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MECHANISMS OF CYTOTOXICITY OF 6-HYDROXYDOPAMINE: ROLES OF OXYGEN FREE RADICALS AND THE EFFECTS OF SCAVENGERS

by

Pauline Gee

B.Sc., Simon Fraser University, 1977

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Mechanisms of Cytotoxicity: Roles of Oxygen Free Radicals and the Effects of Scavengers

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ABSTRACT

The neurotoxicity of 6-hydroxydopamine (6-OHDA) can be attributed to free radicals resulting from its oxidation by O_2, while the specificity of its target is determined by its affinity for the catecholamine uptake systems. Studies of the reaction pathways of the 6-OHDA/O_2 redox couple were undertaken to further our understanding of the mechanisms involved in cellular damage caused by free radicals. Because of the electronic configuration of O_2, single electron transfers to it inevitably lead to the formation of free radical species which can attack cellular components, rendering them irreversibly non-functional. We report that despite favourable thermodynamic considerations, O_2 does not oxidise 6-OHDA directly but requires a co-oxidant. Instead, the initiation reaction involves a ternary reductant-metal-oxygen transition state. When the major oxidising species (the superoxide-metal complex) is removed, the autoxidation can be resolved into initiation and propagation phases. These represent respectively, the time required for the accumulation of secondary catalytic species; and a steady state in which the free radical chain is propagated. To identify and separate the catalytic roles of various co-oxidants or co-reductants in each phase, we determined the effects of combinations of the following scavengers: superoxide dismutase (SOD), diethylenetriaminepentaacetate (DTPA), desferrioxamine, catalase, formate, mannitol, benzoate and glucose. Oxidation of 6-OHDA by H_2O_2 in the absence of air was three orders of magnitude slower. This latter peroxidatic oxidation had an absolute requirement for metal ions so that anaerobic oxidation by H_2O_2 was completely prevented by the simultaneous presence of DTPA and desferrioxamine. In the aerobic

oxidation, SOD and DTPA each provided 96% and 66% inhibition of maximal rates respectively, while the simultaneous presence of DTPA+catalase+SOD inhibited virtually completely. No other combinations of the scavengers were completely effective in preventing the autoxidation. Thus the accumulation of any of several intermediate species allowed molecular oxygen to oxidise 6-OHDA. The following species were capable of playing this catalytic role: O_2^- > Me^{n+} > H_2O_2 > $^{\cdot}MH$ = CO_2^- in order of effectiveness. Neither molecular oxygen itself nor $^{\cdot}OH$ was however effective. The hydroxyl scavengers studied, including glucose, could reactivate the DTPA+catalase+SOD inhibited reaction. It is proposed that the products of hydroxyl scavengers react with metal ion chelates to reinitiate the free radical chain. Thus seemingly innocuous and beneficial cellular substances become part of and propagate the free radical chain and therefore can contribute to the cytotoxicity of 6-OHDA. On the other hand, cytochrome c, a more complex cellular component than glucose, undergoes reversible redox changes during oxidation of 6-OHDA by O_2 without losing its functional integrity.

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PREFACE

The thesis comprises a general introduction outlining the context and goals of the study, followed by four manuscripts containing most of the substance of the thesis, with a final concluding statement. Only studies which were particularly salient to the mechanisms of the oxidation reaction of 6-OHDA and its generation of free radicals are summarised in the introductory statement and it does not reflect the volume of literature concerned with in vivo and behavioural effects related to 6-OHDA toxicity. At the time of writing one of the manuscripts was at the proof stage (Section III), another had been accepted for publication (Section V), while the remaining two (Sections II and IV) have been submitted for publication. Although a detailed description of experimental procedures typical of the studies may be found in the first manuscript (Section II), we have restricted repetition of introductory and experimental material to the minimum necessary for each separately published study to be understood and evaluated autonomously.

In the first manuscript (Section II), we tested all possible combinations of the scavengers studied to separate out participation of oxidising and reducing species, individually and in combination. The following species were found to contribute to the overall rate in decreasing order of effectiveness: O_2^- > Me^{n+} > H_2O_2 > CO_2^- = $^{\bullet}MH$ = glucose radical = benzoate radical. Many of the effects observed under the above (aerobic) conditions concerned the initial step of the

autoxidation and the behaviour of the resultant autocatalytic species, O_2^- or its metal-ion complex.

In the second manuscript (Section III), we describe a combination of scavengers which completely blocked the thermodynamically favourable autoxidation of 6-OHDA by O_2. Furthermore we found that the hydroxyl scavengers, formate, benzoate glucose and mannitol were effective equally in reactivating the DTPA+catalase+SOD (DTPA: diethylenetriaminetetraacetate; SOD: superoxide dismutase) inhibited reaction. These observations indicate that harmless cellular components can interact with free radical species to completely abolish free radical production in the presence of strongly cytotoxic combinations of free radical generators on the one hand, or to increase the toxicity of the free radical generating system on the other.

The third manuscript (Section IV) arises from observations that most free radical damage to living cells has been shown to be due to hydroxyl radicals, and the major source of these is the reduction of hydrogen peroxide in the so called Fenton reaction. (The better known Haber-Weiss reaction is a special case of the Fenton reaction in which superoxide is the reducing agent). Such damage was inhibited to a variable extent by scavengers of superoxide, hydrogen peroxide, or hydroxyl radicals. Surprisingly we found that hydroxyl scavengers could also reactivate free radical production in the presence of combinations of scavengers (e.g. DTPA+catalase+SOD) which had completely inhibited free radical production. We therefore examined

the reduction of H_2O_2 by 6-OHDA in the absence, and in the presence of various concentrations of O_2. As expected, Fenton-type interactions dominate the system anaerobically, and the $^{\bullet}OH$ contributes substantially to the formation of p-quinone product. Whereas metal ions play a major role in the aerobic reaction, they are an absolute requirement for the anaerobic oxidation by H_2O_2.

Thus in the final study, (Section V) we consider interactions of the 6-OHDA/O_2 redox system with an important intracellular redox enzyme, cytochrome c, adapted by evolution to accept electrons from and donate to other redox enzymes. It also undergoes redox reactions with superoxide, hydrogen peroxide, and hydroxyl radicals. We describe a series of fluctuations in the steady state of cytochrome c, alternating between oxidised and reduced as a result of changes in the pseudo-steady state concentrations of 6-OHDA, O_2^-, and H_2O_2.

If there is a unifying theme in this compendium, it is that not only free radical damage, but also free radical yield are influenced by synergistic and moderating interactions. These occur among a complex matrix of competing reactions between the free radical species which propagate in the steady state. The actions of free radical scavengers cannot be interpreted simply in terms of the removal of their target species, but in terms of the consequent modification of the rates of formation of a variety of radicals. In some cases these include the target itself, while in others, species apparently only distantly related to those scavenged.

GENERAL INTRODUCTION

Interest in 6-hydroxydopamine (6-OHDA:2,4,5-trihydroxyphenylethylamine) arose from chemical studies of melanin, a naturally occuring pigment found in most organisms. 6-OHDA was one of the synthetic intermediates found to produce a high yield of 5,6-dihydroxyindole when oxidised by ferricyanide [1]. 5,6-Dihydroxyindole is a common key intermediate in the biological synthesis of melanin from several catechols including dopamine and noradrenaline.

For the past 15 years neurobiologists have used 6-OHDA as a tool in "chemical surgery" to destroy selectively catecholaminergic neurones. Unlike cholinergic transmission, where acetylcholine is hydrolysed by acetylcholinesterase to terminate stimulation, the catecholamines are removed from synapses primarly by reuptake systems situated on the neurones which release them. Since 6-OHDA is a structural analogue of endogenous catecholamines, it is transported and concentrated into catecholaminergic neurons, specifically destroying them while leaving others morphologically and functionally intact. Thus it became a useful tool in studying the physiological role of the sympathetic nervous system, in mapping neuronal tracts in the brain, as well as in unravelling the complex mechanisms of pro-oxidant neurotoxins.

CELLULAR DAMAGE DUE TO AUTOXIDATION OF 6-OHDA

Whereas its neurotoxic specificity is determined by its affinity for the

catecholamine uptake systems, the toxicity of 6-OHDA is directly related to its ease of oxidation. It is generally agreed that 6-OHDA exerts its cytotoxic effects through the generation of free radicals of both oxygen and its quinone derivatives but there are several schools of thought as to the mechanism of damage.

One theory states that the primary avenue of damage is the covalent cross-linking of important macromolecules. Since the quinone products of the autoxidation of 6-OHDA are electrophilic, substances with acessible nucleophilic groups such as sulfhydryl groups are likely targets [2]. A 2-adduct of glutathione covalently bound to the p-quinone of 6-OHDA [3] has been isolated, although less than 0.2% of the bound 6-OHDA was found attached to glutathione and only 8-20% of labelled 6-OHDA taken up by catecholaminergic cells was covalently bound [4,5].

However, others have proposed that hydrogen peroxide, an intermediate of the reduction of molecular oxygen, is the primary species mediating damage caused by free radical species of oxygen generated in the autoxidation of 6-OHDA [6]. In suppport of this hypothesis, catalase protected cultures of neuroblastoma cells while scavengers specific for other intermediates of oxygen reduction were not as effective [7]. Much of the damage by free radicals is nonspecific (such as peroxidation of the lipid membrane of the axon [8]) and is similiar to that induced by other ene-diol reductants such as ascorbate [9]. The relative contributions of these two overall mechanisms to damage remain unclear,

although both most certainly are involved.

ONE ELECTRON TRANSFERS TO OXYGEN

Some of the most interesting features of the autoxidation of 6-OHDA lie in the paradox surrounding molecular oxygen. The benefits of aerobic existence are enormous in terms of energy production when compared to anaerobic existence; however oxygen is also a potential threat to all living systems. Molecular oxygen in its triplet state is remarkably stable, because it has two unpaired electrons of parallel spin. This precludes a direct divalent reduction since insertion of a pair of electrons would result in two electrons sharing the the same orbital with parallel spins. To overcome this spin restriction, oxygen can be bound to a transition metal ion which has its own unpaired electronic spin and it can then undergo a 2 electron reduction to hydrogen peroxide. To eliminate the spin restriction one of the electrons can be raised to a more energetic orbital and its spin inverted, but this involves a very reactive singlet state.

The alternative, a univalent reduction, inevitably leaves one electron unpaired, and thus single electron transfers result in potentially cytotoxic free radical intermediates which are also capable of interacting with other related free radicals in a variety of "incestuous" reactions as follows. The product of the first univalent reduction, the superoxide anion, is fairly unreactive in aqueous environments, however (particularly in the presence of traces of

transition metal ions) it undergoes dismutation to yield hydrogen peroxide and perhaps singlet state oxygen [10], while enzymic dismutation gives hydrogen peroxide and triplet state oxygen (ground state). The superoxide anion also reacts with hydrogen peroxide in the presence of metal ions to produce the hydroxyl radical, whose reactivity is so great that it produces widespread and indiscriminant oxidation of biological molecules. Appendix 1 contains a collation of these elementary reactions of which combinations may or may not occur in reaction pathways depending on environmental conditions (for a comprehensive review, see [11]).

ONE ELECTRON TRANSFERS FROM 6-OHDA

Some quinone intermediates resulting from the autoxidation of 6-OHDA (Appendix 2) have been demonstrated. A few seconds of exposure to air at pH 12-13 produced a quinone species identified by ESR spectrum as a the semiquinone anion [12], found also during anaerobic oxidation by ferric ions [13]. The p-quinone (pKa_1 = 4.5-5.0; pKa_2 = 9.0) exists in equilibrium with its zwitterion at neutral pH's [12,14]. Whereas the two electron oxidation product of the endogenous catecholamine, dopamine is the open chain o-quinone, in the case of 6-OHDA the p-quinone is favoured thermodynamically due to resonance stablisation of its structure. In situ, 6-OHDA exists in redox equilibrium with its p-quinone and was estimated to maintain its integrity for the high affinity uptake pumps of adrenergic neurons for 1.9 h [14]. Endogenous catecholamines undergo 1,4-addition of the Michael type (via a

leucochrome intermediate) to form the adrenochrome with an overall loss of 4 electrons [15]. In contrast, the p-quinone of 6-OHDA is relatively stable and only slowly undergoes 1,2 side chain closure to the aminochrome with elimination of water. The half time of this intracyclisation was estimated to be 38 min [16] and the aminochrome is thought to rearrange to the 5,6-dihydroxyindole or become reduced to 5,6-dihydroxyindoline [17].

Clearly, when a very strong reducing agent (such as 6-OHDA) donates electrons in a univalent pathway to the universal biological electron sink, molecular oxygen, the yield of free radical species is substantial. Any combination of possible radical interactions (Appendix 3) can react with many different cellular constituents causing indiscriminant damage.

SCAVENGERS OF OXYGEN FREE RADICALS

To untangle the complex interactions resulting from the autoxidation of 6-OHDA, scavengers specific for each free radical species of oxygen were used to study the effects of each on the rates of p-quinone formation and oxygen consumption. Superoxide dismutase (SOD) was used to remove O_2^- in the following reaction:

$$2\ O_2^- + 2\ H^+ \longrightarrow H_2O_2;\ (1)$$

In turn, catalase removes H_2O_2 produced either from SOD activity or directly from the autoxidation of 6-OHDA by the following reaction:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2 \cdot \quad (2)$$

Since there are no known enzymes to remove specifically the hydroxyl radical, various scavengers were used. Formate reacts with hydroxyl radicals ($k_2 = 2.7 \times 10^9\ M^{-1}\ s^{-1}$, [18]) to form a carboxylate radical which, in the presence of excess oxygen, produces the superoxide anion in the following sequence of reactions [19]:

$$HCO_2^- + {}^\cdot OH \longrightarrow H_2O + CO_2^{-\cdot} \quad (3)$$

$$CO_2^{-\cdot} + O_2 \longrightarrow CO_2 + O_2^- \quad (4)$$

Other scavengers such as mannitol and benzoate were used when production of O_2^- was not desirable. Glucose, although not traditionally used, has been shown to have hydroxyl scavenging effects [20].

While diethylenetriaminepentaacetate (DTPA), a general chelating agent, was used to eliminate most catalysis involving reduction of transition metal ions [21], the already-reduced DTPA-Fe^{2+} chelate remains active in catalysing Fenton-type reactions [22]:

$$\text{DTPA-}Fe^{2+} + H_2O_2 \longrightarrow {}^\cdot OH + OH^- + \text{DTPA-}Fe^{3+} \quad (5)$$

When these interactions were not desirable, desferrioxamine was added. This chelating agent, specific for iron, is not known to allow bound iron to participate in redox reactions. A drawback to its use however is that at concentrations above 500 µM it can scavenge hydroxyl radicals [23].

INTERMEDIATES OF UNIVALENT REDUCTION OF OXYGEN BY 6-OHDA

I. The Intermediate: H_2O_2

The production of H_2O_2 by 6-OHDA and molecular oxygen was demonstrated by the regeneration of oxygen from disproportionation of H_2O_2 product upon addition of catalase at a given time in the reaction sequence and the following reaction was proposed [6]:

$$\text{6-OHDA} + O_2 \longrightarrow \underline{p}\text{-quinone} + H_2O_2 \cdot \quad (6)$$

However, closer scrutiny revealed that the yield of H_2O_2 was much less than that predicted by the above stoichiometry [24]. Concommitantly, the final amount of p-quinone produced far exceeded that of oxygen consumed and this could be exaggerated under conditions where O_2 becomes limiting [25]. Thus in the absence of O_2, H_2O_2 can oxidize 6-OHDA, although at a much slower rate:

$$\text{6-OHDA} + H_2O_2 \longleftrightarrow \underline{p}\text{-quinone} + 2H_2O \cdot \quad (7)$$

Since catalase of high purity did not inhibit the autoxidation of 6-OHDA, H_2O_2 is not involved in the rate determining steps instrumental to propagation of the free radical chain. Also part of the O_2 or H_2O_2 deficit may be due to the Haber-Weiss interactions producing $^{\cdot}OH$ (Appendix 1) which may react in the manner described above.

Liang et al. [24] made no attempt to quantify the steady state concentrations of H_2O_2 and perhaps for this reason most workers (e.g.

[6]) have routinely assumed the incorrect stoichiometry as stated in Reaction 6. However we have found that the quantitative contribution of H_2O_2 is not constant but intimately dependent on the O_2:6-OHDA ratio. A typical stoichiometry of a reaction under standard conditions - air-saturated phosphate buffer (234 μM O_2) at 25°C, pH 7.00, ionic strength 0.01 - may be:

$$1.46\ \text{6-OHDA} + O_2 \longrightarrow 1.46\ \underline{p}\text{-quinone} + 0.67\ H_2O_2 + 0.66\ H_2O + 0.26\ H^+ \quad (8)$$

Under 1 atm O_2, the $\underline{p}$-quinone:H_2O_2 ratio is 1.2 suggesting that the ratio increases as the O_2 tension decreases. Both the stoichiometry of H_2O_2 production during the progress of the autoxidation and the rate of H_2O_2 consumption under anaerobic, metal ion free conditions require further quanitification. It is important to investigate the kinetics of 6-OHDA:H_2O_2 redox couple and the nature of metal ion catalysis under anaerobic or low O_2 atmospheres.

II. The Intermediate: O_2^-

The involvement of O_2^- was demonstrated by the inhibition of the initial rate of autoxidation by SOD [27]:

$$\text{6-OHDA} + O_2 \longrightarrow \text{semiquinone} + O_2^- + H^+ \quad (9)$$

$$\text{6-OHDA} + O_2^- + H^+ \longrightarrow \text{semiquinone} + H_2O_2 \quad (10)$$

$$\text{semiquinone} + O_2 \longrightarrow \underline{p}\text{-quinone} + O_2^- + H^+ \quad (11)$$

$$\text{semiquinone} + O_2^- + H^+ \longrightarrow \underline{p}\text{-quinone} + H_2O_2 \quad (12)$$

Reactions 10 and 11 constitute a self-propagating sequence where O_2^- consumed is regenerated. In the absence of O_2^-, Reactions 10 and 12 no longer proceed and the initial rate is inhibited in a dose dependent manner by SOD. This inhibition, however is not simple, since SOD actually induces a latent period after which the rate increases as the reaction progresses, indicating that there is an accumulation of some other intermediate which drives the reaction faster as it progresses. This is characteristic of autocatalytic reactions and O_2^- must be a primary catalytic species or involved in the synthesis of the catalytic species. In the presence of SOD some other, more slowly accumulating, catalytic species comes to dominate the reaction mechanism. However, it is unlikely that Reaction 11 actually proceeds since molecular oxygen alone could not oxidize 6-OHDA to the extent of the p-quinone [28]. This is supported by ESR spectral data which failed to show chemical reactivity between O_2^- and semiquinone [29].

The presence of p-quinone and other mixtures available at redox equilibrium eliminated the lag period [30] suggesting that the accumulation of p-quinone products is causing acceleration of the rate near the end of the SOD-inhibited reactions. Perhaps reversal of the dismutation of the semiquinone:

2 semiquinone <---> p-quinone + 6-OHDA (13)

yields autocatalytic conscentrations of the semiquinone. Non-enzymic disproportionation of semiquinone is a major pathway for its elimination

in many reactions such as peroxidase and phenolic subtrates [31], thus Reaction 13 contributes to the high yields of p-quinone not attributable to oxidation by H_2O_2 or O_2.

III. The Production of Singlet Oxygen.

Chemiluminescence observed from the autoxidation of 6-OHDA, was inhibitable by SOD, catalase, ethanol or thiourea [32]. $^1O_2$ may result from spontaneous dismutation of O_2^- or from the Haber-Weiss reaction, however all detectors of $^1O_2$ react with $^\bullet OH$ at a much faster rate, therefore the chemiluminescence could be due to the $^\bullet OH$. At present there are no specific scavengers which conclusively suggest the presence of $^1O_2$. We did not pursue the role of singlet state oxygen in the autoxidation of 6-OHDA.

ROLES OF METAL IONS

With exception of a very few studies, [13,26] the question of metal ion catalysis from trace contaminations has been largely ignored and data must be interpreted with this in mind. The nature of metal ions in the interactions among O_2 species has been well characterized [18,23,33-35]. The addition of Cu^{2+} accelerated the rate of autoxidation of 6-OHDA in a dose dependent fashion and this was completely prevented by ethylenediaminetetraacetic acid (EDTA) [36]. Fe^{2+} was not as efficient as EDTA-Fe^{2+} in accelerating the autoxidation. It also caused a bleaching of the p-quinone which could

be prevented by catalase or ethanol [30]. This enhanced rate was not inhibitable by SOD which implies that the ferrous complex is a better catalyst than O_2^-. Substituting DTPA for EDTA resulted in acceleration but only by 20% of the EDTA-Fe^{2+} rate. In the absence of added metal ions, both EDTA and DTPA were necessary to confer sensitivity to SOD inhibition when no other precautions to eliminate contaminating transition metal ions [30], however in assay systems using deionised distilled or Chelex treated water, metal chelating agents were not necessary for strong inhibition by SOD [26]. The most profound consequence of metal ion catalysis seems to be the generation of $^{\bullet}OH$.

IV. The Generation of $^{\bullet}OH$.

The production of hydroxyl radicals was demonstrated by the evolution of ethylene gas from methional [37] which could be inhibited by other hydroxyl scavengers. Neither H_2O_2 nor O_2^- alone could generate ethylene gas. However, either SOD or catalase was able to partially inhibit the rate of ethylene production so it was thought that both H_2O_2 and O_2^- were involved in the production of $^{\bullet}OH$ via a Haber-Weiss or Fenton type reaction:

$$H_2O_2 + Fe^{2+} \longrightarrow {}^{\bullet}OH + OH^- + Fe^{3+} \quad (14)$$

$$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \quad (15)$$

$$H_2O_2 + O_2^- \longrightarrow {}^{\bullet}OH + OH^- + O_2 \quad (16)$$

Hydroxyl scavengers do not inhibit the rate of autoxidation but appears to slightly accelerate the overall rate under certain conditions [28] probably because they prevent bleaching of the p-quinone. Thus the $^{\bullet}OH$ is not involved prior to the rate limiting steps in the initial autoxidation. However if it promotes polymerisation or intracyclisation of the p-quinone products it may shift the redox equilibrium even further in favour of 6-OHDA oxidation. More importantly, the high reactivity of the hydroxyl radical itself is responsible in causing damage to the cell.

It is generally accepted that some organically chelated metals such as EDTA-Fe^{2+} are more efficient catalysts than free metal ions in generating $^{\bullet}OH$. However the following evidence suggests that the nascent radicals resulting from $^{\bullet}OH$ propagate the chain by facilitating the transition of the metal ion through its redox states. In the simultaneous presence of DTPA, catalase and SOD, the autoxidation was completely inhibited despite the presence of molecular oxygen [28]. However addition of either mannitol or formate for the purposes of scavenging of $^{\bullet}OH$ restarted the reaction. Therefore we postulate that either the semidehydro-mannitol or the carboxylate radical can reduce the DTPA-Fe^{3+} and thus allow iron to cycle through its redox states. Since this revived oxidation exhibited a latent period, O_2^- is probably not involved. Moreover, since the yield of p-quinone reflected utilisation of of recycled molecular oxygen only, H_2O_2 was probably not involved either. We do not know the source of $^{\bullet}OH$ in this system, but presumably a small portion of 6-OHDA oxidized in the aliquot before

addition to the reaction chamber was enough to allow recycling of the chelated iron. Moreover we do not know if these hydroxyl scavengers act in a catalytic or stoichiometric manner since both were present in concentrations over 10 times in excess of the predicted yield of $^{\bullet}OH$ from the control, unhibited autoxidation.

If analogous reactions are involved in idiopathic Parkinsonism, the fact that its progress spans many years is consistent with the slower oxidation rates characteristic of endogenous catecholamines, low intracellular oxygen tensions and tightly chelated metal ions in a mileu of catalase and SOD. Metal ions among other compounds found chelated by endogenous catecholamines are perhaps essential to the process of their concentration for storage in synaptic vesicles [38]. The nature of ligand bound to a metal ion determines both its redox potential and the ease of inner and outer sphere electron transfers. Conversely, binding by metal ions may increase the susceptibility of the participating ligand to metal catalysed oxidation. The ferrous ion chelated by DTPA still participates in Fenton-type reactions however its rereduction was not observed [22].

GENERAL GOALS OF STUDY

While much of the autoxidation of 6-OHDA has been characterised, neither its mechanisms of cytotoxicity nor its oxidation have been unambiguously elucidated. It was hoped that in studying the autoxidation of 6-OHDA, we would establish some general principles of free radical pathology. Not only did we want to identify the conditions which would promote or inhibit the autoxidation but also to quantify the relative contributions of the intermediates in initiating and propagating the free radical chain. The complexities of this autoxidation were in part due to the autocatalytic nature of one of its intermediates, the superoxide anion and in part attributed to the synergistic interactions of contaminating metal ions. The following sections will reveal the rewards as well as the frustrations which accompany any search for generalisations which starts among the seemingly intractable problems of finding a practical approach at the level of observable and measurable phenomena to events occurring in the semi-abstract world of molecular and submolecular interactions.

