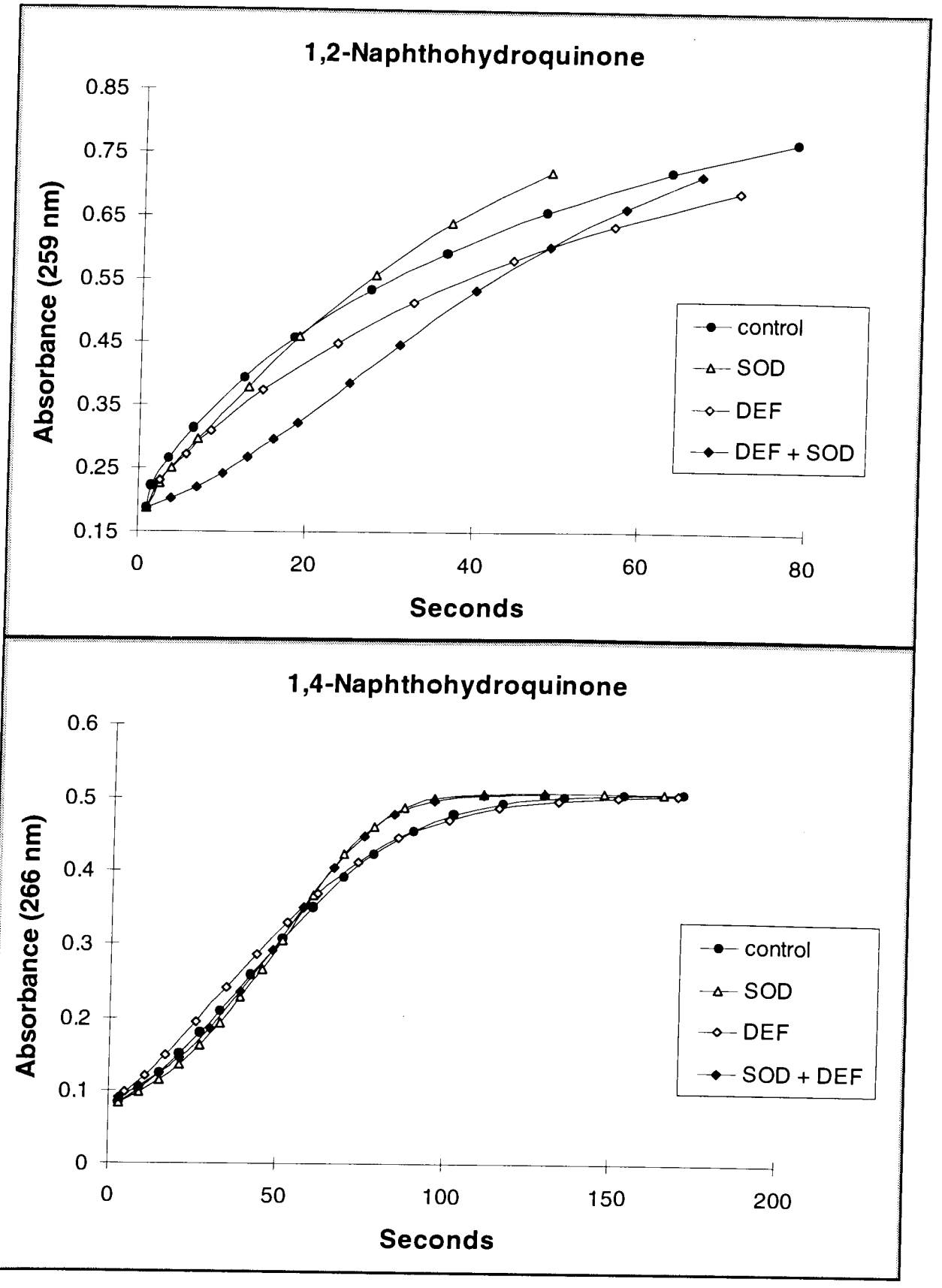

1,4-Naphthohydroquinone + 1,4-Naphthoquinone
Absorbance (266 nm)
1.05
1
0.95
0.9
0.85
0.8
0.75
0.7
0.65
0.6
0.55
0
20
40
60
80
100
Seconds
Control
SOD