APPENDIX 1

ONE ELECTRON TRANSFERS TO MOLECULAR OXYGEN

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

$$O_2^- + H^+ \longleftrightarrow HO_2^\bullet$$

$$O_2^- + O_2^- \longleftrightarrow {}^1O_2 + H_2O_2$$

$$O_2^- + e \longrightarrow O_2^{2-}$$

$$O_2^{2-} + H^+ \longleftrightarrow HO_2^-$$

$$HO_2^- + H^+ \longleftrightarrow H_2O_2$$

$$2(H_2O_2) \longleftrightarrow H_2O + O_2$$

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

$$O^- + H^+ \longrightarrow {}^\bullet OH$$

$$O^- + e \longrightarrow O^{2-}$$

$$O^{2-} + 2H^+ \longrightarrow H_2O$$

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

$$H_2O_2 + e \longrightarrow {}^\bullet OH + OH^-$$

$${}^\bullet OH + e + H^+ \longrightarrow H_2O$$

$$O_2^- + H_2O_2 \longrightarrow {}^\bullet OH + OH^- + {}^1O_2$$

$$O_2^- + {}^\bullet OH \longrightarrow O_2 + OH^-$$

Metal Ion Interactions with Intermediates of the Reduction of Oxygen:

$$Me^{(n+)} + O_2^- \longrightarrow MeO_2^{(n-1)+}$$

$$Me^{(n+)} + H_2O_2 \longrightarrow (MeO_2H)^{(n-1)+} + H^+$$

$$Me^{(n+)} + H_2O_2 \longrightarrow Me^{(n+1)+} + {}^{\bullet}OH + OH^-$$

$$Me^{(n+1)+} + O_2^- \longrightarrow Me^{(n+)} + O_2$$

$$Me^{(n+)} + {}^{\bullet}OH \longrightarrow MeO^{(n-1)+} + H^+$$

APPENDIX 2

ONE ELECTRON TRANSFERS FROM 6-OHDA

$$6\text{-OHDA} \longleftrightarrow sQ^{\bullet} + e + H^+$$

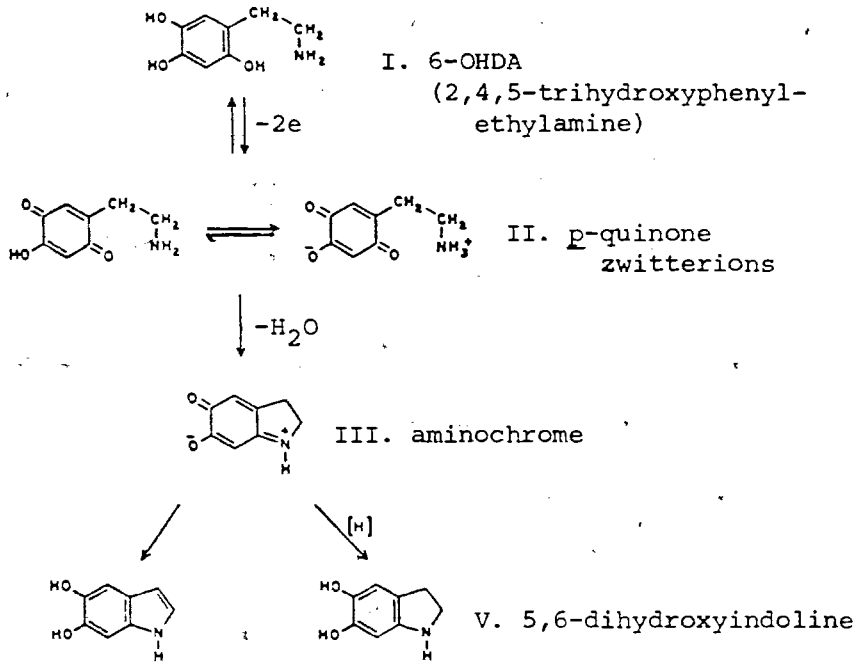
$$2(sQ^{\bullet}) \longleftrightarrow pQ + 6\text{-OHDA}$$

$$sQ^{\bullet} \longleftrightarrow pQ + e + H^+$$

$$pQ \longrightarrow \text{aminochrome} + H_2O$$

aminochrome (via rearrangement) $\longrightarrow$ 5,6-dihydroxyindole

OXIDATION PRODUCTS OF 6-OHDA



IV. 5,6-dihydroxyindole

APPENDIX 3

POSSIBLE ELEMENTARY REACTIONS BETWEEN 6-OHDA AND MOLECULAR OXYGEN

Production of the semiquinone ($sQ^{\bullet}$):

6-OHDA <------> $sQ^{\bullet}$ + e + H^+
O_2 + e -----> O_2^-

6-OHDA + O_2 <------> $sQ^{\bullet}$ + O_2^- + H^+

6-OHDA <------> $sQ^{\bullet}$ + e + H^+
O_2^- + e + $2H^+$ -----> H_2O_2

6-OHDA + O_2^- + H^+ <------> $sQ^{\bullet}$ + H_2O_2

6-OHDA <------> $sQ^{\bullet}$ + e + H^+
H_2O_2 + e + H^+ -----> H_2O + $^{\bullet}OH$

6-OHDA + H_2O_2 -----> $sQ^{\bullet}$ + H_2O + $^{\bullet}OH$

6-OHDA <------> $sQ^{\bullet}$ + e + H^+
$^{\bullet}OH$ + e + H^+ -----> H_2O

6-OHDA + $^{\bullet}OH$ -----> $sQ^{\bullet}$ + H_2O

Production of the para-quinone (pQ):

$$sQ^{\bullet} \quad <\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!> \quad pQ + e + H^{+}$$
$$O_2 + e \quad -\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!> \quad O_2^{-}$$

$$sQ^{\bullet} + O_2 \quad -\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!> \quad pQ + O_2^{-} + H^{+}$$

$$sQ^{\bullet} \quad <\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!> \quad pQ + e + H^{+}$$
$$O_2^{-} + e + 2H^{+} \quad -\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!> \quad H_2O_2$$

$$sQ^{\bullet} + O_2^{-} + H^{+} \quad -\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!> \quad pQ + H_2O_2$$

$$sQ^{\bullet} \quad <\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!> \quad pQ + e + H^{+}$$
$$H_2O_2 + e + H^{+} \quad -\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!> \quad H_2O + {}^{\bullet}OH$$

$$sQ^{\bullet} + H_2O_2 \quad -\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!> \quad pQ + H_2O + {}^{\bullet}OH$$

$$sQ^{\bullet} \quad <\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!> \quad pQ + e + H^{+}$$
$${}^{\bullet}OH + e + H^{+} \quad -\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!> \quad H_2O$$

$$sQ^{\bullet} + {}^{\bullet}OH \quad -\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!-\!\!\!> \quad pQ + H_2O$$

REFERENCES

1. Harley-Mason, J., J. Chem. Soc. (1953):200-203.

2. Saner, A. and H. Thoenen, Mol. Pharm. 7:147-154 (1970).

3. Liang, Y.-O., P.M. Plotsky and R.N. Adams J. Med. Chem. 20:581-583 (1977).

4. Jonsson, G., MED. BIOL. 54:406-420 (1976).

5. Creveling, C.R., A. Rotman and J.W. Daly, In Chemical Tools in Catecholamine Research (ed. G. Jonsson, T. Malmfors and C. Sachs) Vol. 1, pp. 23-32. North-Holland, Amsterdam (1975).

6. Heikkila, R.E. and G. Cohen, Mol. Pharm. 8:241-248 ((1972).

7. Tiffany-Castiglioni, E., R.P. Saneto, P.H. Proctor and J.R. Perez-Polo, Biochem. Pharm. 31(2):181-188 (1982).

8. Davison, A.J., B.D. Wilson, and P. Belton, Biochem. Pharm. in press (1984).

9. Reynolds, C.P. and J.R. Perez-Polo, Neurosci. Lett. 26:151-155 (1981).

10. Halliwell, B. Bull. Europ. Physiopath. Resp.(Suppl) 17:21-28 (1981).

11. Hill, H.A.O. Phil. Trans. R. Soc. Lond. B294:119-128 (1981).

12. Adams, R.N., E. Murrill, R. McCreery, L. Blank and M. Karolczak, Eur. J. Pharm. 17:287-292 (1972).

13. Floyd, R.A. and B.B. Wiseman, Biochem. Biophys. Acta 586:196-207 (1979).

14. McCreery, R.L., R. Dreiling and R.N. Adams, Brain Res. 74:15-21 (1974).

15. Harrison, W.H., W.W. Whisler and B.J. Hill, Biochemistry 7(9):3089-3094 (1968).

16. Blank, C.L., R.L. McCreery, R.M. Wightman, W. Chey and R.N. Adams, J. Med. Chem. 19(1):178-180 (1976).

17. Powell, W.S. and R.A. Heacock, J. Pharm. Pharmac, 25:193-200 (1973).

18. Halliwell, B., FEBS Lett. 92:321-326 (1978).

19. Matheson, M.S., W.A. Mulac, J.L. Weeks and J. Rabani, J. Phys. Chem. 70:2092-2098 (1966).

20. Sagone Jr., A.L., J. Greenwald, E.H. Kraut, J. Bianchine, D. Singh, J. Lab. Clin. Med. 101:97-104 (1983).

21. Chabereck, S., A.E. Frost, M.A. Doran and N.J. Bicknell, J. Inorg. Nucl. Chem. 11:184-196 (1959).

22. Cohen, G. and P.M. Sinet, FEBS Lett. 138:258-260 (1982).

23. Hoe, S., D.A. Rowley and B. Halliwell, Chem.-Biol. Interact. 41:75-81 (1982).

24. Liang, Y.-O., R.M. Wightman and R.N. Adams, Eur. J. Pharm. 36:455-458 (1976)

25. Davison, A.J. and P. Gee, Pacific Slope Biochem. Conf. (1980).

26. Gee, P. and A.J. Davison, Internat. Congr. Biochem. 001-244 (1982).

27. Heikkila, R.E. and G. Cohen, Science 181:456-457 (1973).

28. Gee, P. and A.J. Davison, Fed. Proc. 42:2935 (1983).

29. Borg, D.C., K.M. Schaich, J.J. Elmore, Jr. and J.A. Bell, Photobiochem. Photobiol. 28:887-907 (1978).

30. Sullivan, S.G. and A. Stern, Biochem. Pharm. 30:2279-2285 (1981).

31. Yamazaki, I. and L.H. Piette, Biochem. Biophys. Acta 77:47-64 (1963).

32. Heikkila, R.E. and F.S. Cabbat, Photobiochem. Photobiol. 28:677-680 (1978).

33. Halliwell, B. Biochem. J. 167:317-320 (1977).

34. Halliwell, B., FEBS. Lett. 92:238-242 (1978).

35. Gutteridge, J.M.C., R. Richmond and B. Halliwell, Biochem. J. 184:469-472 (1979).

36. Heikkila, R.E. and F.S. Cabbat, Life Sciences 23:33-38 (1978).

37. Cohen, G. and R.E. Heikkila, J. Biol. Chem. 249:2447-2452 (1974).

38. Granot, J. and K. Rosenheck, FEBS Lett. 95:45-48 (1978).

THE AEROBIC OXIDATION OF 6-HYDROXYDOPAMINE: EFFECTS OF SCAVENGERS OF OXYGEN FREE RADICALS - INDIVIDUALLY AND IN COMBINATION†

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ABSTRACT

Rates of autoxidation of 6-hydroxydopamine were determined in the presence of combinations of: superoxide dismutase (SOD), diethylenetriaminepentaacetate (DTPA), desferrioxamine, catalase, formate, mannitol, benzoate and glucose, by measuring rates of p-quinone (product) formation (at 490 nm) and concomitantly O_2 utilisation (polarographically). Added individually, the scavengers were inhibitory in the following order of effectiveness: SOD (96%) > DTPA (66%) > desferrioxamine (30%). When scavengers were present in combinations, each modified the actions of the other, to the extent that only SOD (93-100%) and desferrioxamine (15-40%) were always inhibitory. The hydroxyl scavengers formate or mannitol alone produced little or no (0-10%) inhibition, but inhibited the autoxidation by 30% in the presence of catalase+SOD. In contrast, in the presence of catalase+SOD+DTPA, they (including glucose) reactivated the completely blocked reaction. DTPA and desferrioxamine increased the latent period induced by SOD, while their inhibition of the rates of autoxidation were much less. Thus in the presence of SOD, the transition metal ions are more essential to the initiation mechanisms than to the propagation steps of the autoxidation.

Index Words:

6-hydroxydopamine oxidation,

free radical intermediates,

scavengers of oxygen radicals

[1]Abbreviations used:

6-OHDA, 6-hydroxydopamine; SOD, superoxide dismutase; ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DTPA, diethylenetraminepentaacetic acid; BSA, bovine serum albumin.

[2]Enzymes:

SOD: Superoxide:superoxide oxidoreductase, EC 1.15.1.1

Catalase: Hyrdrogen-peroxide:hydrogen peroxide oxidoreductase, EC 1.11.1.6

INTRODUCTION

6-Hydroxydopamine, (6-OHDA)[1] by virtue of its affinity for the catecholaminergic uptake systems, is a selective toxin to those neurones which are able to concentrate it to critical levels. Its toxic actions are directly related to its capacity to generate free radical species of molecular oxygen and 6-OHDA quinones. These reactive species are thought to modify cellular components (in part by oxidising or crosslinking free sulphydryl groups), rendering them irreversibly non-functional. Although, many aspects of the autoxidation of 6-OHDA have been characterised, neither the mechanisms of its autoxidation nor its cytotoxicity have been unambiguously elucidated. The current study was therefore undertaken to ascertain the relative importance of the various species of oxygen participating in the propagation chain, and the predominent interactions between them.

Several workers have reported the effects of various individual free radical scavengers upon the 6-OHDA/O_2 reaction (Heikkila & Cohen, 1972, 1973; Floyd & Wiseman, 1979; Sullivan & Stern, 1981). In a few cases two or three scavengers were simultaneously present (Sullivan & Stern, 1981). The current investigation examines the effects of all possible combinations of SOD, catalase, one or more metal ion chelating agent and one or more hydroxyl scavengers. The purpose of such a thorough approach was to identify those combinations of scavengers which would allow examination of the role(s) of a single

free radical species by selective elimination of participation by others. Moreover since many components coexist in cellular systems, it was of interest to determine the effects of a single scavenger in the presence of various combinations of others, with a view to identifying synergistic or moderating interactions among them.

The formation and utilisation of both O_2^- and H_2O_2 in the 6-OHDA reaction have been characterised (Heikkila & Cohen, 1972, 1973; Liang et al., 1976). Although it is unclear as to whether the hydroxyl radical is produced from metal catalysed Haber-Weiss type interactions between H_2O_2 and O_2^-, or directly from oxidation of the 6-OHDA quinone, $^{\cdot}OH$ have been demonstrated by ESR using the spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO; Floyd & Wiseman, 1979). The semiquinone of 6-OHDA was also demonstrated by ESR under anaerobic conditions. It has been established that O_2^- plays a cata lytic role in the 6-OHDA/O_2 reaction (Heikkila & Cohen, 1974; Sullivan & Stern, 1981) while H_2O_2 can substitute for molecular oxygen as an oxidising species (Liang et al., 1976). Since no evidence of reactivity of the semi-quinone with molecular oxygen was found, Borg et al. (1978) suggested that formation of the p-quinone involved dismutation of two semi-quinones. In support of this, oxidised quinone mixtures eliminated a latent (induction) period caused by the removal of the primary autocatalytic species, O_2^- (Sullivan & Stern, 1981)

Electrochemical studies suggest that the open chain p-quinone product

is in redox equilibrium with 6-OHDA and only slowly undergoes 1,2-side chain closure with the elimination of H_2O (Adams et al., 1972). The half-life of this intramolecular cyclisation was estimated to be 38 min at pH 7.4, 37^{o} C (Blank et al., 1976) and the resulting adrenochrome is thought to rearrange to the 5,6-dihydroxyindole or to become reduced to 5,6-dihydroxyindoline (Powell & Heacock, 1973). Although the latter species is also of cytotoxic importance, we have tried to restrict our observations to the earlier phases of the reaction, in particular to the initial two electron oxidation segment of the autoxidation of 6-OHDA.

MATERIALS AND METHODS

REAGENTS

6-Hydroxydopamine hydrobromide salt and DTPA were purchased from the Sigma Chemical Co. (St. Louis, MO). Sodium phosphate buffers were obtained from American Scientific and Chemical (Seattle, WA) and Matheson Coleman and Bell Manufacturing Chemists (Norwood, Ohio). Desferrioxamine (Desferal mesylate) was a gift from CIBA Pharmaceutical Co. (Summit, NJ). Chelex 100 resin and AG501-8X deionising resin were obtained from BioRad Laboratories (Mississauga, Ont., Canada). Sodium formate was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ) while mannitol, glucose (dextrose) and sodium sulfite were obtained from Fisher Scientic Co. (Fair Lawn, NJ). Sodium benzoate was purchased from Anachemia Chemicals Ltd. (Toronto, Canada). Hydrogen peroxide was obtained from BDH Chemicals (Vancouver, Canada). Catalase (bovine liver) was purchased from both Calbiochem-Behring Corp. (88,823 U mg^{-1}; La Jolla, CA) and Sigma Chemical Co. (2500 U mg^{-1}). SOD (bovine blood, 2900 U mg^{-1}), and BSA were obtained from Sigma Chemical Co. All reagents were of the highest purity commercially available at the time of purchase.

The purity of the water was >10 MOhm cm^{-3}, which was further increased >7 times by passage through a Chelex 100 column. The following metal ions from the buffer salts were below the stated values (which

represent the detection limits of inductive coupled plasma emission spectroscopy): copper <79 nM; iron <54 nM; manganese <18 nM; aluminium <741 nM and vanadium <39 nM. Phosphate buffers, pH 8.00, ionic strength 0.04, made from freshly deionized distilled water were also below these detection limits. However, as an extra precaution, acid and base were treated by Chelex 100 resin separately and titrated to the desired pH, since pH changed even after equilibration of the column. DTPA (1.0 mM) was added as a non-specific chelating agent for transition metal ion contaminants (Chabereck et al., 1959). Although 500 μM desferrioxamine, a chelating agent specific for iron, was reportedly sufficient to remove catalysis due to 100 μM iron (Hoe et al., 1982), 1.0 mM was used to be consistent with DTPA. Other scavengers were added in excess of 10 times the amount needed to remove the estimated yield of each radical species. Benzoate (10 mM) and mannitol (10 mM) were also used as hydroxyl scavengers since formate (10 mM) produces O_2^- (in the presence of O_2) which is a catalytic species in 6-OHDA autoxidation. However, at least one pathway of the scavenging reaction of $^{\cdot}OH$ by formate is well established (Klug et al., 1972) and the intermediate radical CO_2^- is converted to O_2^- which can then be specifically scavenged by SOD. Also, formate reacts with $^{\cdot}OH$ almost 3 times faster than mannitol (Halliwell, 1978). Glucose (10 mM) is not as efficient as the other hydroxyl scavengers but was used in some conditions due to its biological importance. Catalase (250 U ml^{-1}) and SOD (250 U ml^{-1}) were used to scavenge hydrogen peroxide and superoxide respectively.

BSA (5 mg ml^{-1}) and boiled SOD were used as protein controls.

ANAEROBIC PREPARATION OF 6-OHDA

Deionized distilled or Chelex 100 treated water was alternately flushed with high purity nitrogen (Linde, Union Carbide Canada Ltd.) which was just previously bubbled through a sodium sulfite solution, and evacuated using a Virtis vacuum evacuator. 6-OHDA was gently dissolved by removing trapped air in the powder under vacuum and replaced with nitrogen. This vial was sealed under a slight positive pressure of nitrogen to minimise exposure to oxygen as aliquots were withdrawn. The absorbance at 490 nm provided an indication of baseline oxidation and the quality of the stock solution (0.79 $\pm$0.03%). The reduced concentration under anaerobic conditions (pH 6.0-7.0) was estimated from the absorbance at 290 nm, while a null difference spectrum from a sample at pH 2.00; and the symmetry of the 290 nm peak were taken as indications of the homogeneity of the preparation.

ASSAY PROCEDURES

Water saturated air at 733 $\pm$1 mm Hg barometric pressure was bubbled gently through buffer in the sample chamber of a YSI Model 53 oxygen monitor for 20 min to achieve an initial concentration of molecular oxygen of 246 $\pm$6 µM. Buffer was then transferred to a cuvette and

appropriate combinations of scavengers were added. After thorough mixing the reaction was initiated by 50 µl 6-OHDA and the formation of p-quinone was followed at 490 nm using a Beckman DB-GT spectrophotometer. The corresponding reaction was monitored for oxygen concentration by a YSI Clark-type oxygen electrode either simultaneously or sequentially. pH was checked in the reaction medium before and after addition of scavengers and at the start and upon completion of the reaction for representative conditions. Data were collected on line through a twelve bit analogue-digital converter and transferred to a IBM 4341 or 3033 computer using a microprocessor data buffer/coupler locally designed and constructed.

DATA ANALYSIS

All subsequent data analyses were performed using APL programmes written by the authors and implemented on a IBM 4341 or 3033 computer. Digitized voltages were converted to micromolar concentrations of p-quinone using the internally determined molar extinction coefficent of 1892 M^{-1} cm^{-1}, and concentrations of molecular oxygen using the Bunsen coefficient for aqueous solutions (Estabrook, 1967). Peak values in first derivative plots of the reactions, were used to determine the time at which the initial rates were maximal and linear regressions were fitted over a suitable interval to determine maximal rates. The induction or latent period, was obtained by measuring the time to the intersection of the

extrapolated lines approximating the initial and maximal velocities respectively (Burton and Ingold, 1981).

RESULTS:

EFFECTS OF INDIVIDUAL SCAVENGERS

The effects of individual scavengers in the absence of others are shown in Figure 1. Clearly SOD provided the greatest inhibition (96%), while substantial inhibition is also induced by the chelating agents, DTPA and desferrioxamine. Neither denatured SOD which had been gently boiled for 40 min nor BSA had any effect on either rate. With respect to p-quinone formation, hydroxyl scavengers (benzoate, glucose, mannitol or formate) or catalase had only a minimal effect, and thus $^{\bullet}OH$ or H_2O_2 are not important intermediates in or prior to the rate determining step. In general the rates of p-quinone production and O_2 consumption agreed reasonably well, but in the presence of either of the above hydroxyl scavengers O_2 utilisation was consistently lower than p-quinone formation, suggesting that either the scavengers themselves or the products of their action act as electron acceptors.

EFFECTS OF SUPEROXIDE DISMUTASE

Comparisons illustrating the effects of SOD in the presence of the mixtures of scavengers tested are shown in Figure 2. In every case SOD not only decreased the maximal rate of autoxidation of 6-OHDA, but it changed the reaction from pseudo-first order to sigmoidal

kinetics. Specifically, SOD inhibited: by 100% when added in the presence of DTPA+catalase; by 98% in the presence of DTPA+catalase+any hydroxyl scavenger or DTPA without catalase (whether or not hydroxyl scavengers were present); and by approximately 96% when added alone, or in the presence of catalase+any hydroxyl scavenger. In contrast, the presence of catalase alone decreased the inhibitory action of SOD to 93%.

The latent period induced by SOD was of the order of 180 s (Figure 3). In contrast, DTPA and/or desferrioxamine did not themselves induce a latent period, but they greatly increased its duration (4 to 15 times) when simultaneously present with SOD. H_2O_2 (500 μM) did not alter the latent period. However, if HO_4^-, not H_2O_2 is the product of SOD activity (Bernofsky & Wanda, 1983), then additions of H_2O_2 would not be expected to reduce the latent period.

Therefore to determine whether the catalytic species might be HO_4^-, aerated mixtures of H_2O_2 (500 μM) were incubated with 250 U ml^{-1} SOD for 2.3 h at 25° C to SOD-inhibited reactions. However, this mixture supposedly containing HO_4^-, did not decrease the latent period or affect maximal rates of oxidation. There was no evidence of a long lived acidic species resulting from the action of SOD on H_2O_2, as reported by Bernofsky and Wanda (1983). This inconsistency may be due to differences in the metal ion content since the reported study was performed in buffers made in deionised distilled water while the

present study used Chelex 100 treated buffers. This suggests that metal ions are necessary for the formation of whatever species induces the change in pH.

EFFECTS OF DTPA

DTPA, known to chelate a variety of transition metal cations to differing degrees (Chabereck et al., 1959), was the next most potent inhibitor (67%) of the autoxidation of 6-OHDA (Figure 4). Its inhibitory effect was increased by 8-50% by the presence of SOD, and decreased by 2-12% in the presence of the other scavengers. In some cases the actions of DTPA were influenced by a concommitant decrease in the pH of the reaction mixture, which consistently shifted from 8.00 ± 0.02 to 7.30 ± 0.05.

Since the autoxidation of 6-OHDA is base catalysed and enhanced at low ionic strengths we investigated the extent to which inhibition by DTPA was due to changes in pH (Figure 5). DTPA made up in sufficient NaOH to eliminate changes in pH failed to decrease the rate of autoxidation, the rate being 102% of the pH matched control and 124% of control adjusted for ionic strength by the addition of NaCl. This relative acceleration induced by the presence of NaOH may be due to the presence of contaminant metal ions in the NaOH. Because of this uncertainty, we followed control reactions at the same pH as that resulting from the addition of DTPA (pH 7.30). Under these conditions,

the slowing attributable to the metal binding properties of DTPA was 17%. The remaining 83% of the inhibition in this instance was a direct effect of pH on the autoxidation of 6-OHDA. Other reports of the action of DTPA in this system have not mentioned the use of special procedures to control pH, and unless this was done, quantitative conclusions regarding the effects of metals should be interpreted with caution. The contributions of iron can be inferred with more confidence from the effects of desferrioxamine, which has been reported to block Fenton reactions as well as the Haber-Weiss interactions, and which caused no significant change in pH.

EFFECTS OF CATALASE

Under conditions where either DTPA or SOD was present, catalase accelerated the autoxidation of 6-OHDA (Figure 6) by 20-45%, in contrast to our own previous (unpublished) studies and those of others (Sullivan & Stern, 1981), where catalase was inhibitory. Much of this difference may be due to the fact that at the relatively high concentrations of 6-OHDA (1.0 mM) used in the current studies, molecular oxygen (246 ± 6 μM) may be limiting. Under these conditions catalase serves to regenerate O_2 which is preferred over H_2O_2 as the electron acceptor under the usual reaction conditions. A further consequence of this recycling was that in the presence of catalase, the net rates determined for oxygen utilisation were generally one-half of the rates of p-quinone production, regardless of what

other scavengers were present. However even at lower concentrations of 6-OHDA (200 μM), catalase had a small stimulatory effect (11% increase; Figure 7). Wherever catalase had an inhibitory effect (10-100%), H_2O_2 or some product derived from it (e.g. $^{\bullet}OH$) must contribute to the autoxidation. This was especially evident in the simultaneous presence of DTPA+SOD.

While catalase obtained from Calbiochem-Behring Corp. was used in all experiments, we compared its effects with catalase purchased from Sigma Chemical Co. on this single occasion. As illustrated in Figure 7, the preparation from Sigma Chemical Co. had an inhibitory effect, while the more active preparations from CalBiochem-Behring did not when present alone. The inhibitory effect from the Sigma preparation was most likely due to trace contaminations of SOD (Halliwell, 1973). Any effects due to catalase before and after this point refer only to the preparations from Calbiochem-Behring Corp.

EFFECTS OF HYDROXYL SCAVENGERS

These scavengers had both inhibitory and stimulatory effects depending on which other scavengers were present. Moreover, the effects observed for formate (Figure 8) were not the same as for mannitol (Figure 9). Generally formate was inhibitory when mannitol was present (5-30%) but it accelerated the reaction when hydroxyl yield was expected to be greater due to Fenton-type reactions, such as in

the presence of DTPA (5-10%). Under the latter conditions •OH is involved only after the rate limiting step since the hydroxyl scavengers did not produce significant inhibition. Formate reacts with •OH at diffusion limits to produce the carboxylate radical which is converted to O_2^- and CO_2 after the rate limiting step, therefore it recycles •OH to O_2^- (Klug et al., 1972). Mannitol on the other hand, with no identified radical intermediate, competes with formate for the hydroxyl radical, since each was mutually inhibitory (5-30%) in the presence of the other. While the stimulatory effects of the formate and mannitol (Figures 8 and 9) can hardly be accounted for on the basis of their actions as hydroxyl scavengers, reactive derivatives resulting from their scavenging action presumably promote the autoxidation.

EFFECTS OF SCAVENGERS WHEN PRESENT IN VARIOUS COMBINATIONS

From the various combinations of scavengers it was possible to choose conditions in which a particular species would be most prominent, with minimal contamination or interaction with other intermediates. Thus Figure 10 summarises the rates attributable to the catalytic presence of just one or two free radical intermediates. The particular scavenger mixtures used (see legend) were selected on the basis of the following arguments.

Although DTPA does not preclude all participation of metal ions, the

simultaneous presence of catalase should preempt $^{\cdot}OH$ generation by Fenton-type reactions by removing H_2O_2. Formate recyles any remaining $^{\cdot}OH$ or that produced directly from the autoxidation of 6-OHDA back to O_2^-. Thus, a combination of DTPA+catalase+formate leaves O_2^- as the primary intermediate. This can be seen to contribute 83% to autoxidation of 6-OHDA (Figure 10). Since the effect of its removal by SOD (96%), is greater that its apparent contribution, O_2^- most certainly has a catalytic role both in its own generation and its utilisation.

In the combination DTPA+formate+SOD, the consequences of DTPA and formate are similiar to that in the previous scavenger combination, while SOD converts O_2^- to H_2O_2. Thus H_2O_2 remains as the primary species. Although the portion of the autoxidation attributable to H_2O_2 is barely discernable when compared to O_2^-, it is 50 times greater than that observed in H_2O_2 (500 μM) in the absence of molecular oxygen. This indicates that much of the observed participation of H_2O_2 is catalysed by the presence of species of O_2 rather than due to direct oxidation of 6-OHDA by H_2O_2. Under most circumstances, $^{\cdot}OH$ contributes little to the autoxidation. This follows from the evidence that hydroxyl scavengers were inhibitory only under special circumstances, and that the rates in the scavenger combination, DTPA+SOD (which permitted the simultaneous presence of both H_2O_2 and $^{\cdot}OH$) were not greater than that of H_2O_2 alone. However, using the scavenger mixture DTPA+formate, the effects from the

simultaneous presence of H_2O_2 and O_2^- (88%) were close to the simple sum of effects from H_2O_2 alone (2%) and O_2^- alone (83%). Again $^{\bullet}OH$ does not contribute to the autoxidation by O_2^- since the effect of O_2^- (83%) is not increased in the scavenger combination, DTPA+catalase (81%) which allows the simultaneous existence of these two species.

Although the assay medium was relatively free from metal ions, their contribution was estimated in the combination, formate+catalase+SOD. Under these conditions, O_2^- is converted to H_2O_2, which is converted to O_2 and H_2O while any $^{\bullet}OH$ formed is recycled to O_2^-. Thus the remaining oxidation should be due to catalysis by ambient traces of metal ion contaminants. This contribution makes up 5% which is close to the inhibition (7%) provided by the simultaneous presence of chelating agents, DTPA and desferrioxamine.

The total contributions of all listed species when individually present account for 90% of the control rates. Synergistic interactions between the various reaction intermediates and unknown species account for some the effects found when only one species is removed (Figure 1).

REACTIVATION OF THE REACTION IN THE PRESENCE OF DTPA+CATALASE+SOD

While the above combination of scavengers virtually prevented the autoxidation of 6-OHDA, the reaction was partly released from inhibition by the further addition of formate or mannitol (Gee & Davison, 1984). Clearly glucose or benzoate also reactivated the autoxidation to about the same degree as formate or mannitol (Figure 11). Desferrioxamine when substituted for DTPA in combination with catalase+SOD did not completely block the autoxidation implying that iron is not the only metal active as a catalyst.

Since the inhibitory action of DTPA in some cases was due to its acidity, it was important to estimate the pH effect in the totally inhibitory scavenger combination DTPA+catalase+SOD. When pH was adjusted to 8.00, this combination did not prevent the autoxidation and the contribution by DTPA in a chelating capacity was only 23% (see bars labelled D.NaOH+C+S and C+S:pH 8.0) However, when pH was adjusted to 7.30, (C+S:pH 7.3) the maximal rates were inhibited by 67%. While the remaining 10% has not been accounted for, this residual effect cannot be attributed merely to a pH dependent increase in SOD activity (Lawrence & Sawyer, 1979) in view of the presence of SOD in considerable excess.

DISCUSSION

ROLES OF O_2^- IN INITIATION AND PROPAGATION

The consequences of the addition of SOD are reflected in dramatically decreased maximal rates (Figure 2), and to an even greater extent in the induction of substantial latent periods (Figure 3). The inhibition by SOD indicates a primary role for O_2^- as the major species involved in the usual autocatalytic propagation of the reaction. The absence of a latent period when SOD is not present indicates rapid recycling of effective amounts of O_2^- during its catalytic actions under the usual reaction conditions. The latent period induced by SOD represents the additional time required for the accumulation of alternative catalytically reactive intermediates catalyse the reaction in the absence of O_2^-.

CATALYTIC ROLES OF REACTION INTERMEDIATES OTHER THAN O_2^-

Many of the known oxygen radical intermediates can be excluded. Despite the slowing of the reaction by catalase in the presence of DTPA+SOD, the reactive species is not likely to be H_2O_2, since the addition of H_2O_2, did not eliminate the latent period induced by SOD (Sullivan & Stern, 1981). Moreover it is not likely to be HO_4^-.

Having excluded catalytic roles for $^{\bullet}OH$ or H_2O_2 (Figures 6, 8, and 9)

in the presence of SOD, the most likely candidate for the role of autocatalytic intermediate remains the semiquinone and/or p-quinone of 6-OHDA (Sullivan & Stern, 1981). On this basis, in the presence of SOD the reaction (Borg et al., 1978),

2 semiquinone -----> 6-OHDA + p-quinone (1)

may be rate limiting in the linear (steady state) phase of the autoxidation. Nevertheless, the decrease in the maximal velocity caused by the further addition of DTPA and/or desferrioxamine (Figure 2) suggests that metal ions are involved in generating the autocatalytic species. In view of the need to overcome the negative charges on the two reacting semiquinone radicals, this role must reflect in part dispersal of the negative charges by bridging metal ions. In addition, formation of a metal ion-semiquinone complex should raise the redox potential of the semiquinone to a point where it is a more effective oxidising species.

ROLES OF METAL IONS IN INITIATION AND PROPAGATION

In the absence of SOD, there is no evident need for metal ions for initiation, but in its presence, both desferrioxamine and DTPA have a much more profound effect on the initiating reactions than on the propagating reactions. Thus they increased the latent period by 100 to 300%, regardless of which other scavengers were present (Figure 3). This suggests that chelation of metal ions prevents participation of those metal-radical complexes which are responsible for the

involvement of the semiquinone, and that these are especially crucial when O_2^- is removed.

Metal ions may partially substitute for superoxide as a major catalytic species or vice versa. Thus, in the current studies which used Chelex treated buffers profound inhibition (about 96%) by SOD was consistently observed, without the need to add any metal chelating agent. The fact that the sum of the inhibitions by DTPA and SOD consistently total over 100%, suggests synergism between metal ions and O_2^-. This is evident also in that inhibition by SOD is greater in the presence of DTPA, and conversely, inhibition by DTPA or desferrioxamine in enhanced by the presence of SOD (Figures 2 and 4). This contrasts with other observations that DTPA was necessary to sensitise the autoxidation to inhibition by SOD (Sullivan & Stern, 1981).

This is also consistent with reports that the predominant species capable of directly oxidising 6-HODA in the initiating phases of the reaction is a metal-oxygen complex, which in the later propagating steps gives place to a metal-superoxide complex (Gee & Davison, 1984). The reactions involved in the initiation phase of the autoxidation may be:

$$\text{6-OHDA} + O_2/Me^{n+} \longrightarrow \text{semiquinone} + O_2^-/Me^{n+} + H^+ \quad (2)$$

$$\text{6-OHDA} + O_2^-/Me^{n+} + H^+ \longrightarrow \text{semiquinone} + H_2O_2 + Me^{n+} \quad (3)$$

where reaction (3) is fast relative to (2) which is rate limiting. The lack of inhibition by the scavenger combinations, formate+catalase (Figure 8) or mannitol+catalase (Figure 9) exclude direct participation of $^{\bullet}OH$ or H_2O_2 in the initiation or propagation phases of the aerobic oxidation.

ROLES OF H_2O_2

6-OHDA is oxidised by the H_2O_2 produced in its own oxidation. Its utilisation as an electron acceptor increases as O_2 becomes less available. However oxidation by H_2O_2 anaerobically is over three orders of magnitude slower than that of O_2 under the same conditions. This explains why removal of H_2O_2 by catalase (Figures 6 and 7) does not inhibit the aerobic oxidation. Catalase was in some cases slightly stimulatory due in part to increased O_2 concentration resulting from regeneration from H_2O_2, even when O_2 is present in some excess (Figure 7).

The failure of catalase to inhibit the autoxidation of 6-OHDA contradicts both our own previous observations and those of others (Sullivan & Stern, 1981). Since we had previously used catalase purchased from Sigma, we compared it to the catalase (from Calbiochem-Behring) used in all other experiments throughout the current study (Figure 7). The inhibition due to the Sigma catalase

(36%) in view of its much lower specific ativity may well be attributable to contamination by traces of SOD activity (Halliwell, 1973), or to contaminating metal ions.

INHIBITORY EFFECTS OF •OH SCAVENGERS

Since the major sources of •OH are the Fenton and (related) Haber-Weiss reactions, it is paradoxical that the greatest inhibitory effects of formate and mannitol occurred when both catalase and SOD both present (Figures 8 and 9). However catalase has a relatively low Km for H_2O_2, (Halliwell, 1981) so that side reactions would compete effectively with catalase for H_2O_2 at relatively low concentrations. Moreover, superoxide in high concentrations may remove •OH (McCord and Fridovich 1973), so that while SOD would inhibit •OH production in the Haber Weiss reaction, it would in effect increase its yield from Fenton reactions. Thus in the presence of SOD and catalase, the yield of •OH may be increased and at the same time its participation more evident than when the other oxidising species are more abundant. It is also evident in Figures 8 and 9 that formate enhanced the inhibitory actions of mannitol and vice versa, implying some interaction between the two organic radicals, the semi-dehydromannitol and carboxylate radicals.

STIMULATORY EFFECTS OF HYDROXYL SCAVENGERS:

Since all of the hydroxyl scavengers studied were able to reactivate the blocked reaction in the presence of DTPA+catalase+SOD, the most likely explanation involves the reduction of the DTPA-metal complex so as to allow it again to cycle through its redox states (Gee & Davison, 1984). Although DTPA forms relatively stable chelates with many transition metal ions, DTPA-Fe^{2+} will undergo oxidation in Fenton-type reactions (Sinet & Cohen, 1982):

$$\text{DTPA-}Fe^{2+} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- + \text{DTPA-}Fe^{3+} \quad (4)$$

DTPA blocks the cyclic aspects of the Haber-Weiss reaction since O_2^-, is not able to reduce DTPA-Fe^{3+} resulting from the transfer of an electron from ferrous iron to H_2O_2. Thus the semi-dehydromannitol or other hydroxyl scavenger radicals may permit redox cycling of the iron, despite its chelation by DTPA as follows:

$$\text{DTPA-}Fe^{3+} + {}^{\bullet}MH \longrightarrow M + H+ + \text{DTPA-}Fe^{2+} \quad (5)$$

Alternatively organic radicals resulting from interactions with $^{\bullet}OH$, may act as co-oxidants with molecular oxygen to oxidise 6-OHDA directly. Even benzoate (the hydroxyl scavenger least likely to participate in redox side-reactions; Winston & Cederbaum, 1983) or glucose (not usually considered among the hydroxyl scavengers) were able to reactivate the DTPA+catalase+SOD inhibited oxidation. Although it is only a moderately effective $^{\bullet}OH$ scavenger when compared to formate or mannitol, its abundance in biological systems increases the importance of whatever glucose species is capable of propagating

the chain in free radical reactions.

Despite the use of the highest quality reagents available, and precautions taken to have chelating agents in large excess, the activation effect may be due to contaminating metal ions. In this regard, it is clear that although iron is probably involved, it is not the only metal ion involved. This follows from the fact that while desferrioxamine increased the inhibition by 59% above that provided by catalase+SOD, it was not as effective as DTPA which completely blocked the reaction.

SUMMARY

While SOD afforded the greatest inhibition, the initiation step probably involves a molecular-oxygen complex which reacts with 6-OHDA in a metal-oxygen-6-OHDA ternary transition state. Once the autocatalytic species O_2^- is thus formed, its metal complex is responsible for propagating the autoxidation of 6-OHDA. In the absence of O_2^-, the semiquinone becomes the major species in the propagation reaction, and not HO_4^-. H_2O_2 and/or $^{\bullet}OH$ do not contribute to any appreciable degree to either initiation or propagation unless O_2 and/or O_2^- are unavailable. Under conditions that prevent the autoxidation of 6-OHDA, all hydroxyl scavengers, including glucose, reactivate the reaction.

REFERENCES

Adams, R.N., Murrill, E., McCreery, R., Blank, L. & Karolczak, M., 1972, 6-Hydroxydopamine, a new oxidative mechanism, Eur. J. Pharmacol. 17, 287-292.

Bernofsky, C. & Wanda, S.-Y.C., 1983, Reduction of cytochrome c by hydrogen peroxide and its inhibition by SOD, Biochem. Biophys. Res. Commun. 111, 231-238.

Blank, C.L., McCreery, R.L., Wightman, R.M., Chey, W., Adams, R.N., Reid, J.R. & Smisssman, E.E., 1976, Intracyclization rates of 6-hydroxydopamine and 6-aminodopamine analogs under physiological conditions, J. Med. Chem. 19, 178-180.

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

Burton, G.W. & Ingold, K.U., 1981, Autoxidation of biological molecules. 1. The antioxidant activity of Vitamin E and related chain-breaking phenolic antioxidants in vitro, J. Am. Chem. Soc. 103, 6472-6477.

Chabereck, S., Frost, A.E., Doran M.A. & Bicknell, N.J., 1959, Interaction of some divalien metal ions with diethylenetriaminepentacetic acid, J. Inorg. Nucl. Chem. 11, 184-196.

Estabrook, R.W., 1967, Polargraphic assay of respiratory control, Methods in Enzymology X, pp. 41-47.

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

Gee, P. & Davison, A.J., 1984, 6-Hydroxydopamine does not directly reduce molecular oxygen but requires a co-reductant, Arch. Biochem. Biophys. 231, (in press).

Halliwell, B., 1978, Superoxide-dependent formation of the hydroxyl radicals in the presence of iron chelates. Is it a mechanism for hydroxyl radical production in biochemical systems, FEBS Lett. 92, 321-326.

Halliwell, B., 1973, Superoxide dismutase: a contaminant of bovine catalase, Biochem. J. 135, 379-381.

Halliwell, B., 1981, Free radicals, oxygen toxicity and aging, in: Age Pigments ed. A. Sohal, (Elsevier/North-Holland Biomedical Press U.K.) pp. 1-62.

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

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

Hoe, S., Rowley D.A. & Halliwell, B., 1982, Reactions of ferrioxamine and desferrioxamine with the hydroxyl radical, Chem-Biol. Interact. 41, 75-81.

Klug, D., Rabani, J. & Fridovich, I., 1972, A direct demonstration of the catalytic action of superoxide dismutase through the use of pulse radiolysis, J. Biol. Chem. 247, 4839-4842.

Lawrence, G.D. & Sawyer, D.T., 1979, Potentiometric titrations and oxidation-reduction potentials of manganese and copper-zinc superoxide dismutases, Biochemistry 18, 3045-3050.

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

Powell, W.S. & Heacock, R.A., 1973, The oxidation of 6-hydroxydopamine, J. Pharm. Pharmacol. 25, 193-200.

Sullivan, S.G. & Stern, A., 1981, Effect of superoxide dismutase and catalase on the catalysis of 6-hydroxydopamine and 6-aminodopamine autoxidation by iron and ascorbate, Bioch. Pharmacol. 30, 2279-2285.

Winston, G.W., Harvey, W., Beri L. & Cederbaum, A.I., 1983, The generation of hydroxyl an dalkoxyl radicals from the interaction of ferrous bipyridyl with peroxides. Differential oxidation of typical hydroxyl-radical scavengers, Biochem. J. 216, 415-421.

LEGENDS TO FIGURES

FIGURE 1: EFFECTS OF INDIVIDUAL SCAVENGERS ON THE AUTOXIDATION OF 6-OHDA:

Reactions were carried out in air-saturated (246 $\pm$6 uM O_2) phosphate buffer pH 8.00, ionic strength of 0.04, at 25° C. 1.0 mM 6-OHDA was used to initiate each reaction. DTPA or Des (desferrioxamine), 1.0 mM; benzoate, glucose, formate or mannitol, 10 mM; catalase and SOD, 250 U ml^{-1}. SOD was gently boiled for 40 min and added directly to the assay medium. BSA (bovine serum albumin) was added to concentrations comparable to protein weight as SOD (4.32 mg ml^{-1}). Error bars repesent one S.D.

FIGURE 2: INHIBITION BY SUPEROXIDE DISMUTASE (in the presence of other scavengers):

Reaction conditions were the same as in Figure 1. D, DTPA; Des, desferrioxamine; F, formate; M, mannitol; C, catalase; "None" represents SOD alone. "Percentage inhibition" by a scavenger means the percentage decrease in maximal rate induced by addition of the scavenger to a cuvette already containing the mixture of other scavengers indicated by the inscription on the horizontal axis. Where acceleration occurred, negative bars indicate percentage increase in rate. Error bars repesent one S.D.

FIGURE 3: EFFECT OF DTPA ON THE LATENT PERIOD INDUCED BY SOD (in the presence of other scavengers):

Reaction conditions were as in Figure 1. F, formate; M, mannitol; C, catalase; Des, desferrioxamine. "None" represents SOD ± DTPA, while "boiled SOD" was used as the control condition. The bar which approaches infinitely long latent period represents >12 min. Bars represent latent periods p-quinone formation and the error bars repesent one S.D.

FIGURE 4: INHIBITION INDUCED BY DPTA (in the presence of other scavengers):

Reaction conditions were as in Figure 1. F, formate; M, mannitol; C, catalase, S, SOD; Des, desferrioxamine. "None" represents DTPA alone.

FIGURE 5: ROLE OF pH IN EFFECT OF DTPA:

Reaction conditions were as in Figure 1 except that the pH and ionic strength were: ●, Control: pH 8.00, ionic strength 0.04; ■, pH 7.30, at control ionic strength (0.04); △, ionic strength 0.07 (using NaCl), at control pH (8.00); O, DTPA previously adjusted to pH 8.00 (by NaOH), ionic strength 0.07; □, DTPA: pH changed from 8.00 to 7.30, after addition of DTPA, ionic strength 0.04.

FIGURE 6: INHIBITON INDUCED BY CATALASE (in the presence of other scavengers):

Reaction conditions were as in Figure 1. D, DTPA; F, formate; M, mannitol; S, SOD; "None" represents catalase alone.

FIGURE 7: LACK OF INHIBITION BY CATALASE (whether O_2 was limiting or not):

Reaction conditions were as in Figure 1 except that: ●, Control conditions at 1.0 mM 6-OHDA; ■, catalase from Calbiochem-Behring Corp. in the presence of 1.0 mM 6-OHDA; while Δ, catalase from Sigma Chemical Co. (250 U ml^{-1}) in the presence of 1.0 mM 6-OHDA. O, 200 uM 6-OHDA in the absence of catalase; while □, is in the presence of catalase from Calbiochem-Behring (200 uM 6-OHDA). Catalase from Calbiochem was used in all other conditions in the present studies.

FIGURE 8: INHIBITION INDUCED BY FORMATE (in the presence of other scavengers):

Reaction conditions were as in Figure 1. D, DTPA; M, mannitol; C, catalase S, SOD. "None" represents formate alone. The bar labeled DCS represents reactivation of autoxidation of 6-OHDA by formate from an immeasurable rate to that approximately equal to one-third of the rate in the presence of SOD alone.

FIGURE 9: INHIBITION INDUCED BY MANNITOL (in the presence of other scavengers):

Reaction conditions were as in Figure 1. D, DTPA; F, formate; C, catalase S, SOD. "None" represents mannitol alone. The bar labelled DCS represents reactivation of autoxidation of 6-OHDA by mannitol from an immeasurable rate to that approximately equal to formate activation under the same conditions.