Figure 11


1,2-Naphthohydroquinone
Absorbance (259 nm)
0.85
0.75
0.65
0.55
0.45
0.35
0.25
0.15
0
20
40
60
80
Seconds
control
SOD
DEF
DEF + SOD
1,4-Naphthohydroquinone
Absorbance (266 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
50
100
150
200
Seconds
control
SOD
DEF
SOD + DEF

Chapter 11.
General Conclusions and Perspectives

This thesis explored the mechanisms of oxygen reduction by hydroquinones from three main perspectives: a) the influence of mitochondrial respiratory impairments on the autoxidation of reduced quinone intermediates, b) the participation of metals, ligands, and active oxygen intermediates in the autoxidations of 6-hydroxydopamine and ascorbate, and c) the influences of thermodynamic, kinetic, and steric factors on the mechanisms of reaction of different hydroquinones with oxygen. These studies revealed some of the complexities and diversity of mechanisms of reaction with oxygen, revealed interactions between different antioxidants, and produced some potentially useful generalizations.

From the studies on submitochondrial particles, we conclude that hypoxia itself does not increase the autoxidative redox cycling of mitochondrial quinones, at least under the conditions studied. Conditions which loosen cytochrome *c* however, such as occur in hypoxia/reperfusion, can decrease functional respiration without decreasing autoxidative redox cycling of respiratory intermediates. On a theoretical basis, we surmise that certain mutations to mitochondrial electron transfer components which impair functional respiration may increase the steady-state level of autoxidizable intermediates, and/or destabilize reduced intermediates toward oxygen, and escalate oxidative stress to the cell.

In the autoxidation of 6-hydroxydopamine, we conclude that observations of strong inhibition by superoxide dismutase alone are complicated by metal-binding by the apoprotein, and that superoxide dismutase and certain metal chelators inhibit autoxidation synergistically. In the absence of metal chelators, reduction of oxygen occurs largely by sequential transfer of electrons within a ternary 6-OHDA:metal:oxygen complex. Ligands which prevent formation of a ternary complex force an outer-sphere $O_2{\cdot}^-$-propagated pathway for reduction of oxygen, and render oxidation inhibitable by superoxide dismutase. With different metal-ligand complexes, steric factors contribute to the effectiveness and mechanism of catalysis, but an optimal reduction potential for reduction and reoxidation is a primary determinant for redox cycling catalysis. We also conclude that cooperation between

metal-binding antioxidants, such as urate, and chain-breaking antioxidants such as ascorbate and superoxide dismutase, provides synergistic antioxidant activity to inhibit metal-catalysed oxidations. Natural selection may have optimized such synergisms to help protect against pro-oxidant cytotoxins.

From the autoxidations of selected benzenediols, benzenetriols, and naphthalenediols we conclude that the reduction of oxygen proceeds by an interplay of three main pathways: a) metal-catalysed inner sphere transfer of electrons, b) outer sphere, $O_2{\cdot}^-$-propagated oxidation, and c) comproportionation-propagated oxidation. The extent to which any pathway predominates depends on the properties of the hydroquinone (eg. reduction potentials, metal affinity, aromaticity) and the reaction conditions (eg. presence of metal ions, pH, presence of metal-binding or chain-breaking antioxidants). As a result, the influence of superoxide dismutase on hydroquinone autoxidation depends on the quinone reduction potentials, the affinity for and availability of metals, and the contribution of comproportionation.

Questions Arising

Predictably, the universe responded to our perturbations in ways that were not always easy to explain. The studies presented in this thesis answered several questions, but raised others which will require further experimentation to resolve.