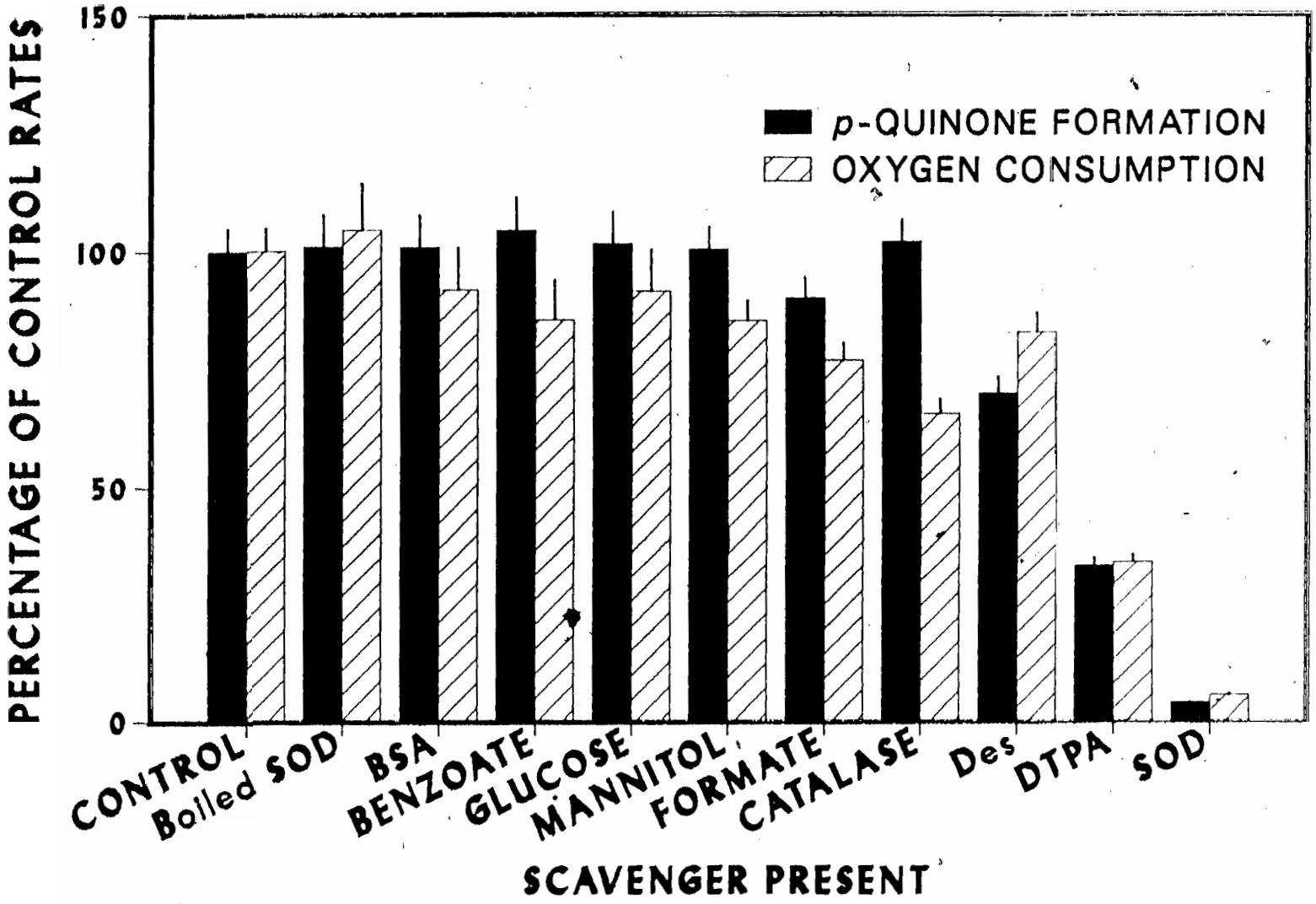
FIGURE 10: CONTRIBUTION OF SELECTED OXIDATIVE INTERMEDIATES IN THE AUTOXIDATION OF 6-OHDA (in the presence of 246 $\pm$ 6 uM O_2):

Reaction conditions were as in Figure 1 except the control rates used for comparisons at pH 7.30 since DTPA was present in all but one condition illustrated. The following combinations of scavengers were chosen to best represent contributions by individual species: O_2^-, DTPA+formate+catalase; Me^{n+}, formate+catalase+SOD; H_2O_2, DTPA+formate+SOD; $^{\bullet}OH$, DTPA+catalase+SOD; H_2O_2+$^{\bullet}OH$, DTPA+SOD; $^{\bullet}OH$+O_2, DTPA+catalase; H_2O_2+$^{\bullet}OH$, DTPA+formate. Error bars repesent one S.D.

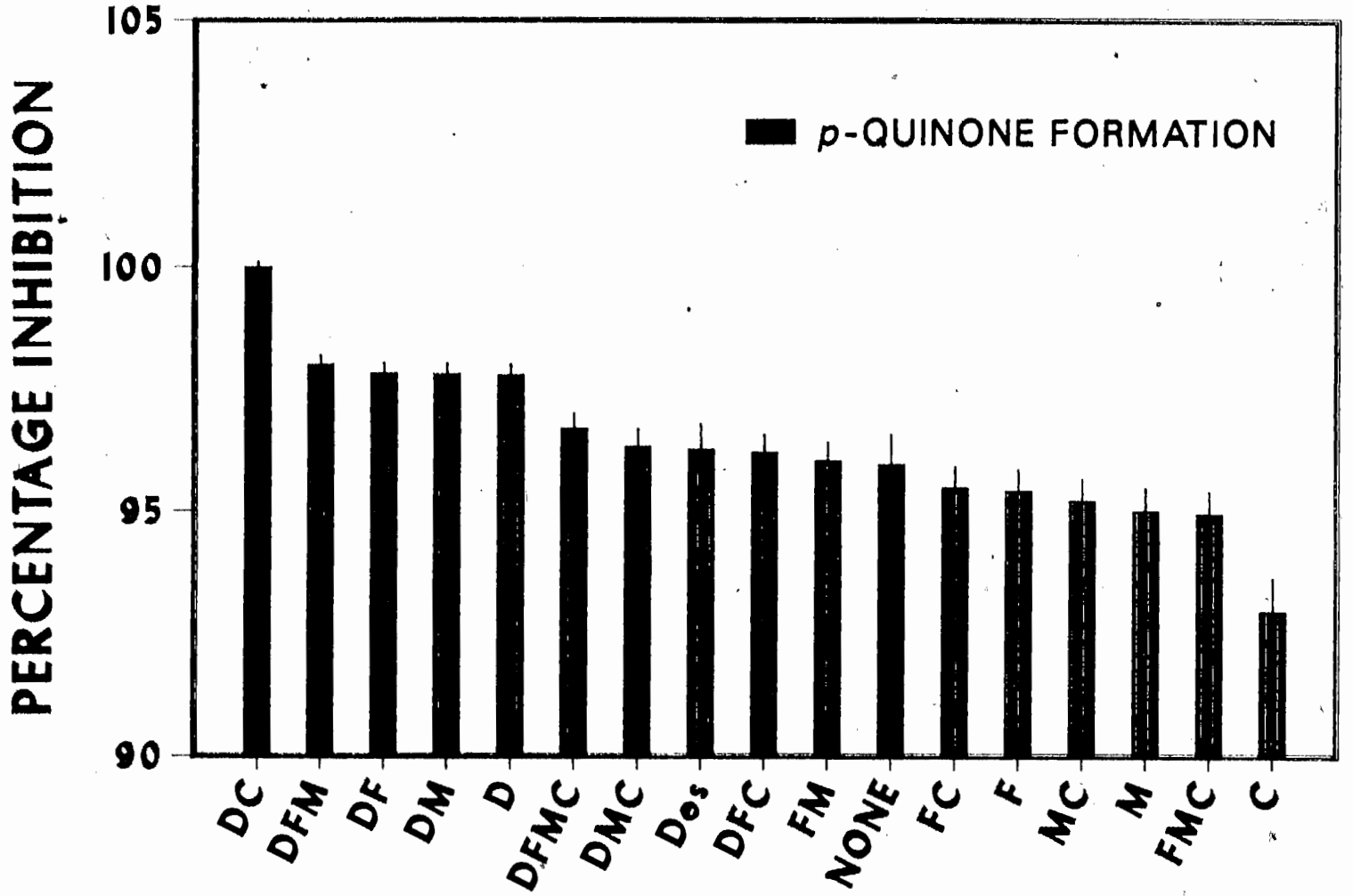
FIGURE 11: ACTIVATION OF D+C+S INHIBITED AUTOXIDATION BY HYDROXYL SCAVENGERS (mannitol, formate, glucose or benzoate):

Reaction conditions and concentrations were as in Figure 1. "D+C+S+None" represents the extent of inhibition by DTPA+catalase+SOD in the absence of any hydroxyl scavengers. "Des+C+S" represents substitution of desferrioxamine for DTPA in the presence of catalase+SOD, while D.NaOH+C+S repesents DTPA corrected for pH in combination with catalase+SOD. The combination C+S, catalase+SOD, were assayed at both pH 7.30 and pH 8.00. Error bars represent one S.D.

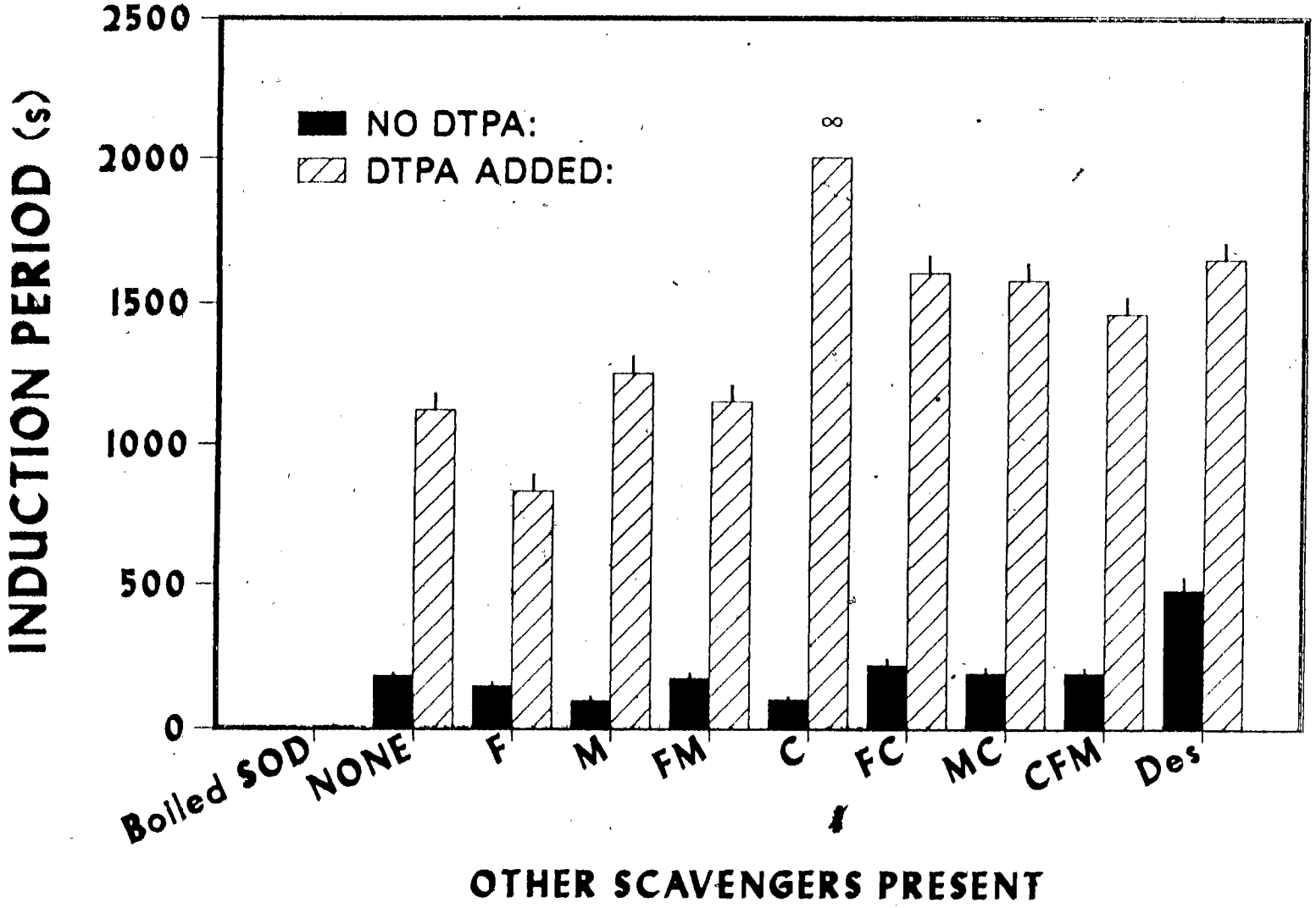
EFFECTS OF INDIVIDUAL SCAVENGERS



INHIBITION INDUCED BY SUPEROXIDE DISMUTASE



EFFECTS OF DTPA ON THE LATENT PERIOD INDUCED BY SOD



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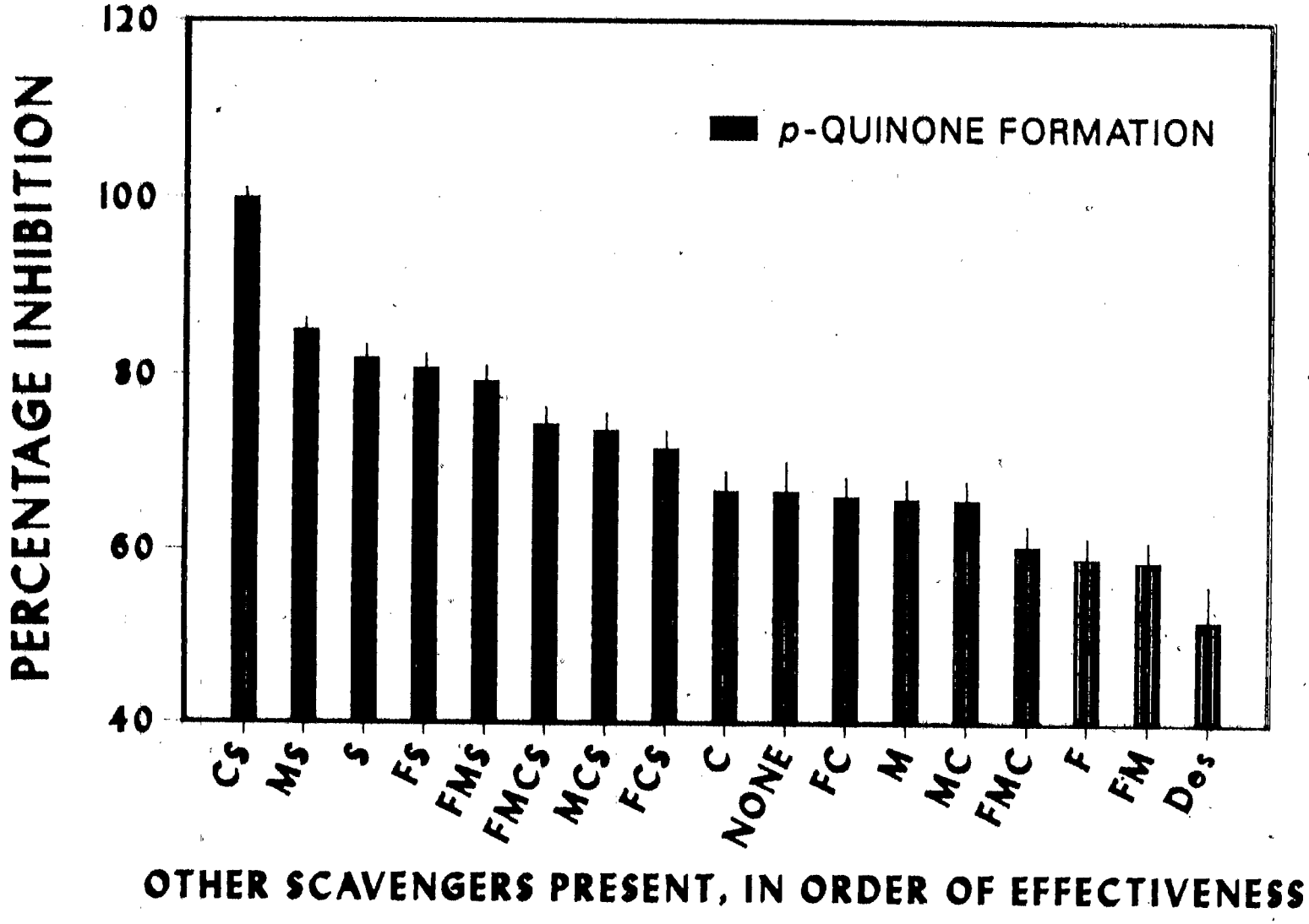
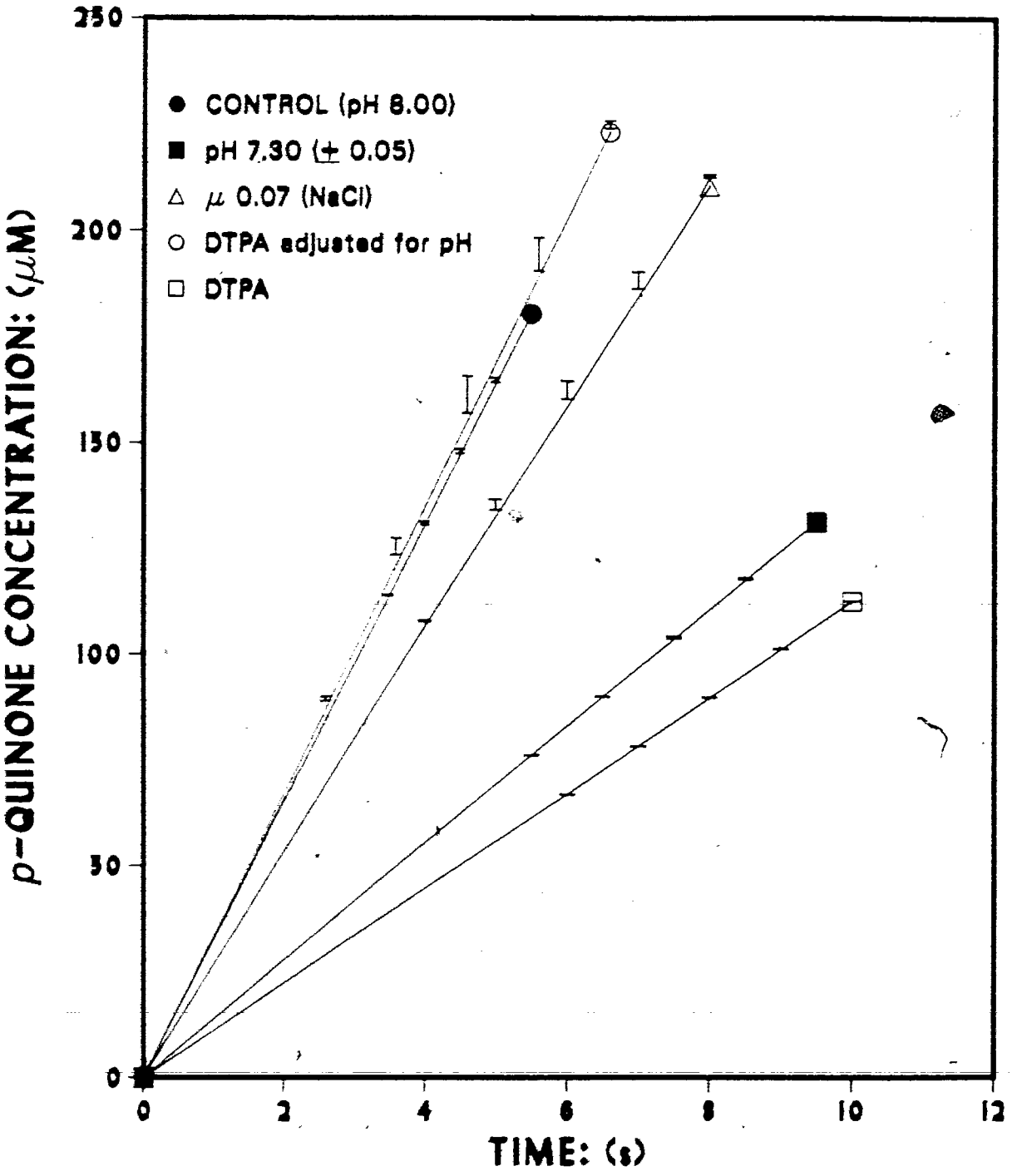
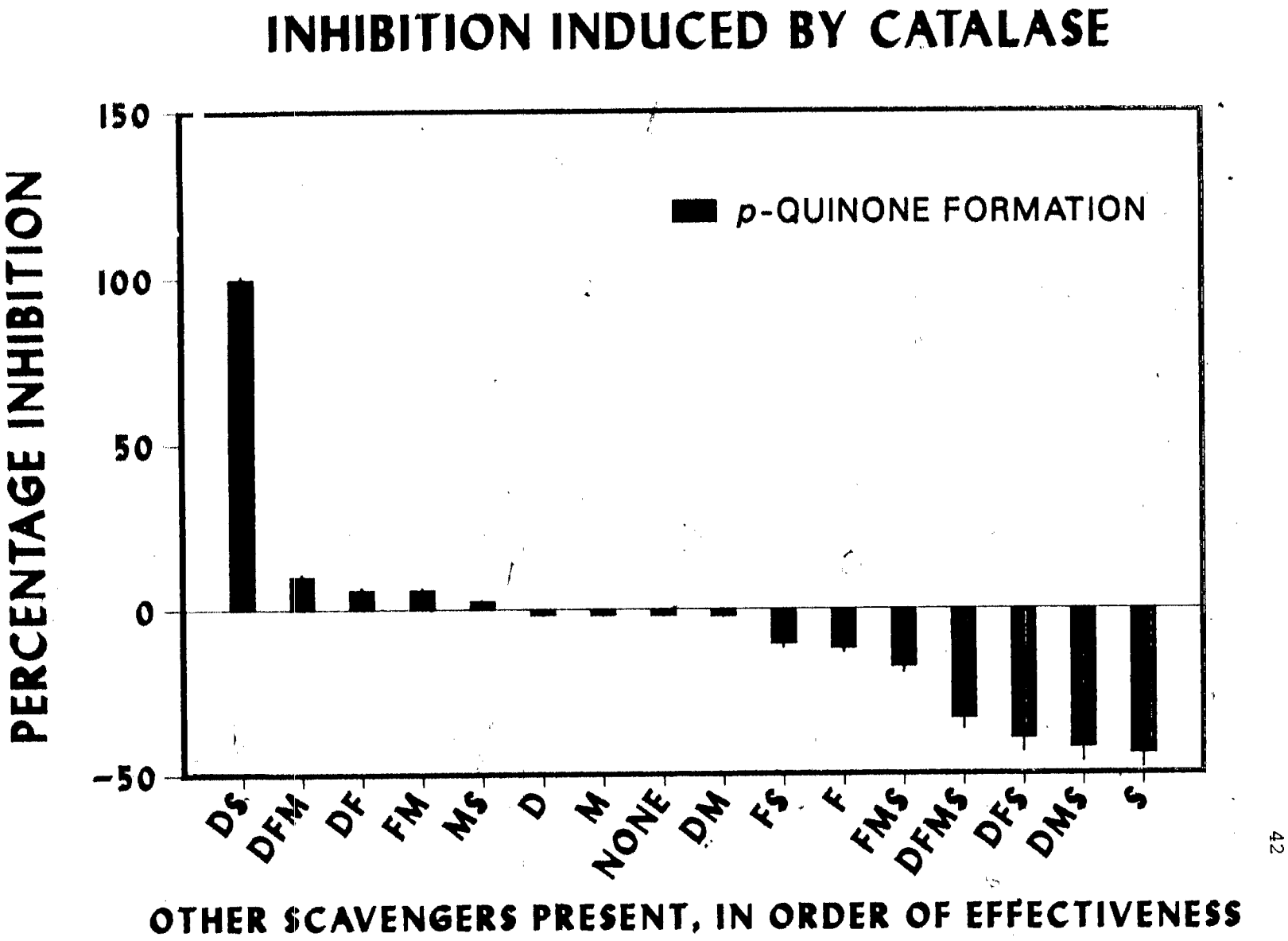


ROLE OF pH IN DTPA EFFECT




INHIBITION INDUCED BY CATALASE
PERCENTAGE INHIBITION
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100
50
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-50
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DFM
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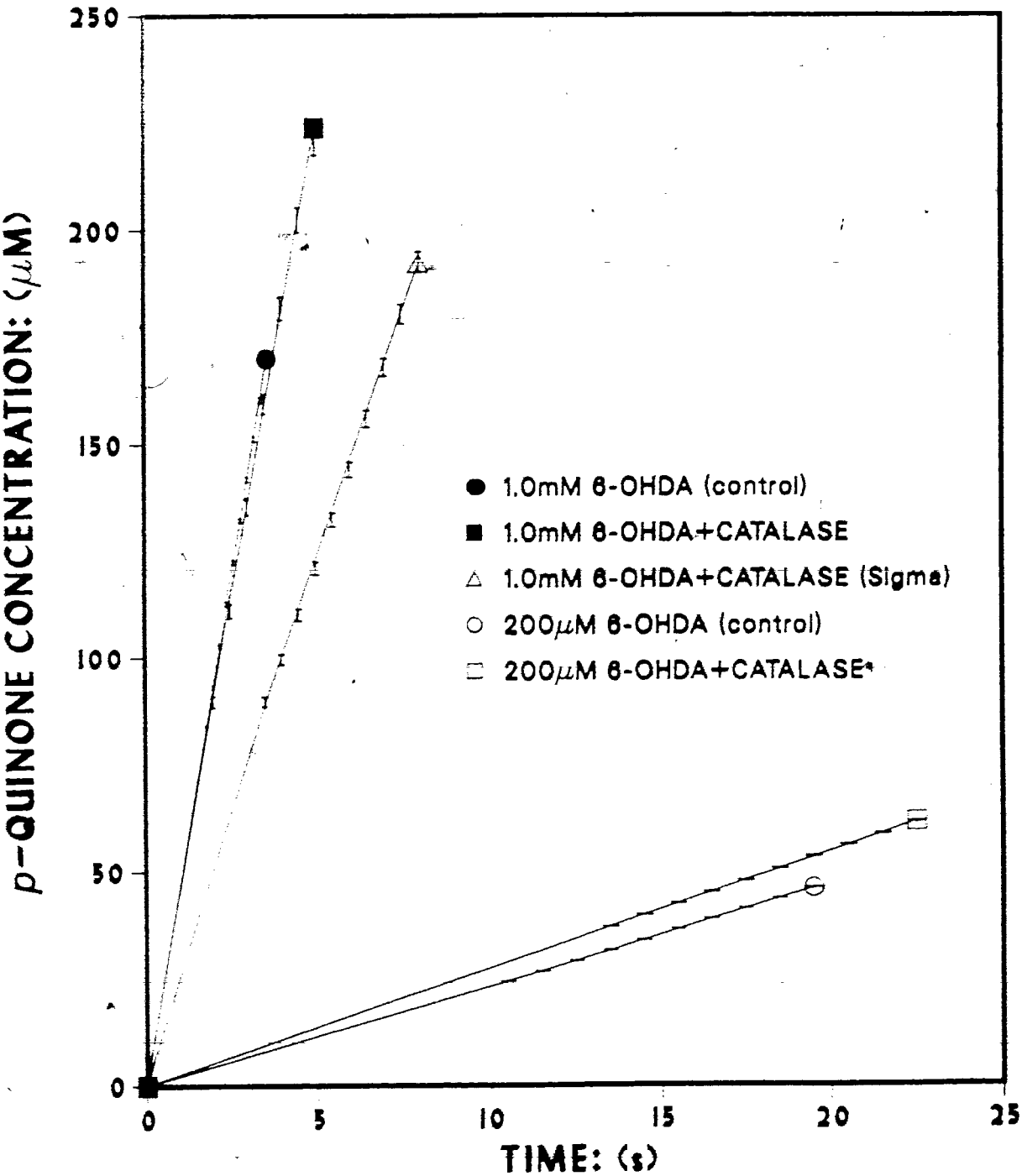
OTHER SCAVENGERS PRESENT, IN ORDER OF EFFECTIVENESS

LACK OF INHIBITION BY CATALASE



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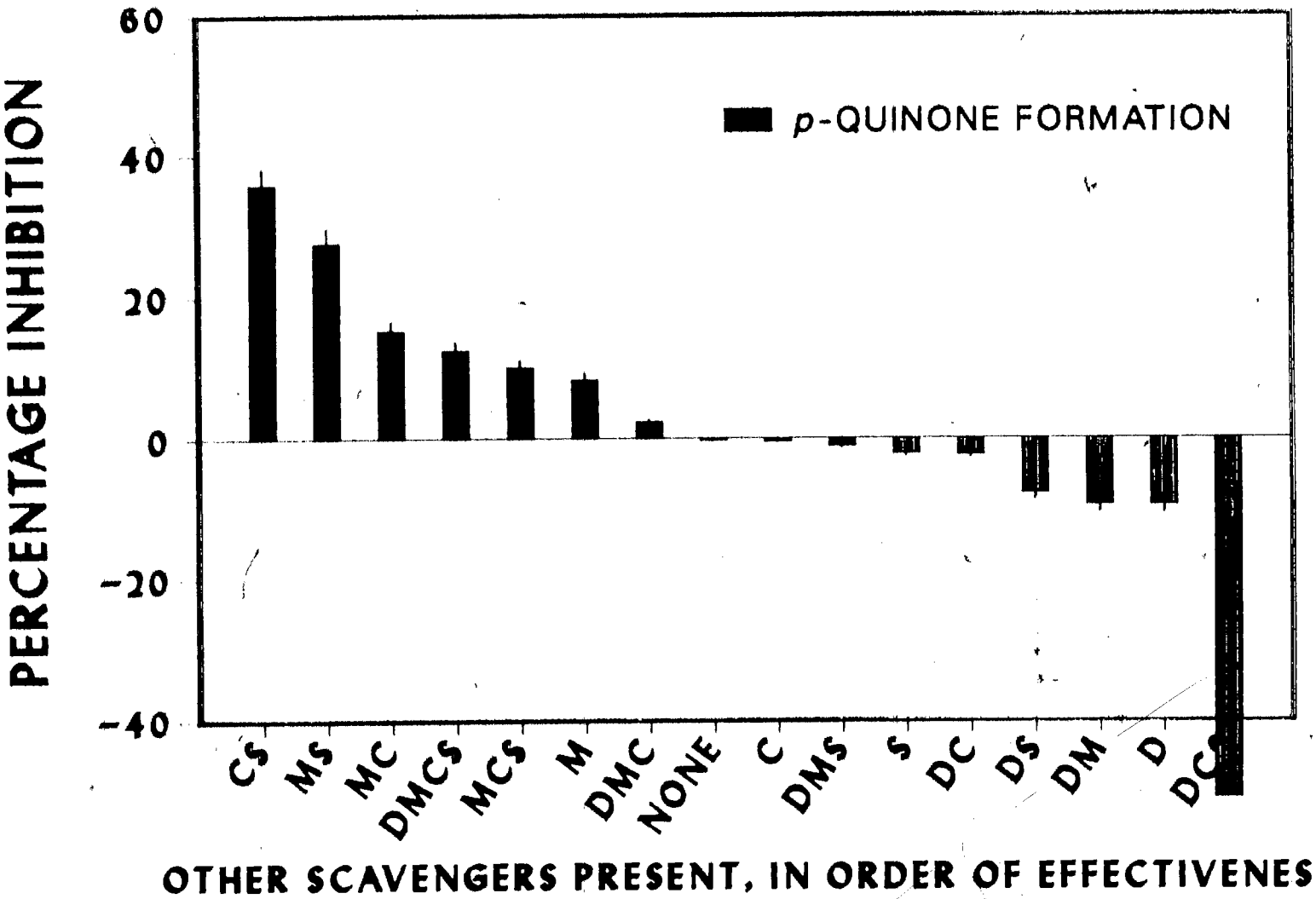

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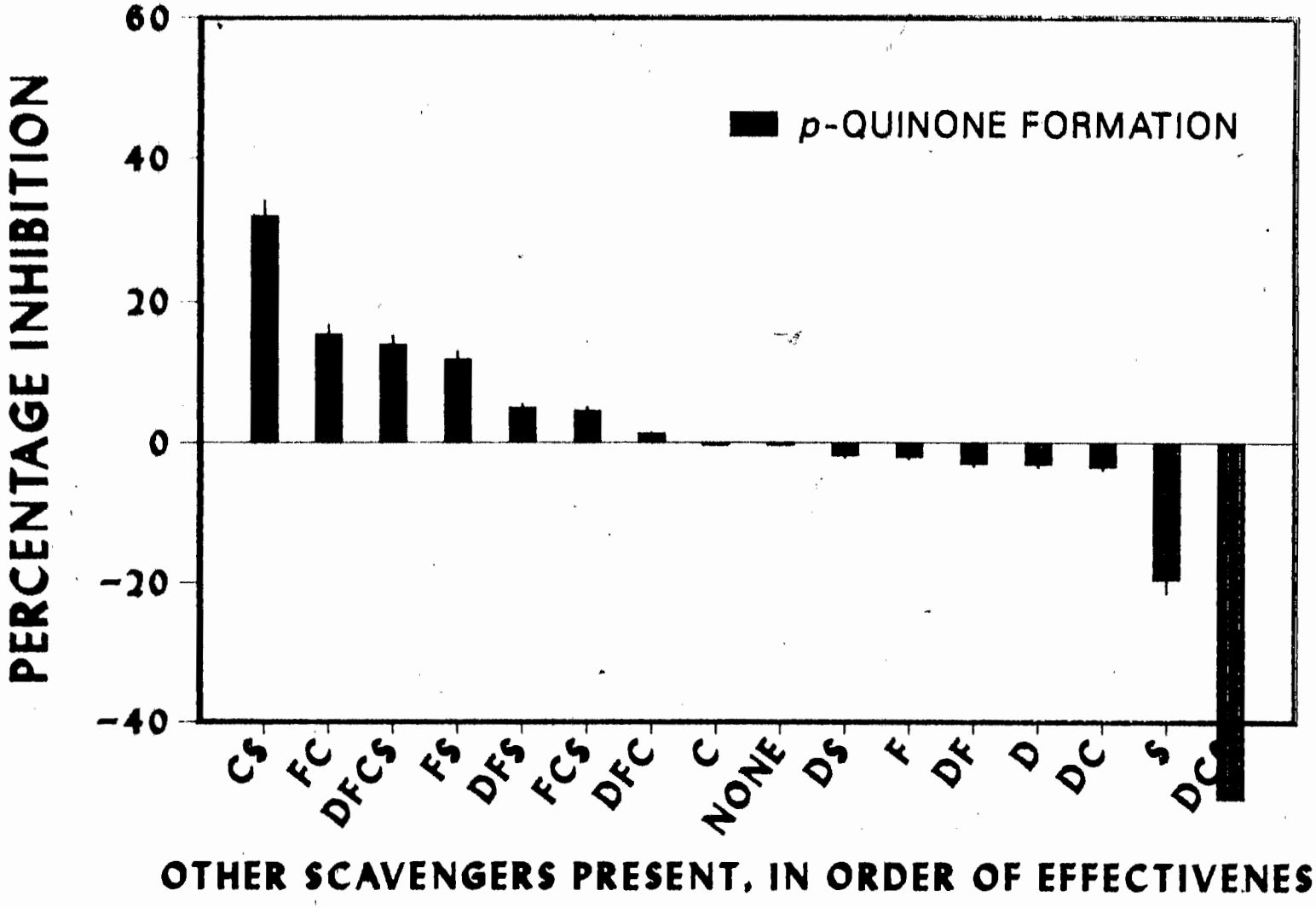
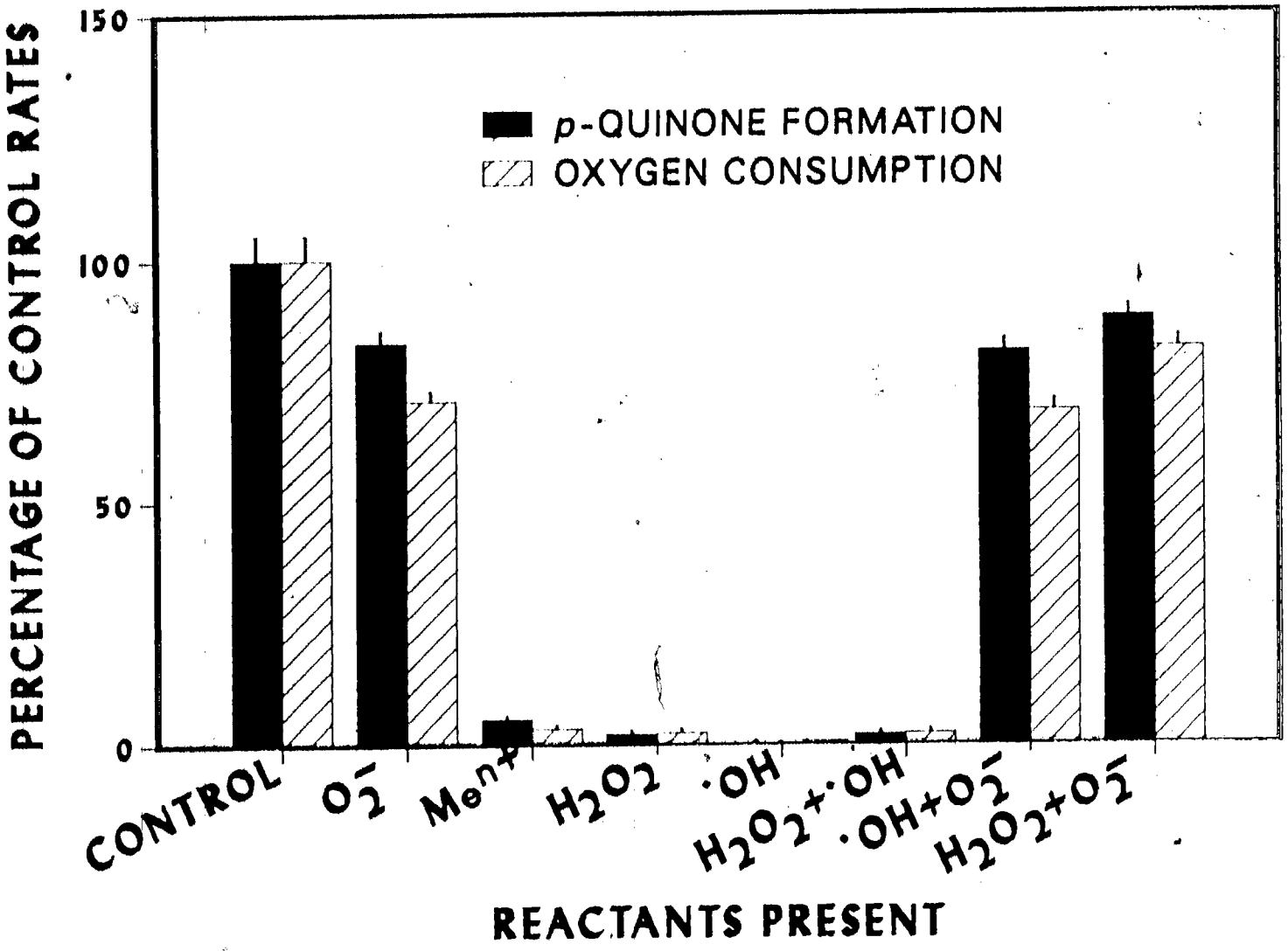
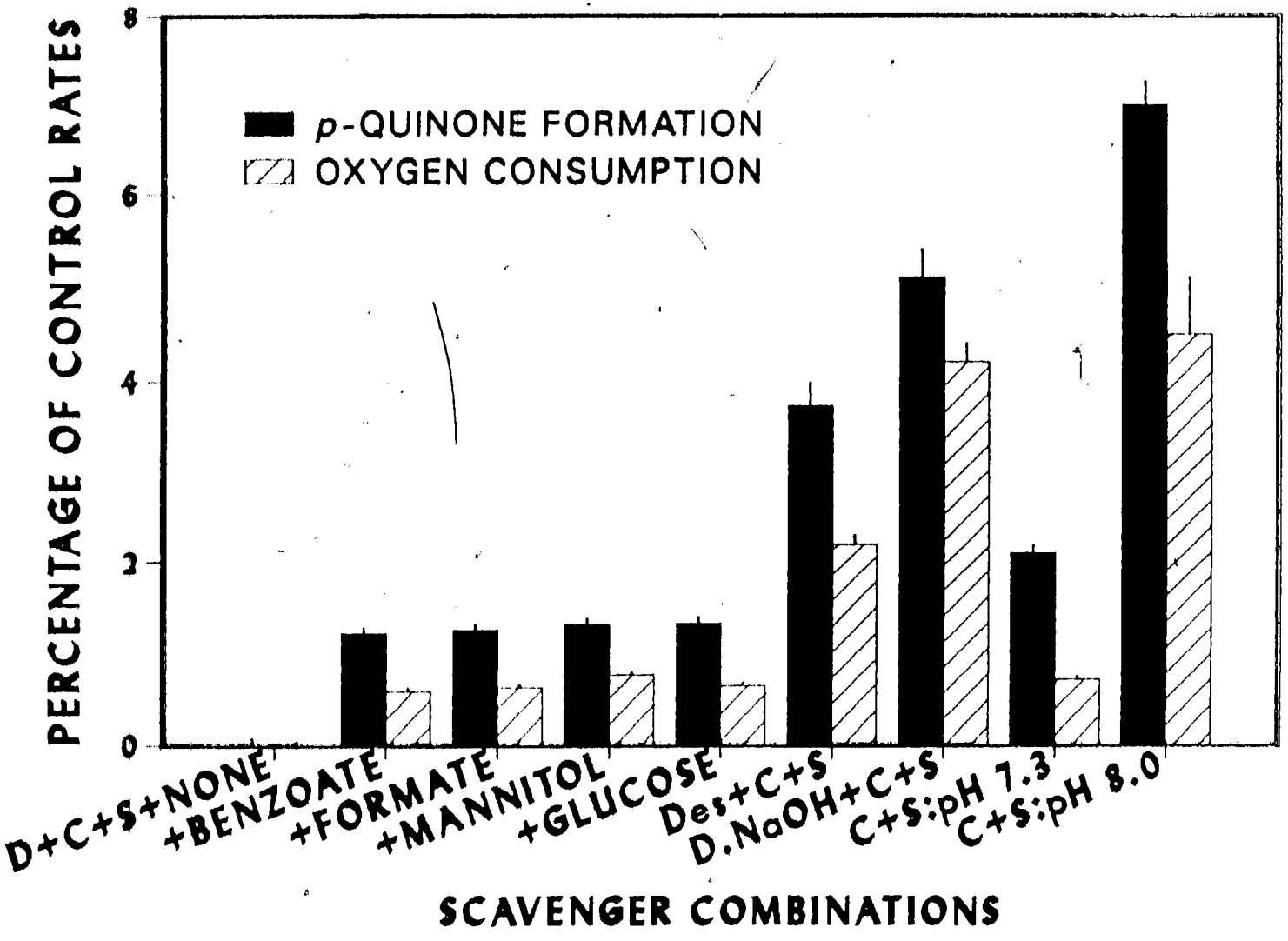


CONTRIBUTION OF SELECTED REACTION INTERMEDIATES



ACTIVATION OF THE D+C+S INHIBITED CONDITION



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6-Hydroxydopamine does not Reduce Molecular Oxygen Directly, but Requires a Co-Reductant[1]

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The autoxidation of 6-hydroxydopamine (6HODA) was virtually blocked ($k_2 < 10^{-15}$ M^{-1} s^{-1} at pH 8.0, ionic strength 0.04) by the simultaneous presence of diethylenetriaminepentaacetic acid (DTPA), catalase, and superoxide dismutase (SOD). No quinone product or oxygen consumption was detectable after 12 min under these conditions. Thus, if 6HODA is to react with molecular oxygen at a measurable rate, some other redox species is required as a coreductant. The subsequent addition of formate or mannitol proved capable of overcoming the total inhibition induced by the mixture of catalase, SOD, and DTPA. The simplest interpretation of the data is that most of the autoxidation of 6HODA, as commonly observed, involves successive reduction of a series of metal-bound species of oxygen; the actual transfer of electrons occurring within a ternary reductant-metal-oxygen transition state.

6-Hydroxydopamine (6HODA),[4] a neurotoxin specific toward catecholaminergic neurons, has been the subject of much investigation (1–8). While it is generally accepted that its neurotoxicity results from its spontaneous oxidation by molecular oxygen, neither the mechanisms of its autoxidation nor its neurotoxicity have been unambiguously elucidated.

Molecular oxygen meets the thermodynamic requirements for the oxidation of many organic compounds, but quantum mechanical aspects of its electronic structure diminish its kinetic reactivity. In that the molecular orbitals of lowest available energy contain two unpaired electrons with parallel spin, electron transfers to oxygen must occur by a series of single electron transfers to overcome spin restrictions. Thus all autoxidations proceed by complex pathways.

While the reaction between 6HODA and oxygen may seem to proceed smoothly to completion under the usual reaction conditions, the mechanism is complex, as evidenced by the current results which show that it is not capable of truly spontaneous oxidation by molecular oxygen alone. In its autoxidation, the transfer of a single electron in the initial elementary step is contingent upon the presence of at least one of several reactive radical intermediates. The 6HODA/O_2 reaction has been shown to be partially inhibited by superoxide dismutase (SOD) (3, 9, 10), diethylenetriaminepentaacetate (DTPA) (8–10), or, under some conditions, by catalase (9). The current study reports selected data from a comprehensive study we have undertaken of interactions between all possible combinations of the above and other scavengers.

While the immediate actions of catalase and SOD are well defined, the effects of

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[4] Abbreviations used: 6HODA, 6-hydroxydopamine; SOD, superoxide dismutase; DTPA, diethylenetriamine pentaacetate.

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DTPA are more complex. DTPA is a relatively nonspecific chelator of divalent metal ions (11), including iron. At least in the case of iron, the effect of DTPA is to decrease the redox potential so that, while the iron can still readily be oxidized, its reduction is prevented except by the strongest reducing agents. Thus, while DTPA blocks most of those autoxidations which are mediated by redox cycling of transition metals, the DTPA-Fe^{2+} chelate remains able to participate in Fenton-type reactions (12). For some compounds, such as cytochrome *c*, autoxidation at a significant rate requires the presence of coreductants or transition metal ion catalysts. Catalysis of its autoxidation by copper(bis)histidine involves participation of a ternary oxygen-metal-reductant complex (13). From the effects of individual scavengers and combinations of scavengers reported here, the various free radical species involved function not merely as reaction intermediates, but also as catalysts which play the role of obligatory coreductants of oxygen.

MATERIALS AND METHODS

6-Hydroxydopamine hydrobromide (Sigma Chemical Co.) was prepared anaerobically to yield a final concentration of 1.0 mM. This method of preparation resulted in a preparation containing an initial concentration of *p*-quinone product equal to 7.9 ± 0.3 μM. The use of relatively high concentrations of 6HODA and alkaline assay conditions provided reaction rates high enough to allow determination of rate constants even in the presence of those scavengers which dramatically slowed the reaction.

Diethylenetriaminepentaacetic acid, also from Sigma, was dissolved in aerated phosphate buffer while other reagents, catalase from bovine liver (CalBiochem-Behring Corp., 38,323 U mg^{-1}), superoxide dismutase from bovine blood (Sigma, 2900 U mg^{-1}), sodium formate (J. T. Baker Chemical Co.), and mannitol (Fisher Scientific Co.) were freshly prepared in aerated, distilled, deionized water. Ion contamination of the water was negligible (>1 MOhm cm^{-1}). Although iron present as a result of contamination from the buffer salts (American Scientific and Chemical, and Matheson Coleman and Bell Manufacturing Chemists) was estimated to have an upper limit of 0.69 μM, with a further 0.02 μM from the formate salt, analysis by inductively coupled plasma emission spectroscopy revealed that actual concentrations of transition metal ions were below the limits of detection, namely 54 nM (iron).

The formation of quinone product(s) of 6HODA was monitored spectrophotometrically at 490 nm while O_2 consumption was followed by a Clarke-type O_2 electrode. Reaction conditions are described in the legends to figures. All data were collected on-line through an analog-digital converter locally designed and based on a Motorola 6802 chip, and transferred to an IBM 4341 computer. All subsequent data analyses were performed by APL programs written by the authors.

RESULTS

Despite the presence of abundant oxygen, the simultaneous presence of DTPA, catalase, and SOD prevented the aerobic oxidation of 6HODA. This is shown by the absence of either *p*-quinone product formation (Fig. 1a), or oxygen utilization (Fig. 1b). The small initial decrease in oxygen concentration corresponds exactly to that predicted from the dilution of aerated buffer by the anaerobic 6HODA stock solution. Addition of a further aliquot of 6HODA, which is indicated in Fig. 1a (at 6.7 *min*) as the small instantaneous deflection of the curve upward (due to trace amounts of preformed product), failed to induce any further autoxidation by either criterion.

It was confirmed that if any of the three scavengers was omitted, substantial rates of formation of *p*-quinone and of oxygen consumption ensued and continued until oxygen was completely exhausted. Surprisingly, if either mannitol or formate was added, the oxidation recommenced (Fig. 2).

We have carried out a comprehensive study of the effects of scavengers on these reactions, in which every possible combination of the above scavengers, collectively and individually was determined. Figure 3 summarizes the small selection of these rates which are relevant to our conclusions.