A notable curiosity is the accelerating action of desferrioxamine on autoxidations of 1,2,4-benzenetriol, catechol, and gallate, compared to an inhibitory action on autoxidations of 6-hydroxydopamine, 2,3,4-trihydroxybenzoate, and pyrogallol. 1,2,4-Benzenetriol, for example, has a similar structure to 6-hydroxydopamine, and being a weaker reductant, would expectedly be *less* able to reduce ferrioxamine. Possible factors contributing to this anomaly include differences in pKa and/or affinity of the hydroquinone for metals, differences in metal-ion contamination of the reagent, or differential participation of desferrioxamine (or a nitroxide radical thereof) in the reaction.

Another question of interest is the difference observed between superoxide dismutase and heat-denatured superoxide dismutase in their maximal inhibition of ascorbate autoxidation. One might expect the inhibition to plateau at the same level, once all catalytic metals are bound to protein. A possible explanation is that denaturation exposes a higher affinity site which inhibits a metal from participating as a redox catalyst more strongly than those sites available on the native enzyme. Further experiments are needed to test this and other possible explanations.

A paradox of potential importance raised in these studies is that, despite being a stronger reductant, ascorbate reduces oxygen much more slowly than does 6-hydroxydopamine or 1,2,4-benzenetriol (see Table). One possible explanation is the much lower affinity of ascorbate for metals. Whatever the reason, this difference presumably has important physiological implications.

	$E_{m7}Q/QH_2$ (mV)	Oxygen Consumption (μM/min)
ascorbate	+54	3.5
6-hydroxydopamine	+92	99.4
1,2,4-benzenetriol	+106	40.9

Table: *Reduction potentials and rates of oxygen reduction for ascorbate, 6-hydroxydopamine, and 1,2,4-benzenetriol.* Rates of oxygen consumption were measured polarographically in air-saturated phosphate buffer, pH 7.4, 25°C, on addition of 0.25 mM reductant. Reduction potentials are midpoint potentials at pH 7.0 versus the normal hydrogen electrode as reported in the literature. The reduction potential for ascorbate can be found in Koppenol and Butler 1985 or Clark 1960. The reduction potential for 6-hydroxydopamine is interpolated from pH 6.87-8.3 values reported by Adams et al 1972, Blank et al 1976, Borchardt et al 1976, Borg et al 1978, Graham et al 1978, and Senoh and Witkop 1959. The reduction potential for 1,2,4-benzenetriol is from Clark 1960.

Implications and Future Directions

The evaluations of mitochondrial respiratory impairments on oxygen activation described in this thesis raise some important implications and directions for future research. In particular, it will be important to determine the extent to which either mitochondrial mutations, or loosening of cytochrome *c*, produce oxidative stress *in vivo*. This includes both experimental confirmation of increased oxygen activation with mitochondrial mutations, and analyses of the extent to which this increase is expressed in pathology. With aging for example, we see an increase in mitochondrial mutations and an increase in mitochondrial release of active oxygen, but no causal relationship has been established. Similarly, with myopathies due to mitochondrial mutations, we see evidence of oxidative stress in *some* cases, but no causal relationship has been apparent, or as yet explored. In reperfusion injury, there is evidence that mitochondria are one source of oxidative damage, but the mechanisms behind, and extent of this contribution provide avenues for further investigation.

The synergisms between metal-binding and chain-breaking antioxidants revealed in this thesis also bear some potentially meaningful implications. First, they imply that observations of a response, or lack of response, from one antioxidant alone may give an incomplete picture. In situations showing a benefit from superoxide dismutase, such as in reperfusion injury or organ transplantations, care must be taken to control for synergistic binding of metals by the protein. Researchers or clinicians finding a lack of benefit from desferrioxamine need to be aware of potential pro-oxidant actions of desferrioxamine, and of the need for cooperation with a chain-breaking antioxidant to fully realize its antioxidant potential. An appropriate combination of metal-binding and chain-breaking antioxidants may give the most benefit. The current studies reveal that urate may be a "natural" alternative to other metal-binding antioxidants, although with other potential drawbacks (eg. gout).