In all reactions, the presence of catalase decreased the net rates and amounts of O_2 consumed to approximately one-half of those for *p*-quinone formation, reflecting (predictably) the regeneration of O_2 from H_2O_2. In the absence of other scavengers, mannitol, formate, or mannitol plus formate had no measurable effects on the

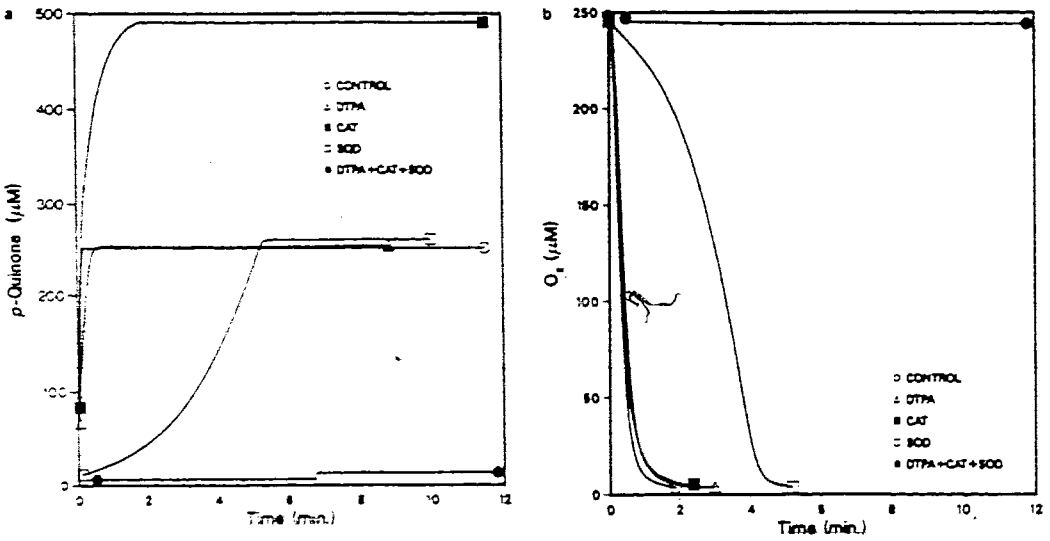

FIG. 1. Progress of the autoxidation of 6HODA. Effects of scavengers. Reactions were carried out in air-saturated phosphate buffer (246 μM O_2), pH 8.00, ionic strength 0.04, 25°C. Final concentrations of catalase and SOD were 250 U ml^{-1}, and DTPA was 1.0 mM. (a) Production of p-quinone; (b) consumption of oxygen.

rates of the control reactions. This excludes kinetically significant participation of hydroxyl radicals prior to the rate-determining step. In the presence of other scavengers, they were sometimes stimulatory and sometimes inhibitory.

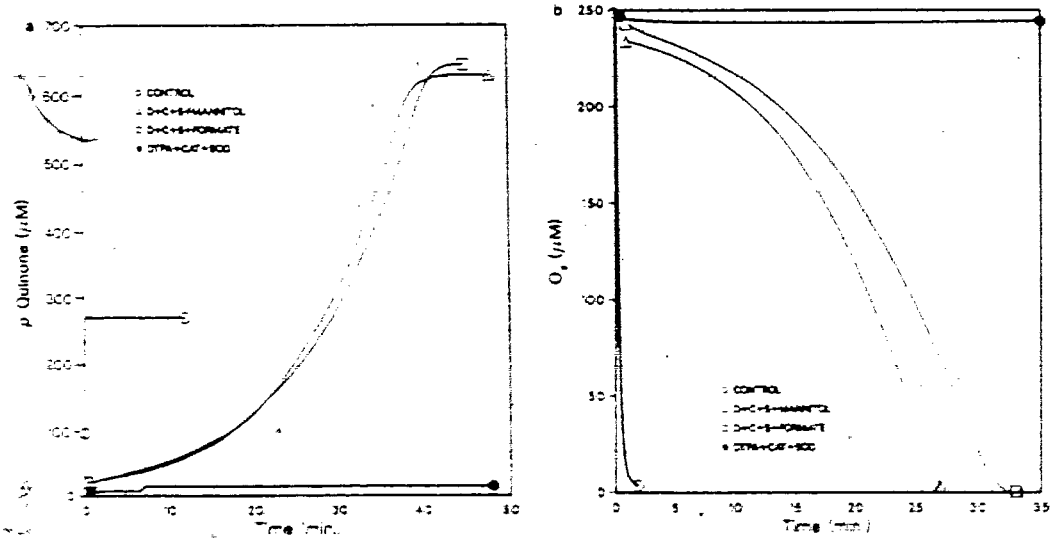

FIG. 2. Progress of the autoxidation of 6HODA. Reversal by formate or mannitol of the inhibition produced by the simultaneous presence of catalase, SOD, and DTPA. Reaction conditions were as in Fig. 1. Final concentrations of formate and mannitol were 10 mM. D, DTPA; C, catalase; S, SOD. a, Production of p-quinone; b, consumption of oxygen.

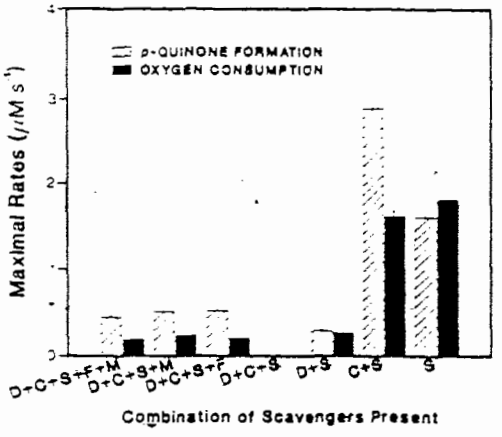

FIG. 3. Rates of autoxidation of 6HODA. Comparison of effects of scavengers. Reaction conditions were as in Fig. 1. The control rate for *p*-quinone formation is not shown but was 46 ± 2.2 μM s^{-1}. Error bars represent standard deviations of the rates plotted. D, DTPA; C, catalase; S, SOD; F, formate; M, mannitol.

DISCUSSION

The loss of reactivity of 6HODA toward molecular oxygen caused by the presence of scavengers of the major coreductants reflects the complexity of the reaction pathway required to circumvent the severe kinetic constraints in the process, which is nevertheless highly favored thermodynamically. Thus, in the simultaneous presence of DTPA, SOD, and catalase, a mixture of oxygen and 6HODA coexists for at least 12 min without any detectible transfer of electrons occurring between them. In comparison, in the absence of these scavengers, oxidation proceeded to virtual completion within 15 s.

The inhibition of both O_2 consumption and 6HODA oxidation by DTPA + catalase + SOD excludes the elementary reaction

$$6\text{HODA} + O_2 \rightarrow \text{semiquinone} + O_2^- + H^+ \quad [1]$$

as a major participant in the autoxidation of 6HODA. Therefore, the involvement of metal ions must precede this step. However, the data also rule out significant participation of metal ions in the elementary reaction

$$6\text{HODA} + Me^{n+} \rightarrow \text{semiquinone} + Me^{(n-1)+} + H^+. \quad [2]$$

Although this reaction has been shown to occur slowly in the absence of oxygen (5), the massive inhibition by SOD alone rules out its possible role as an alternative first step for any major pathway for the autoxidation of 6HODA. The reasoning is that, since the presence of O_2^- would not be essential to the aerobic oxidation of the resulting reduced metal ion, this reaction would not be inhibited by superoxide dismutase. Thus, if Reaction [2] did occur, the overall reaction would not be as strongly inhibited by superoxide dismutase, as is shown in figs. 1 and 2. Consequently, the most plausible candidate for the role of a direct oxidant of 6HODA is a metal-oxygen complex, initially a metal-superoxide complex (superoxide dismutase is not only inhibitory but also causes a substantial induction period), and later a more highly reduced metal-oxygen species, including the metal-peroxide complex.

The simplest explanation of the observation that the addition of either mannitol or formate to the inhibited reaction releases the transfer of electrons from 6HODA to oxygen is that these scavengers generate species reactive enough to reduce the ferric-DTPA complex. Presumably these species are the semi-dehydromannitol (14) and carboxylate (15) radicals, respectively. The resulting ferrous-DTPA complex can undergo both oxidation by H_2O_2 in Fenton-type reactions (12) and cyclic rereduction by radical species of the hydroxyl scavengers mannitol or formate. In many systems, reactions with hydroxyl scavengers are thought to be terminating reactions. However, in the current studies both mannitol and formate promote propagation of the free radical chain. In the 6HODA/O_2 system; their effects are not always as predictable and clear-cut as usually assumed.

REFERENCES

1. HARLEY-MASON, J. (1953) *J. Chem. Soc.* 200.
2. ADAMS, R. N., MURRILL, E., MCCREERY, R., BLANK,

L., AND KAROLSZAK, M. (1972) *Eur. J. Pharmacol.* 17, 287.
3. COHEN, G., AND HEIKKILA, R. E. (1974) *J. Biol. Chem.* 249, 2447.
4. GRAHAM, D. G., TIFFANY, S. M., BELL, W. R., JR., AND GUTKNECHT, W. F. (1978) *Mol. Pharmacol.* 14, 644.
5. FLOYD, R. A., AND WISEMAN, B. B. (1979) *Biochim. Biophys. Acta* 586, 196.
6. JONSSON, G. (1980) *in* Annual Review of Neuroscience (Cowam, W. M., Hall, Z. W., and Kandel, E. R., eds.), Vol. 3, pp. 169–187, Ann. Revs., Palo Alto.
7. TIFFANY-CASTIGLIONI, E., SANETO, R. P., PROCTOR, P. H., AND PEREZ-POLO, J. R. (1982) *Biochem. Pharmacol.* 31, 181.
8. HEIKKILA, R. E., AND CABBAT, F. S. (1981) *Biochem. Pharmacol.* 30, 2945.
9. SULLIVAN, S. G., AND STERN, A. (1981) *Biochem. Pharmacol.* 30, 2279.
10. GEE, P., AND DAVISON, A. J. (1983) *Fed. Proc.* 42, 2935.
11. CHABEREK, S., FROST, A. E., DORAN, M. A., AND BICKNELL, N. J. (1959) *J. Inorg. Nucl. Chem.* 11, 184.
12. COHEN, G., AND SINET, P. M. (1982) *FEBS Lett.* 138, 258.
13. DAVISON, A. J. (1968) *J. Biol. Chem.* 243, 6064.
14. MCCORD, J. M., AND FRIDOVICH, I. (1973) *Photochem. Photobiol.* 17, 115.
15. KRIG, D., RABANI, J., AND FRIDOVICH, I. (1972) *J. Biol. Chem.* 247, 4839.

ANAEROBIC OXIDATION OF 6-HYDROXYDOPAMINE BY H_2O_2: EFFECTS OF SCAVENGERS OF OXYGEN FREE RADICALS

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Abbreviations:

6-OHDA, 6-hydroxydopamine; SOD, superoxide dismutase;

DTPA, diethylenetriaminepentaacetic acid;

SUMMARY

H_2O_2, a product of the aerobic autoxidation of 6-hydroxydopamine, is also consumed as a reactant, contributing progressively more to the oxidation as the concentration of O_2 becomes limiting. H_2O_2 is a less effective oxidant than O_2, since the anaerobic peroxidatic oxidation of 6-hydroxydopamine is slower than the aerobic oxidation by three orders of magnitude. The hydroxyl radical scavengers generally inhibited the anaerobic peroxidation. Thus mannitol inhibited by 13-40%, glucose by 41-62%, and benzoate by 15-100%, showing that $^{\cdot}OH$ catalyses the oxidation. A specific role for iron in the reaction as normally observed was shown by the strongly inhibitory action of desferrioxamine (76 to 91%), regardless of which other scavengers were present. The further addition of diethylenetriaminepentaacetate (DTPA), benzoate or formate to desferrioxamine inhibited the reaction completely. In contrast, the presence of DTPA alone, accelerated the reaction by 160%, augmenting the catalytic actions of transition metal ions. This acceleration is in part due to stimulation by DTPA of production of $^{\cdot}OH$ (by Fenton-type reactions), since it was partially prevented by the hydroxyl scavengers, benzoate (32% inhibition) and glucose (41%). Thus DTPA inhibits the participation of metals other than iron, but potentiates the catalytic role of iron, in the reduction of hydrogen peroxide. The semidehydromannitol radical, can reduce the DTPA-Fe^{3+} chelate directly, since mannitol accelerated the DTPA stimulated

rate (55%). Superoxide dismutase unexpectedly accelerated the reaction (by 57-84%). This activation was seen regardless of which other scavengers were present. These effects are explained in part in terms of potentiating or moderating interactions among the reactive intermediates which propogate the overall reaction.

INTRODUCTION

The cytotoxicity of 6-hydroxydopamine (6-OHDA) can be attributed largely to free radical production during its oxidation by molecular oxygen. Since catecholaminergic uptake systems fail to distinguish the structure of 6-OHDA from those of the endogenous catecholamines, it is concentrated to lethal concentrations by sympathetic neurones [1,2]. Once inside the neurones, autoxidation of 6-OHDA generates a complex mixture of reactive species both of its quinone and of molecular oxygen. These species react irreversibly and somewhat indiscriminantly with cellular components, eventually leading to destruction of the nerve terminals, and in some cases of the cell body itself.

While the aerobic oxidation of 6-OHDA has been extensively characterised [3-8] its oxidation by H_2O_2 has been largely neglected, in part due to the slowness of this reaction. However, while H_2O_2 contributes very little to aerobic oxidation, its participation as an oxidising species increases when O_2 becomes limiting. In fact, the amount of H_2O_2 remaining after completion of the two electron oxidation is always less than that predicted by the generally accepted stoichiometry [9]:

$$\text{6-OHDA} + O_2 \longrightarrow \underline{p}\text{-quinone} + H_2O_2 \quad (1).$$

Because of its relative stability (measurable biological lifetime)

in comparison with other intermediates in the reduction of O_2, H_2O_2 not only has the opportunity to attack cellular components in the immediate environment but may diffuse some distance from its site of generation. Since its removal was found to be beneficial, H_2O_2 is thought to be a major contributing species in pro-oxidant mediated damage. For example, catalase provided nearly complete protection against 6-OHDA toxicity in cultured neuroblastoma cells [10]. Moreover, resistance to 6-OHDA toxicity by some cell types was strongly correlated to levels of endogenous peroxidases. Surprisingly, in the same series of experiments, superoxide dismutase (SOD) did not protect, but merely delayed 6-OHDA-induced destruction of neuroblastoma cells, although SOD profoundly inhibits (96%) the aerobic oxidation itself [11]. Similiarly, the clastogenic activity resulting from autoxidation of caffeic acid is largely attributed to H_2O_2, since catalase provided substantial protection against mutagen induced genotoxicity in Chinese hamster ovary cells [12].

However, H_2O_2 itself is not very damaging when added directly to several systems which were sensitive to H_2O_2 produced during irradiation [13,14] or enzymically [15]. H_2O_2, unlike molecular oxygen, lacks those spin restrictions which present large kinetic barriers against concerted two electron reduction and has a relatively low redox potential so that it can act as an oxidising and reducing agent. For example, H_2O_2 can consecutively [16] or simultaneously [17] reduce ferricytochrome c and oxidise

ferrocytochrome c. In the presence of O_2 and transition metal ions, H_2O_2 may be oxidised to O_2^- or may be reduced in Fenton-type interactions to produce $^{\cdot}OH$, which has a redox potential comparable to that of molecular oxygen but lacks its kinetic hindrances. Thus while the reactivity of H_2O_2 alone cannot account for the cytotoxicity observed, $^{\cdot}OH$, a product of its oxidation in metal catalysed Fenton-type reactions may be responsible for much of the damage. The "Haber-Weiss cycle" is a special case of the Fenton reaction in which O_2^- is the species which provides the reducing equivalents to recycle the iron catalyst. However, in cellular systems, many reducing participants are available (e.g. ascorbate) and as yet there is not body of data to evaluate the importance of these substances.

Powerful pro-oxidants like 6-OHDA may not only generate H_2O_2, but once formed they may themselves act as Fenton donors to increase the toxicity of H_2O_2 by reducing it to $^{\cdot}OH$. Since oxygen is not directly involved in Fenton interactions and may be involved in competing reactions, this latter role is best studied anaerobically. The current investigation was therefore undertaken to determine the involvement of Fenton-type interactions in the aerobic and anaerobic oxidations of 6-OHDA. More particularly we wished to examine the roles of metals in the reduction of H_2O_2 by 6-OHDA and to determine the extent of participation of $^{\cdot}OH$ and other free radicals.

MATERIALS AND METHODS

REAGENTS

6-Hydroxydopamine hydrobromide and diethylenetriaminepentaacetic acid were purchased from the Sigma Chemical Co. (St. Louis, MO). Sodium phosphate buffers were obtained from American Scientific and Chemical (Seattle, WA) and Matheson Coleman and Bell Manufacturing Chemists (Norwood, Ohio). Desferrioxamine (Desferal mesylate) was a gift from CIBA Pharmaceutical Co. (Summit, NJ). Chelex 100 resin and AG501-8X deionising resin were obtained from BioRad Laboratories (Mississauga, Ont., Canada). Sodium formate was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ) while mannitol, glucose (dextrose) and sodium sulfite were obtained from Fisher Scientic Co. (Fair Lawn, NJ). Sodium benzoate was purchased from Anachemia Chemicals Ltd. (Toronto, Canada). Superoxide dismutase (bovine blood, 2800-2900 U mg^{-1}) was obtained from Sigma Chemical Co. Hydrogen peroxide was obtained from BDH Chemicals Canada Ltd. (Vancouver, B.C.) All reagents were of the highest purity commercially available at the time of purchase.

Fresh double deionised distilled water (>7 X 10 megaohm cm^{-3}) was passed through a Chelex 100 column. The following metal ions from the buffer salts were below the stated values (which represent detection limits of inductive coupled plasma emission

spectroscopy): copper <79 nM; iron < 54 nM; manganese <18 nM; aluminium <741 nM and vanadium <39 nM. Residual levels of metal ions in the Chelex treated buffers were determined by atomic absorption for the following species: copper <1.6 nM (below detection limit); iron 31.3-32.23 nM; manganese 8.2 nM; and aluminum 23 nM. Phosphate buffer, pH 8.00, ionic strength 0.04, was made from acid and base which had been seperately treated by Chelex 100 resin. These were titrated against each other to the desired pH since pH of any premixed buffer changed upon passage through the column, even after prior equilibration of the column with the same buffer.

In some reactions, DTPA (1.0 mM) was added as a non-specific chelating agent for transition metal ion contaminants [18] while desferrioxamine (1.0 mM) was added to chelate iron specifically. Other scavengers were added in excess of 10 times the amount needed to remove the estimated yield of each radical species. Benzoate (10 mM) was used to scavenge $^{\bullet}OH$ since it reportedly has only minimal redox interactions [19]. Formate (10 mM) was used to detect any leakage of air into the anaerobic system, since the immediate product from $^{\bullet}OH$ scavenging by formate is CO_2^- which in the presence of O_2 produces O_2^- [20] a catalytic species in the autoxidation of 6-OHDA [21]. Thus, an increase in the rate of oxidation of 6-OHDA caused by the addition of formate was expected if any O_2 was present. In view of the dubious specificity of $^{\bullet}OH$ scavengers, mannitol (10 mM) and glucose (10 mM) were also used,

to allow comparison of a range of hydroxyl scavengers. Superoxide dismutase (SOD, 250 U ml^{-1}) was used to scavenge any superoxide formed in the course of the reaction or preformed in the stock solution prior to deoxygenation.

ANAEROBIC PREPARATION OF REAGENTS

Chelex 100 treated water or buffer was repeatedly flushed with high purity nitrogen (Linde, Union Carbide Canada Ltd.; scrubbed with sodium sulphite) and then evacuated using a Virtis vacuum evacuator. Trapped air in the 6-OHDA powder was gently removed under vacuum and replaced with nitrogen, during dissolution. This vial was sealed under a slight positive pressure to minimise exposure of oxygen as aliquots were withdrawn. The premature presence of pquinone products was monitored from initial absorbance at 490 nm. This measure provided an indication both of baseline oxidation and of the quality of the stock solution which was uniformly less than 1% oxidised (0.79 $\pm$ 0.03%). The initial concentration of 6-OHDA under anaerobic conditions (pH 6.0-7.0) was estimated from the absorbance at 290 nm, while a null difference spectrum over the visible range from a sample at pH 2.00, and the symmetry of the 290 nm peak were taken as indications of the homogeneity of the preparation. All other reagents were deoxygenated and sealed under a slight positive pressure of nitrogen.

ASSAY PROCEDURES

Buffers were continously and gently bubbled with nitrogen previously scrubbed with sodium sulphite and saturated with water vapour. Aliquots with appropriate dilutions were transferred to an anaerobic cuvette fitted with a stopcock. These were bubbled for 8-10 min after which 0.05 ml aliquots of scavengers were added and bubbled for at least 5 min more to ensure equilibration. The cannula was then taken out of the solution and secured to the stopcock in such a way as to gently supply a continuous flow of nitrogen over the surface of the medium, throughout the reaction. The reaction was then initiated by 0.05 ml 6-OHDA and formation of p-quinone product was followed at 490 nm using a Beckman DB-GT spectrophotometer. pH was checked in the reaction medium before and after addition of scavengers and also at the start and upon completion of the reaction for representative conditions. Data were collected on-line through a twelve bit analogue-digital converter and transferred to an IBM 4341 or 3033 computer using a microprocessor data buffer/coupler locally designed and constructed.

DATA ANALYSES

All subsequent data analyses were performed using APL programmes written by the authors and implemented on a IBM 4341 or 3033 computer. Digitised voltages were converted to micromolar

concentrations of p-quinone using the internally determined molar extinction coefficent of 1892 M^{-1} cm^{-1}. Linear regressions over appropriate segments of the reaction profiles were used to determine initial rates of reaction [22].

RESULTS

STOICHIOMETRY OF THE 6-OHDA/O_2 REACTION WITH RESPECT TO H_2O_2 AND O_2

The conventional stoichiometry (Reaction 1) predicts ratios of 1:1:1:1 for p-quinone produced : H_2O_2 produced : 6-OHDA oxidised : O_2 consumed. These ratios were not observed. At low concentrations of oxygen and of 6-OHDA we did indeed find a ratio of 0.99 for H_2O_2 produced per O_2 consumed (slope of left hand portion of the curve in Fig. 1 is 0.99), but neither the amounts of 6-OHDA consumed nor p-quinone produced corresponded to that predicted on the basis of either O_2 consumption or H_2O_2 production. Even at lower concentrations of 6-OHDA, the total amounts of O_2 consumed and H_2O_2 remaining upon completion of the reaction were only 89% of those predicted by a two electron oxidation (slope of the left hand portion of the curve is 0.89 in Fig. 2). When concentrations of 6-OHDA (>250 μM) exceed that of O_2, the yield of H_2O_2 at completion of the autoxidation was considerably less than the predicted stoichiometry, in partial agreement with Liang et al. [9]. Thus, 250 μM 6-OHDA corresponds to that needed to consume all of the oxygen initially present in an air-saturated aqueous solution. The commencement of substantial utilisation of H_2O_2 (strongly negative slope of the H_2O_2 curve above 250 μM 6-OHDA in Fig. 2) then, coincided with the exhaustion of molecular oxygen (horizontal portion of the O_2 curve).

COMPETITION BETWEEN H_2O_2 AND O_2 FOR THE PROVISION OF OXIDISING EQUIVALENTS

That H_2O_2 is a much less effective oxidant of 6-OHDA than O_2 was confirmed by the observation that the oxidation of 1.0 mM 6-OHDA by 500 µM H_2O_2 under anaerobic conditions required approximately 5.6 h for completion (while only 10 s was required for complete oxidation at 250 µM O_2; not shown). Reactions were usually run to "completion", which for our purposes was defined as the point at which the rates of oxygen utilisation and p-quinone formation were less than 1% of the maximal rate. At concentrations of 6-OHDA above 500 µM, no detectible H_2O_2 remained upon completion of the reaction (termination of H_2O_2 curve Fig. 2).

In the presence of excess 6-OHDA, disappearance of H_2O_2 could be demonstrated by the addition of catalase at successive time intervals. During the first 5 min after cessation of O_2 consumption, the amount of H_2O_2 remaining decreased from approximately 190 µM to 160 µM. Care was taken therefore not to obtain anomalously low estimates of H_2O_2 production as a result of delays in the addition of catalase. It should be noted in contrast that, under anaerobic but otherwise similar conditions the amount of H_2O_2 consumed in the production of p-quinone was less than 2 µM over the same time interval (estimated from Control curve, Fig. 3). Hence H_2O_2 was consumed at a much greater rate in the presence of O_2 than anaerobically.

EFFECTIVENESS OF INDIVIDUAL SCAVENGERS IN THE ANAEROBIC OXIDATION OF 6-OHDA BY H_2O_2

The hydroxyl scavengers, benzoate and mannitol provided 12-15% inhibition of the anaerobic peroxidation. Thus an equivalent portion of the oxidation may be attributed to direct participation of $^{\bullet}OH$ (Fig. 4). Since desferrioxamine inhibited the reaction by 76%, catalysis by iron was substantial. Although some of the inhibition may be due to its capacity to scavenge $^{\bullet}OH$ [23], hydroxyl scavengers had comparatively small effects. Since all other solutions were treated by Chelex 100 resins, any residual iron probably comes from the H_2O_2 reagent, which cannot be treated by deionising resins. In contrast to the inhibition by desferrioxamine, DTPA played a stimulatory role, accelerating the the peroxidation by 160% (Figs. 3 and 4). Surprisingly, SOD stimulated the anaerobic oxidation by 57% (Figs. 3 and 4), in direct contrast to its strong inhibitory effect in the oxidation of 6-OHDA by molecular oxygen.

EFFECTS OF HYDROXYL SCAVENGERS IN THE PRESENCE OF DTPA OR DESFERRIOXAMINE

The hydroxyl scavengers further increased the inhibitory effect of desferrioxiamine (Fig. 5) by 40-100%. Specifically both formate (Fig. 4) and benzoate (Fig. 5) completely inhibited the anaerobic peroxidation indicating that in the absence of iron, $^{\bullet}OH$ comprises an essential link in the radical chain which propagates the

reaction. If the system was contaminated with oxygen some residual oxidation would have been seen in these latter systems. It was not. Mannitol (40%) and glucose (62%), which appear to participate in other reactions to promote p-quinone formation [8], were also inhibitory under these conditions (Fig. 5) but did not completely prevent the peroxidation.

In the presence of DTPA, not all hydroxyl scavengers tested were inhibitory. Glucose (41%) and benzoate (32%) decreased the stimulation by DTPA, but surprisingly, mannitol (otherwise inhibitory) further accelerated the peroxidation by 55% (Fig. 5). Not only did desferrioxamine decrease the stimulation induced by DTPA, but the anaerobic oxidation of 6-OHDA by H_2O_2 was completely blocked when both metal-chelating agents were present (Fig. 5). This implies an absolute requirement for at least one of the transition metal ions present as trace contaminants in the reaction medium.

EFFECTS OF SUPEROXIDE DISMUTASE

Whereas SOD alone increased the peroxidation of 6-OHDA by 57% (Fig. 4) it enhanced the stimulation by DTPA by 77% and relieved the inhibition induced by desferrioxamine by 84% (Fig. 5). The explanation that H_2O_2 destroyed SOD is not tenable, since there was no measurable decrease in the inhibition of the aerobic oxidation of 6-OHDA by SOD which had been incubated with H_2O_2 for

2.3 h under comparable conditions. Although this stimulation may be due to traces of free copper released from SOD, the total content from SOD added was estimated to be 53 nM. DTPA present at 1 mM would be expected to chelate copper as well as other transition metal ions. However in the presence of SOD, DTPA accelerated the peroxidation by 190%, while desferrioxamine (specific for iron) decreased the SOD stimulation by 72%.

DISCUSSION

COMPETITION BETWEEN O_2 AND H_2O_2 IN THE OXIDATION OF 6-OHDA

Reduction of O_2 by 6-OHDA is a two electron process in the presence of excess O_2, and a four electron process in the presence of excess 6-OHDA. Thus, H_2O_2 is an end product of the reaction if and only if 6-OHDA is limiting. The minimal concentration of 6-OHDA required to allow complete consumption of O_2 initially found in air-saturated buffers by a four electron transfer mechanism is 500 $\pm$ 30 µM.

ROLES OF TRANSITION METAL IONS

The strongly inhibitory effects of desferrioxamine (76%) confirm the importance of traces of iron in the mechanism of the reaction as normally observed. The remaining 24% of the oxidation is catalysed by non-iron transition metals, since the further addition of DTPA resulted in complete inhibition. That DTPA produces 100% inhibition when desferrioxamine is present implies that the stimulatory effects of DTPA (in the absence of desferrioxamine) were absolutely dependent on the presence of iron susceptible to chelation by DTPA. In other words, in the case of iron, chelation by DTPA stimulates interaction with H_2O_2, whereas in the case of the other transition metal ions chelation by DTPA has an overwhelmingly inhibitory effect. The stimulation induced by DTPA can in part be explained by the catalysis of Fenton-type

interactions by iron chelate to generate $^{\bullet}OH$:

$$H_2O_2 + DTPA\text{-}Fe^{2+} \longrightarrow {}^{\bullet}OH + OH^- + DTPA\text{-}Fe^{3+} \quad (2).$$

ROLES OF HYDROXYL RADICALS

In the simultaneous presence of desferrioxamine and either benzoate or formate, the reaction was inhbited completely. Clearly H_2O_2 alone does not react directly with 6-OHDA, but requires the presence of either transition metal ions, or hydroxyl radicals, or both. In the absence of kinetically accessible iron, the reaction becomes even more dependent on the presence of $^{\bullet}OH$, so that in the presence of desferrioxamine the hydroxyl scavengers increased the inhibition in the following order of effectiveness: mannitol (40%) < glucose (62%) < formate (100%) = benzoate (100%). The latter two values show that the catalytic effects of the non-iron transition metals are prevented by the presence of formate or benzoate, indicating that they depend on hydroxyl radical-mediated recycling of some important reaction intermediate(s). Similarly, chelation of transition metal ions by DTPA increases the contribution of the $^{\bullet}OH$ mediated processes in the reaction mechanism. This follows from the observations that in the presence of DTPA, glucose or benzoate inhibited by 32% or 41% respectively.

The role of $^{\bullet}OH$ as an important intermediate in the peroxidatic oxidation of 6-OHDA contrasts strongly with its non-involvement in

the autoxidation (by O_2). This implies a sequential single electron transfer mechanism from 6-OHDA to H_2O_2, even though H_2O_2 is capable of concerted two-electron reductions.

The stimulatory action of mannitol in the presence of DTPA, resembles its stimulatory effects in the presence of DTPA, or DTPA+catalase [11] or DTPA+catalase+SOD in the aerobic reaction [8] in which acceleration was attributed to the reactivity of semi-dehydromannitol radicals toward the iron-DTPA complex. The semi-dehydromannitol radical produced in the scavenging action of mannitol may promote p-quinone production by reducing the DTPA-Fe^{3+} complex formed in Reaction 2:

$$\text{DTPA-Fe}^{3+} + {}^{\bullet}\text{MH} \longrightarrow \text{DTPA-Fe}^{2+} + \text{M} + \text{H}^{+} \quad (3)$$

which would allow recycling of the iron catalyst.

ROLES OF O_2^- IN THE H_2O_2 OXIDATION OF 6-OHDA

The finding that SOD (which removes O_2^-) enhanced the rate of p-quinone formation was particularly surprising, since O_2^- accelerates the autoxidation of 6-OHDA. Moreover, O_2^- participates in both the spontaneous and the metal catalysed disproportionation of H_2O_2 under aerobic conditions [24]. Nevertheless SOD has been shown to stimulate several $^{\bullet}OH$ mediated processes in which O_2^- has been shown to scavenge $^{\bullet}OH$ [24,25]. On this basis, the stimulatory actions of SOD reflect in part, preemption of the removal of catalytically effective amounts of

$^{\cdot}OH$ by $O_2^{\cdot-}$. That the stimulatory effects of SOD were enhanced by the addition of DTPA, (in much the same proportions as the inhibitory effects of hydroxyl scavengers were stimulated by them) supports this contention.

COMPARISON OF THE OXIDATION OF 6-OHDA BY O_2 AND BY H_2O_2

The oxidation of 6-OHDA by H_2O_2 is similar to its oxidation by molecular oxygen in the following respects: (1). the reaction is substantially inhibited by desferrioxamine; (2). the oxidation is completely prevented by a mixture of selected scavengers, indicating that 6-OHDA is not directly oxidised by either H_2O_2 or O_2.

There are however major differences in these two reactions. Hydroxyl scavengers on their own inhibit the peroxidation, but not the autoxidation; DTPA which consistently accelerated the oxidation by H_2O_2, slightly retarded the oxidation by O_2; SOD consistently stimulated the reaction with H_2O_2, while it strongly inhibited the reaction with O_2. Finally, the peroxidatic reactions were monotonic regardless of what combinations of scavengers were present, while all aerobic reactions in the presence of SOD were sigmoidal indicating that accumulation of an intermediate was not required in or prior to the rate limiting step.

Summarising the arguments made in this section, it follows that transition metal ions (particulaly iron) are crucial to the propagation of both reactions. In the case of oxidation by H_2O_2, $^{\bullet}OH$ (and not O_2^-) is an important species in the steady state by maintaining an effective concentration of the actual electron acceptors, and DTPA increases the catalytic effectiveness of iron. In contrast, in the case of oxidation by O_2, O_2^- (and not $^{\bullet}OH$) is an important propagating species, and DTPA decreases the effectiveness of metal catalysis.

ACKNOWLEDGEMENTS

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REFERENCES

1. Saner, A. and Thoenen, H., (1971) Mol. Pharmacol. 7, 147-154
2. Sachs, C. and Jonsson, G., (1972) J. Neurochem. 19, 1561-1575
3. Cohen, G. and Heikkila, R.E. (1974) J. Biol. Chem. 249, 2447-2452
4. Adams, R.N., Murrill, E., McCreery, R., Blank, L. and Karolczak, M. (1972) Eur. J. Pharmacol. 17, 287-292
5. Borg, D.C., Schaich, K.M., Elmore, J.J., Jr. and Bell, J.A. (1978) Photochem. Photobiol. 28, 887-907
6. Floyd, R.A. and Wiseman, B.B., (1979) Biochim. Biophys. Acta 586, 196-207
7. Sullivan, S.G. and Stern, A. (1981) Biochem. Pharmacol. 30, 2279-2285
8. Gee, P. and Davison, A.J. (1984) Arch. Biochem. Biophys. 231, in the press
9. Liang, Y.O., Wightman, R.M. and Adams, R.N. (1976) Eur. J. Pharmacol. 36, 455-458
10. Tiffany-Castiglioni, E., Saneto, R.P., Proctor, P.H. and Perez-Polo, J.R. (1981) Biochem. Pharmacol. 31, 181-188
11. Gee, P. (1983) Ph.D. thesis, Simon Fraser University, Burnaby, British Columbia, V5A 1S6, Canada
12. Hanham, A.F., Dunn, B.P. and Stich, H.F. (1983) Mutation Res. 116, 333-339

13. Kong, S. and Davison, A.J. (1981) Biochim. Biophys. Acta 640, 313-325

14. Klebanoff, S.J. (1956) J. Gen. Physiol. 41, 725-753

15. Alder, H.J. (1963) Radiat. Res. Suppl. 3, 110-129

16. Davison, A.J. and Hulett, L.G. (1971) Biochim. Biophys. Acta, 226, 313-319

17. Bernofsky, C. and Wanda, S.-Y.C. (1983) Biochem. Biophys. Res. Comm. 111, 231-238

18. Chabereck, S., Frost, A.E., Doran, M.A. and Bicknell, N.J. (1959) J. Inorg. Chem. 11, 184-496

19. Winston, G.W., Harvey, W., Beri, L. and Cederbaum, I. (1983) Biochem. J. 216, 415-421

20. Klug, D., Rabani, J. and Fridovich, I., (1972) J. Biol. Chem. 247, 4839-4842

21. Heikkila, R.E. and Cohen, G. (1973) Science 181, 456-457

22. Davison, A.J. and Burr, A.H. (1979) in Kinetic Data Analysis (Endrenyi, L. ed.), pp. 59-72, Plenum Press, New York

23. Hoe, S., Powley, D.A. and Halliwell, B (1982) Chem.-Biol. Interact. 41, 75-81

24. Davison, A.J. and Kaminsky, L.S. (1974) in Proc. Hyperbaric O_2 Congress (Trapp, W., Banister, E.W., Davison, A.J. and Trapp, P., eds.), pp. 642-649, Simon Fraser University, Vancouver, Canada

25. McCord, J.M. and Fridovich, I. (1973) Photochem. Photobiol. 17, 115-121

LEGENDS TO FIGURES

FIG. 1: STOICHIOMETRY OF H_2O_2 YIELD AFTER AEROBIC OXIDATION OF 6-OHDA:

Reactions were carried out in phosphate buffers at pH 8.00, ionic strength 0.04, 25°C which were saturated with air (246.2 $\pm$ 0.3 µM O_2). Concentrations of H_2O_2 were determined from O_2 regeneration by catalase (250 U ml^{-1}). Points represent increasing concentrations of 6-OHDA from 50 to 400 µM by 50 µM increments followed by 500 µM and 1 mM. Error bars represent one standard deviation.

FIG. 2: ROLE OF H_2O_2 IN AEROBIC OXIDATION OF 6-OHDA:

Reaction conditions were as in Fig. 1. O—O, represents levels of H_2O_2 remaining at the end of the reaction while □—□, indicates the total amount of O_2 used for a given remaining at the end of the reaction while Curve 2 indicates initial concentration of 6-OHDA. Error bars represent one standard deviation.

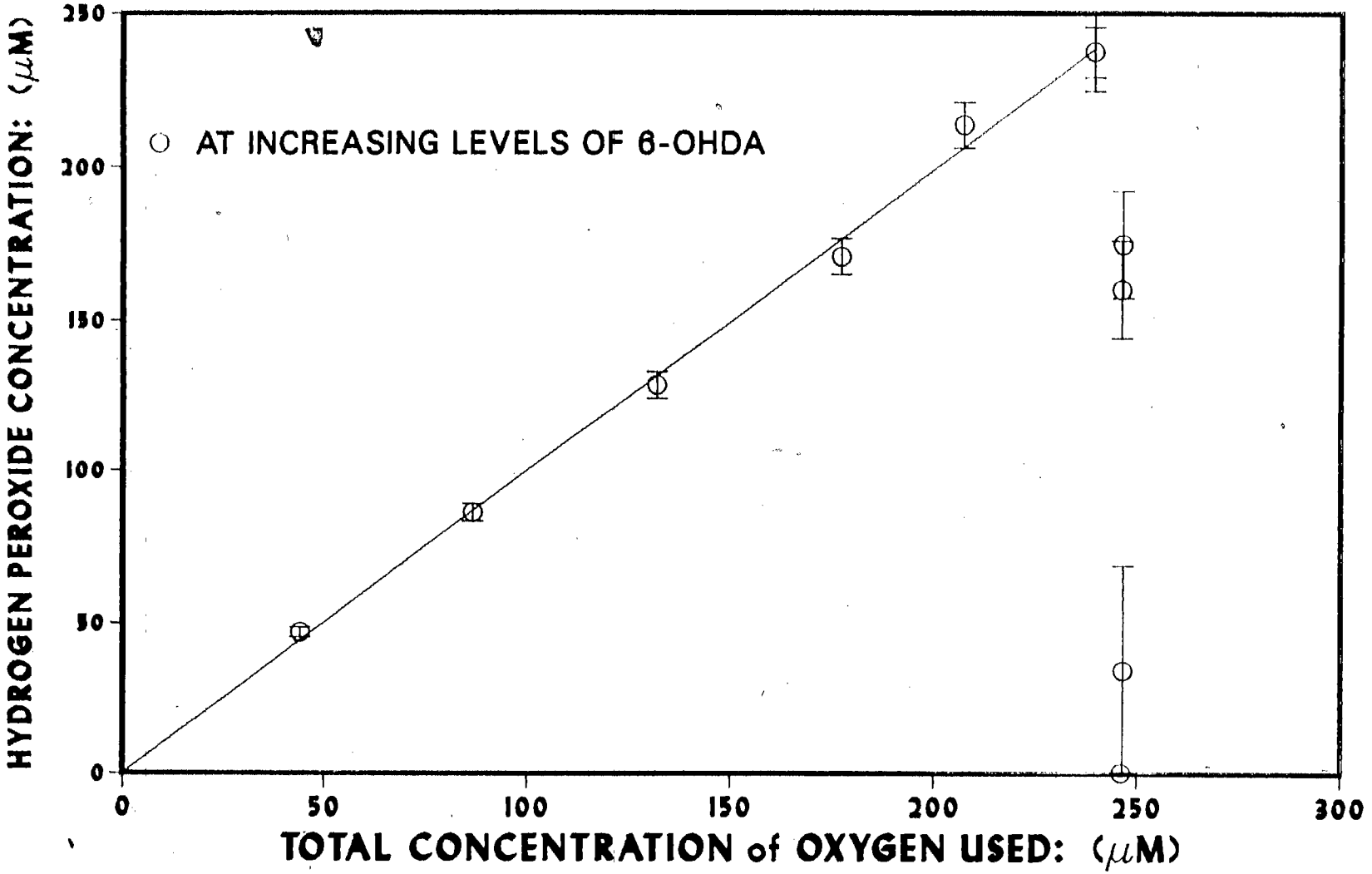
FIG. 3: EFFECTS OF SCAVENGERS ON THE ANAEROBIC OXIDATION OF 6-OHDA BY H_2O_2:

Reaction conditions were as in Fig. 1 except the reaction medium was equilibrated with 100% nitrogen and contained 500 μM H_2O_2 instead of 246 μM O_2. Catalase was not used in anaerobic conditions. ●—●, represents the control rate of oxidation of 6-OHDA (1 mM) by H_2O_2; △—△, addition of mannitol (10 mM) which is fairly representative of the effect of hydroxyl scavengers; □—□, inhibition by desferrioxamine (1 mM) ; ■—■, acceleration by DTPA (1 mM); and O—O, profound stimulation by SOD (250 U. ml^{-1}). Error bars represent one standard deviation.

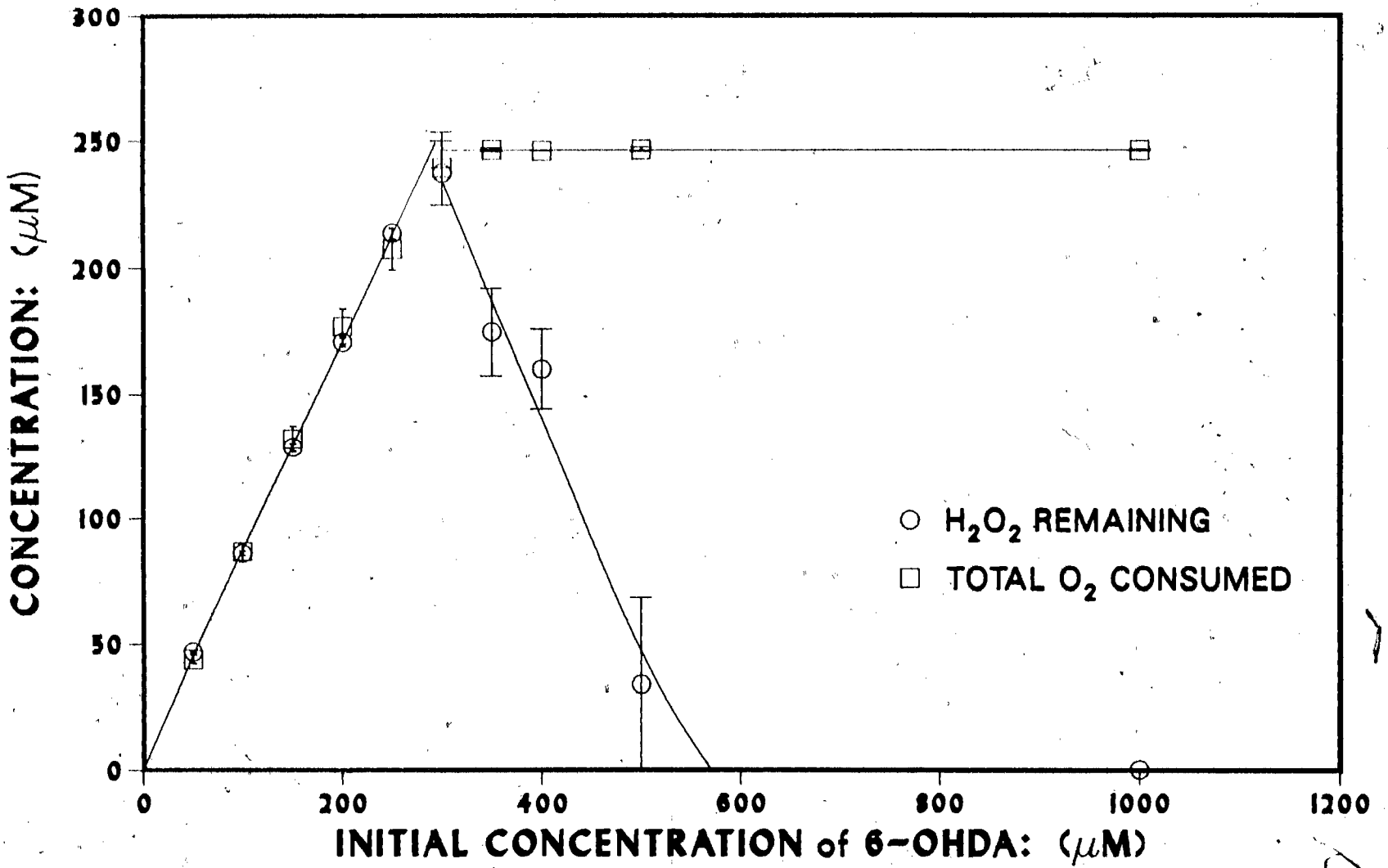
FIG. 4. SUMMARY OF EFFECTS OF INDIVIDUAL SCAVENGERS ON THE ANAEROBIC OXIDATION OF 6-OHDA:

Reaction conditions were as in Fig. 3. DES, desferrioxamine; DES+DTPA, simultaneous presence of desferrioxamine and DTPA. No scavengers or chelating agents were present in the control conditions. Error bars represent one standard deviation.