Interactions between different antioxidants are becoming increasingly recognized (eg. the vitamin E / vitamin C cycle). From a biological perspective, it is apparent that antioxidants have been selected to act in concert, with each one supporting and sparing the other. The

current studies add interactions between urate, ascorbate, and superoxide dismutase to the web of synergistic interactions.

The current studies helped clarify contrasting actions of superoxide dismutase. Experiments on different hydroquinone autoxidations revealed an interplay between thermodynamic and kinetic explanations for stimulatory and inhibitory actions of superoxide dismutase. A set of generalized theorems are offered which may help explain and predict responses to superoxide dismutase in different situations. Experiments on other autoxidations (eg. anthraquinones, other naphtho- or benzo- quinones), and in more biological systems are needed to determine the extent to which these principles apply generally, and to unveil other factors which may influence the actions of superoxide dismutase.

Researchers have previously concluded that one protective action of superoxide dismutase is to remove semiquinones, by accelerating their reaction with oxygen. The current results imply that, although superoxide dismutase accelerates reduction of oxygen by some hydroquinones, and thus may temporarily increase oxidative stress, the disposition of these quinones is such that superoxide dismutase may assist redox pathways for conjugation and excretion. The influences of superoxide dismutase on detoxification and excretion of cytotoxic quinones could be an interesting direction for future studies.

The studies on autoxidation of different hydroquinones revealed structure-activity relationships and reaction conditions which influence the mechanisms of reaction with oxygen. These studies represent an initial systematic exploration of some of the factors involved, and need to be expanded to additional quinones and other reaction conditions (eg. lipid environment). Such information is potentially useful in both biomedical and industrial applications. Retrospectively, this knowledge may help us better understand the mechanisms of action, or side effects, of quinone drugs or environmental toxins. Prospectively, it is hoped that these, and future studies, provide fundamental knowledge useful in predicting the antioxidant or pro-oxidant activity of a particular quinone, in designing new quinone drugs, and in optimizing therapeutic treatments for exposure to cytotoxic quinone or aromatic hydrocarbon xenobiotics.

References

Adams, R.N.; Murrill, E.; McCreery, R.; Blank, L.; Karolczak, M. 6-Hydroxydopamine, a new oxidation mechanism. *Eur. J. Pharmacol.* **17**:287-292; 1972

Blank, C.L.; McCreery, R.L.; Wightman, R.M.; Chey, W.; Adams, R.N.; Reid, J.R.; Smissman, E.E. Intracyclization rates of 6-hydroxydopamine and 6-aminodopamine analogs under physiological conditions. *J. Medicinal Chem.* **19**:178-180; 1976

Borchardt, R.T.; Reid, J.R.; Thakker, D.R. Catechol-O-methyltransferase. 9. Mechanism of inactivation by 6-hydroxydopamine. *J. Medicinal Chem.* **19**:1201-1209; 1976

Borg, D.C.; Schaich, K.M.; Ellmore, J.J.Jr.; Bell, J.A. Cytotoxic reactions of free radical species of oxygen. *Photochem. Photobiol.* **28**:887-907; 1978.

Clark, W.M. *Oxidation-Reduction Potentials of Organic Systems.* The Williams and Wilkins Company, Baltimore; 1960.

Graham, D.G.; Tiffany, S.M.; Bell, W.R.Jr.; Gutknecht, W.F. Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells in vitro. *Molec. Pharmacol.* **14**:644-653; 1978.

Koppenol, W.H.; Butler, J. Energetics of interconversion reactions of oxyradicals. *Adv. Free Rad. Biol. Med.* **1**:91-131; 1985.

Senoh, S.; Witkop, B. Formation and rearrangements of aminochromes from a new metabolite of dopamine and some of its derivatives. J. Am. Chem. Soc. 81:6231-6235; 1959