STOICHIOMETRY OF H_2O_2 REMAINING AFTER AEROBIC OXIDATION OF 6-OHDA



ROLES OF H_2O_2 IN AEROBIC OXIDATION OF 6-OHDA



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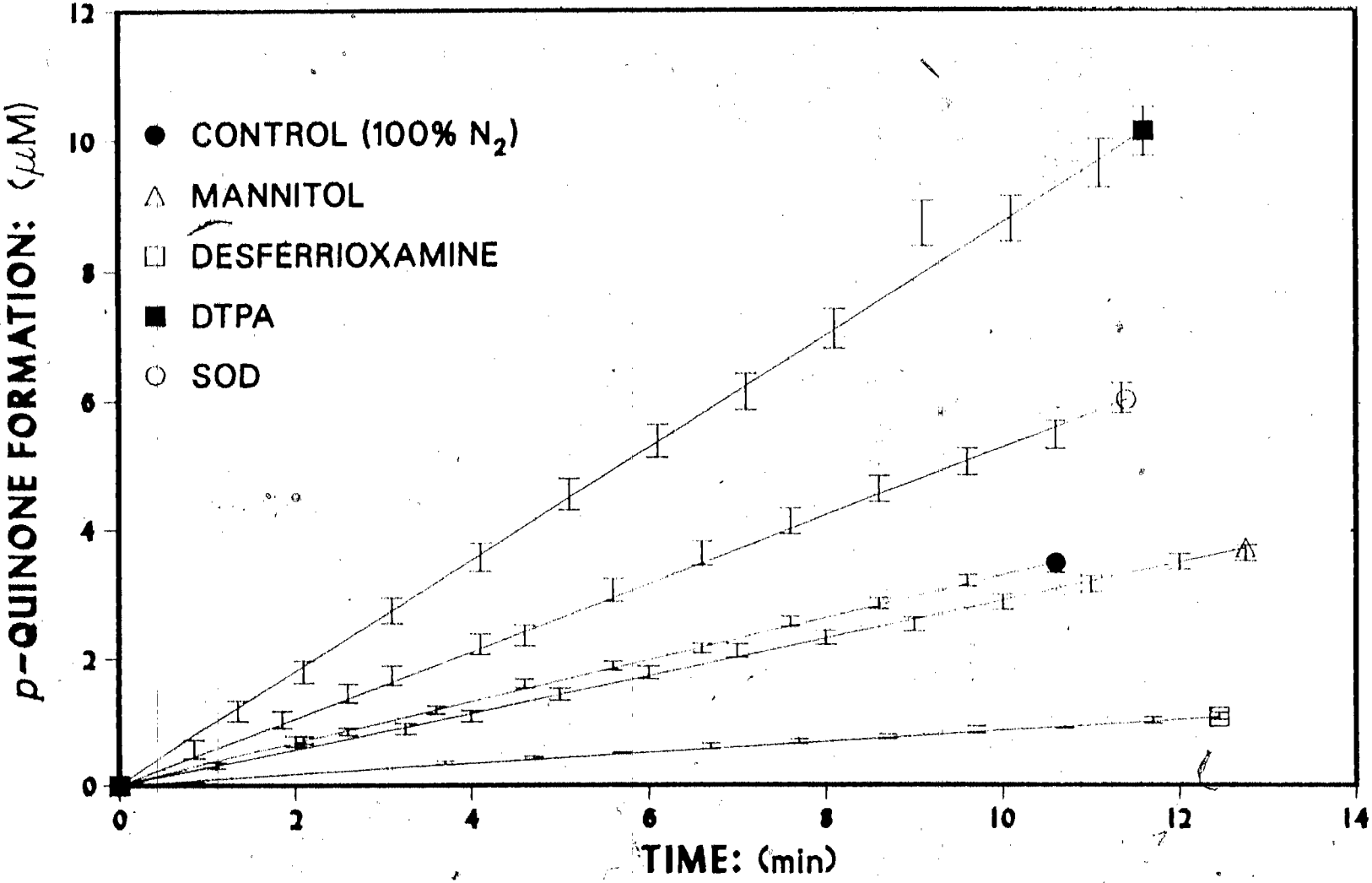
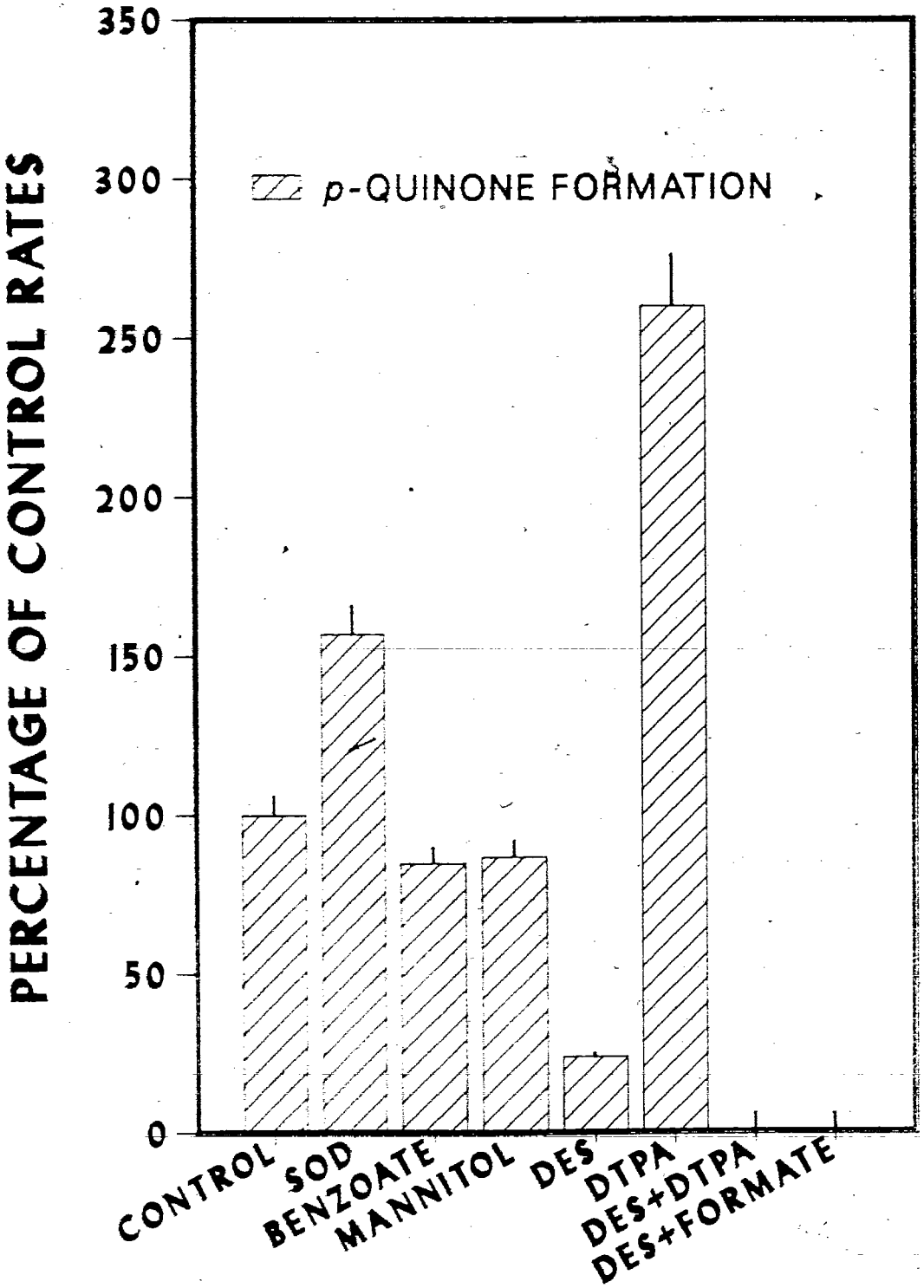
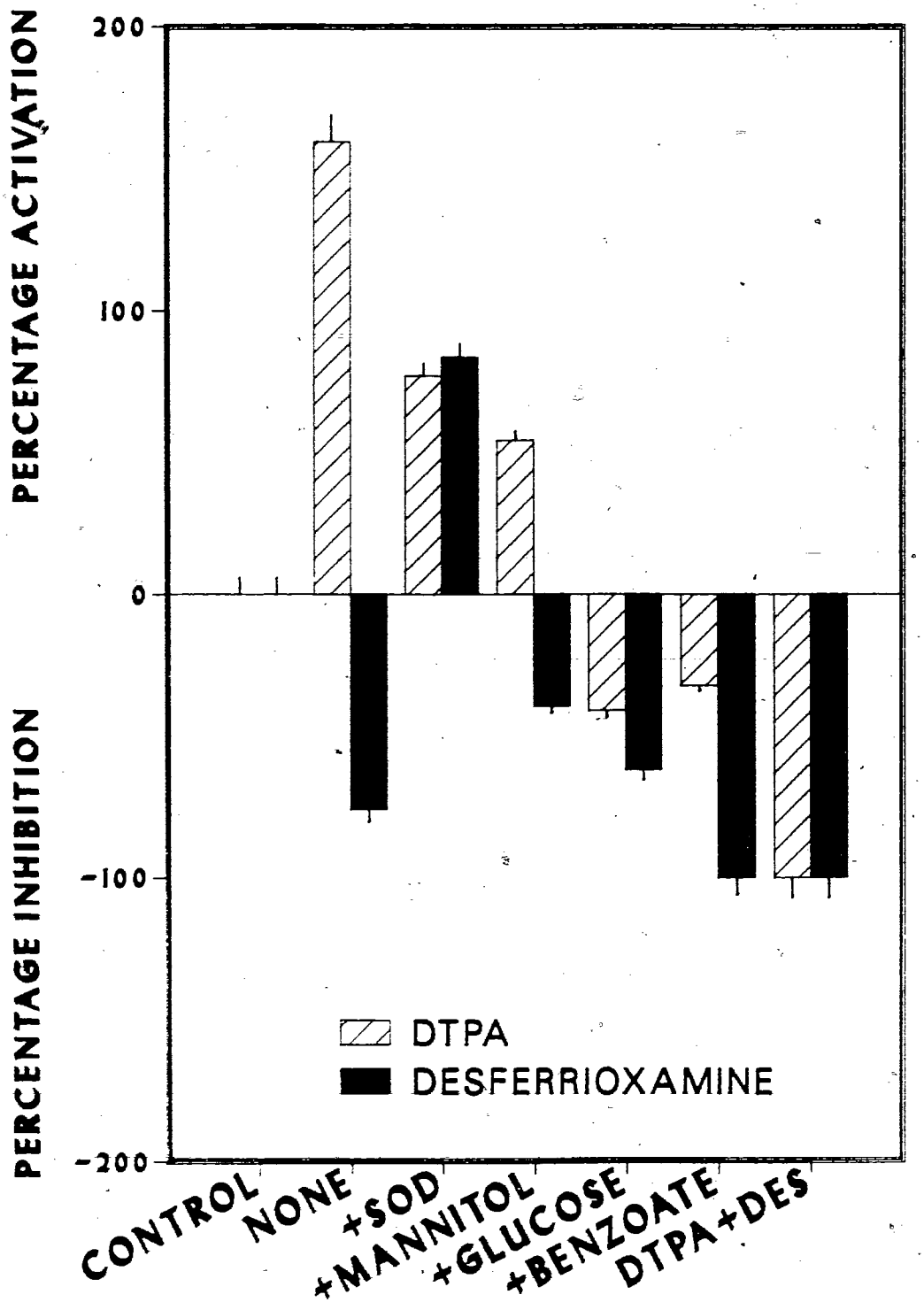


EFFECT OF SCAVENGERS ON: THE H_2O_2 OXIDATION OF 6-OHDA



EFFECTS OF SCAVENGERS IN THE PRESENCE OF DTPA AND DESFERRIOXAMINE



REDOX STATE OF CYTOCHROME C IN THE PRESENCE OF THE 6-HYDROXYDOPAMINE/OXYGEN COUPLE: OSCILLATIONS DEPENDENT ON THE PRESENCE OF HYDROGEN PEROXIDE OR SUPEROXIDE[1]

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FOOTNOTES

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

ABSTRACT

The reduction of ferricytochrome c in the presence of 6-hydroxydopamine/O_2 mixtures was examined under various reaction conditions. As the autoxidation of 6-hydroxydopamine progressed to completion, there were fluctuations in the net redox reactivity between reducing and oxidizing steady states. This was reflected in a sequence of damped oscillations in the redox state of cytochrome c. Corresponding to the time when 6-hydroxydopamine was 75-100% exhausted, re-oxidation of the ferrocytochrome c occurred (prevented by catalase or catalase plus superoxide dismutase). After the H_2O_2 in turn was mostly consumed the next phase commenced in which the cytochrome c became reduced for a second time. This reductive phase was 52% inhibited by superoxide dismutase. In the subsequent and final phase of the process a progressive oxidation of cytochrome c lasting at least 24 h was observed. Of the initial reduction of ferricytochrome c, at most 37% can be attributed to direct reduction by 6-hydroxydopamine or its semiquinone. This initial net reduction of cytochrome c was inhibited 51% by superoxide dismutase and 41% by catalase. However, since either catalase or superoxide dismutase inhibited the autoxidation of 6-hydroxydopamine by at least as much as they slowed the reduction of cytochrome c, their effects in slowing the reduction of cytochrome c resulted largely from the decreased PRODUCTION of those free radicals which reduce ferricytochrome c,

and only in part from accelerated REMOVAL. Elimination of the actions of transition metal ions (whether by passage of the buffer solutions through Chelex 100 resins or by addition of desferrioxamine to the reaction medium) slowed both the re-oxidation and re-reduction by up to 96%. Addition of mannitol decreased the rate of the first re-oxidation by 25% and increased the rate of the re-reduction by 7%. In general, the oscillations are explicable in terms of changes in the steady state levels of O_2^- and H_2O_2, with metal ions playing a major role and hydroxyl radicals a minor role in both the re-oxidation and re-reduction.

INTRODUCTION

In view of their presumed roles in the neurotoxicity of 6-hydroxydopamine (6-OHDA)[3] it was of interest to investigate the production of O_2^- and H_2O_2 in the reaction between 6-OHDA and molecular oxygen as it progressed to completion. It is well established that the 6-OHDA/O_2 reaction produces H_2O_2, O_2^- [1] and subsequently $^{\bullet}OH$ [2] either directly or from "Haber-Weiss-like" interactions between H_2O_2 and O_2^- in the presence of metal ions. However the events which result from (and result in) these processes are enormously complex, and remain poorly understood. For example, the O_2^- produced reacts catalytically with 6-OHDA, and this autocatalysis influences the rates at which O_2 is consumed and reactive intermediates including O_2^- are generated [3]. A variable fraction of the H_2O_2 produced is again consumed as an electron acceptor in the peroxidatic oxidation of 6-OHDA [4]. Moreover interactions between radicals produce some unexpected effects: e.g. McCord and Fridovich [5] have shown that the addition of superoxide dismutase (SOD) promotes the oxidation of ferrocytochrome c by $^{\bullet}OH$, reportedly by pre-empting the competing reaction:

$$^{\bullet}OH + O_2^- \longrightarrow O_2 + OH^-.$$

Thus the O_2^- generated may serve, not only as a source of $^{\bullet}OH$, but

also as an $^{\cdot}OH$ scavenger.

In systematic studies to determine what conditions promoted or inhibited the autoxidation of 6-OHDA, we found that transition metal ions introduced a further level of complexity. While the autoxidation was slightly inhibited by metal chelating agents, the presence of SOD greatly amplified the inhibitory actions of desferrioxamine or diethylenetriaminepentaacetate (DTPA) [6].

The simultaneous presence of catalase, SOD and DTPA produced 100% inhibition. This evidence supports the contention that 6-OHDA is not truly autoxidizable but requires a co-oxidant in addition to molecular oxygen [7] and that electron transfer occurs within a ternary oxygen-metal-6-OHDA complex [8]. The pattern of these radical-metal ion interactions depends on the nature and concentrations of transition metal ions present and their ligands. The implications are discussed in detail elsewhere [7-8].

As an important intracellular redox reagent we undertook to study the interactions of cytochrome c with intermediates generated by the autoxidation of 6-OHDA in relation both to the reported effects of 6-OHDA on energy metabolism [9], and to the interactions of ferri- and ferrocytochrome c with the radicals involved. In particular, since ferricytochrome c is reduced by

either O_2^- [10] or semiquinone radicals [11] while ferrocytochrome c is susceptible to oxidation by H_2O_2 [12], we wished to examine the steady state levels of reduction of cytochrome c as the 6-OHDA/O_2 reaction proceeded to completion, and to learn the roles of the various free radical intermediates in determining these levels.

Instead of attaining a steady state however, fluctuations in the net redox status of the system occurred such that an unusual series of oscillations in the redox state of cytochrome c was observed. These oscillations are largely explicable in terms of changes in the steady state levels of O_2^- and H_2O_2 as the various reactants become depleted. Although oscillations are not uncommon in thermodynamically open systems, most reactions in closed systems proceed smoothly to equilibrium or until one of the reactants is exhausted. The occurrence of oscillations in the present (closed) system results from an interaction between the particular reaction conditions chosen and an unusual combination of reactivities of the intermediates in the reaction mixture, as discussed hereunder.

Our limited goal for this study was to explain the oscillations by determining the factors responsible for them. To do so we sought to identify what intermediates were necessary at each stage in order for the oscillations to occur. Thus oscillations and the

conditions promoting them were examined by varying the reaction conditions and by inhibiting various phases of the sequence by SOD or catalase. We also tested the role of hydroxyl radicals in the oscillations by adding mannitol, and the role of transition metal ions by carrying out selected reactions in Chelex 100 treated buffers (with and without added iron salts) and by adding desferrioxamine.

MATERIALS AND METHODS

REAGENTS

Cytochrome c (Type III, horse heart: minimum purity 95%) and DTPA were from the Sigma Biochemical Co., and desferrioxamine was a gift from CIBA Pharmaceuticals. 6-Hydroxydopamine hydrobromide salt (Sigma) was made up anerobically in deoxygenated phosphate buffer pH 7.00, ionic strength 0.2 and checked for purity by the symmetry of the 290 nm peak. 6-OHDA stock solutions were prepared immediately before use and stored under 1 atmosphere of high purity nitrogen (Linde, Union Carbide Canada Ltd.) in rubber capped vials. Precautions taken against the admission of oxygen to the 6-OHDA stock solution included boiling and evacuating rubber caps prior to use. In addition, the vials were initially placed under a slight positive pressure of nitrogen and maintained in this state by the introduction of a volume of nitrogen at least equal to any volume of liquid removed. The substantial exclusion of oxygen from the 6-OHDA stock solution was confirmed by the absence of significant concentrations of the end products of its oxidation as measured by the lack of absorbance at 490 nm at the start of each assay.

Catalase, from beef liver (2500 U mg^{-1} protein) was from Sigma Chemical Co.; and SOD, from beef erythrocytes (2900 U mg^{-1}

protein) was from Miles Laboratories Ltd. Catalase and SOD activities of the reagents were confirmed by direct assay of the stock solutions. SOD (10 U ml^{-1}) and catalase (50 U ml^{-1}) were added to assay systems at final concentrations sufficient to remove at least ten times the flux of radicals expected if all the 6-OHDA present were to be oxidized within a 10 min period.

BUFFER SOLUTIONS: METAL ION CONTAMINANTS

Distilled deionized water was used for preparation of stock buffer solutions. Ion contamination of the water was negligible (>10 Mohm cm^{-3}). Transition metal ions were present largely as a result of contamination from the buffer salts (American Scientific & Chemical and Matheson Coleman & Bell). To determine the roles of these traces of metal ions some buffer solutions were passed through Chelex 100 resin (BioRad Laboratories). Acid washed plasticware was used for the Chelex 100 treated reagents. Since Chelex 100 treatment modified the pH of buffers, these were made up by mixing solutions of buffer salts which had been individually passed through the Chelex columns until the desired pH was reached.

Analysis of these purified buffer solutions by inductively coupled plasma emission spectroscopy or atomic absorption spectrophotometry revealed that actual concentrations of the

following transition metal ions were as follows: copper (<1.60 nM), iron (31.3 nM), manganese (8.19 nM), aluminum (23.0 nM), and vanadium (<39.0 nM). Although the iron content represented only 1.8 ug l^{-1}, some reactions were nevertheless inhibitable by desferrioxamine, so that even these trace amounts may not be negligible.

ASSAY PROCEDURES

A spectrophotometer cuvet with 2.45 ml of phosphate buffer of the stated pH and ionic strength (containing any desired mixtures of scavengers) was placed in a water bath at 25°C and bubbled for 30 min with oxygen saturated with water vapor at the same temperature. The cuvet was then quickly transferred to a thermostatically controlled cell chamber of a Beckman DB-GT spectrophotometer equipped with a Beckman 10 inch strip chart recorder, and facilities for on-line acquisition of data by an IBM 3033 mainframe. The reaction was then initiated by addition of 0.05 ml of the anaerobic 6-OHDA stock solution also at 25°C. The progress of the reaction was followed at 490 nm until ferricytochrome c was added. Unless otherwise stated, this was done when oxidation of 6-OHDA was 30% complete to allow prior measurement of initial autoxidation rate, and subsequent readings were taken alternately at 490 nm and 550 nm for approximately 5 s at 5 s intervals. We confirmed that pH did not change

significantly during the assay procedures by measuring pH in the reaction cuvets after bubbling with oxygen, and upon termination of the reaction.

Molar absorptivities for the p-quinone of 6-OHDA were determined specifically for conditions used (1677 (± 14) M^{-1} cm^{-1}), c.f. zero for 6-OHDA itself at 490 nm, while 21,100 M^{-1} cm^{-1} was used for the reduced-oxidized difference spectrum of cytochrome c at 550 nm [13]. Reaction conditions and final concentrations of reagents in the cuvet are stated in legends to figures.

DATA ANALYSES

Concentrations of the oxidized quinone product(s) of 6-OHDA and ferri- and ferrocytochrome c were calculated by simultaneous equations which determined the relative contributions of each product to absorbances measured at 490 nm and 550 nm. These equations were solved and rates were determined using programs written by the authors (in APL) and implemented on an IBM 3033 computer. Pseudo-first order rate constants were determined using multiple linear regression analysis [14].

RESULTS

The sequence of changes in the redox state of cytochrome c shown in Fig. 1, reflects fluctuations in the net redox status of the 6-OHDA/O_2 mixture. While ferricytochrome c was initially reduced rapidly by either 6-OHDA or the early intermediates in its aerobic autoxidation, these intermediates did not reach either an equilibrium or a steady state level. Instead a defined and limited sequence of damped oscillations in the redox state of cytochrome c ensued. The amplitude of oscillation was more pronounced at low ionic strength, at higher pH values, and in oxygen- rather than air-saturated buffer, and in the presence of transition metal ions. In addition to the initial reduction of ferricytochrome c, the sequence of events comprised: subsequent re-oxidation, re-reduction, and a final slow progressive oxidation by molecular oxygen. The successive phases in the redox state as the oscillation progresses will now be considered in turn.

FIRST REDUCTIVE PHASE

The initial reduction of cytochrome c, is due both to the direct reducing action of 6-OHDA, and to intermediates in the reduction of O_2. The simultaneous presence of SOD and catalase decreased the rate of the reduction of cytochrome c by 63% (Fig. 2). The use of Chelex 100 treated buffers, or the addition of

desferrioxamine also caused a significant decrease in the rate of reduction of cytochrome c. Addition of SOD alone decreased the initial rate of reduction of cytochrome c by 51%, but as indicated by Fig. 3, the effects of SOD cannot be attributed solely to the scavenging of a given amount of O_2^- produced in the reduction of molecular oxygen by 6-OHDA. The decrease in the rate of autoxidation of 6-OHDA (86%) by SOD suggests that the decrease it causes in the reduction of cytochrome c is attributable not merely to increased REMOVAL of reducing radicals, but to an even greater extent to decreased PRODUCTION. Nevertheless, direct reduction of cytochrome c by 6-OHDA was confirmed, since the reaction readily progressed to completion under anaerobic conditions, at a rate equivalent to that in Chelex 100 treated buffer saturated with oxygen at 1 atmosphere (not shown).

Such a combined direct and indirect action for SOD is supported by a similar but lesser inhibition (41%) of the initial rate of reduction of ferricytochrome c by catalase. Catalase in this instance can be acting only by slowing production of reductive intermediates, since it is not likely to remove any species capable of directly reducing ferricytochrome c (although the possibility of traces of SOD activity in the catalase preparation must be kept in mind [15]).

The end of this phase coincides with the time at which 6-OHDA concentrations approach zero. For this reason the oscillations were not observed below pH 7, because (from the measured concentrations of 6-OHDA) the slowness of oxidation of 6-OHDA led to its persistence throughout the time scale of the observations in concentrations sufficient to produce a net reducing environment.

FIRST OXIDATIVE PHASE.

After the ferricytochrome c had become 64% reduced (and corresponding to the time when 6-OHDA was 74-100% oxidized) a slow re-oxidation of the ferrocytochrome c commenced (Fig. 1). This phase results from the presence of H_2O_2 produced in the autoxidation of the 6-OHDA since the re-oxidation is completely abolished by the presence of catalase in the reaction mixture (Fig. 4). The substantial inhibition of the re-oxidation observed when SOD is added, must be attributed to the indirect effect of SOD in decreasing the yield of H_2O_2 from the autoxidation of 6-OHDA (Fig. 3), since the direct action of SOD in removing O_2^- is to increase the yield of peroxide in stoichiometric proportion.

This phase was slowed (by 76%), and its amplitude was dramatically decreased when Chelex 100 treated buffer was used, indicating a major role for transition metal ions (Fig. 5). Both the amplitude

and the rate of this phase of the oscillation were restored, to or above control levels by the addition of $FeSO_4$ to a final concentration of 10 uM. Further addition of desferrioxamine to the $FeSO_4$ reaction (to 50 uM) decreased the rate of this phase by 96%. The rate of this phase was also decreased by 25% when the hydroxyl scavenger mannitol was added (Fig. 5).

SECOND REDUCTIVE PHASE.

When the H_2O_2 was largely consumed, a phase commenced in which the ferricytochrome c again became reduced, this time reaching 76% reduction. SOD inhibited this re-reduction by 52% implicating O_2^- as a major reductant (Fig. 6). This re-reduction in the presence of H_2O_2 could be duplicated in a system which initially contained (in addition to ambient oxygen) no redox reagents other than ferricytochrome c and H_2O_2 (Fig. 7).

O_2^- is thought to be generated in the spontaneous or metal catalysed disproportionation of H_2O_2 [12, 16-18] and the presence of residual traces of H_2O_2 during this phase provides a source of O_2^- for ferricytochrome c reduction. A role for transition metal ions in this phase is shown by the substantial inhibition caused by the substitution of Chelex treated buffers, and the restoration of the rate to significantly above that of the control reaction by the addition of $FeSO_4$ (Fig. 8). A role for iron in this phase

of the oscillation is confirmed by the almost complete inhibition produced by the presence of desferrioxamine (Fig. 8). In the same figure, it can be seen that addition of mannitol to the reaction medium accelerated this phase by a marginal 7%. In the simpler system, consisting of ferricytochrome c and H_2O_2 in the absence of 6-OHDA, desferrioxamine or DTPA STIMULATED the net reduction of cytochrome c (not shown). This surprizing result is explicable on the basis that in this system desferrioxamine inhibits the re-oxidation more than it inhibits the re-reduction. Clearly the two systems are not completely analogous, and additional experiments over a range of concentrations of reagents, metals, and scavengers will be needed before all the variables can be defined.

FINAL OXIDATIVE PHASE.

The subsequent (final) phase of the process is most simply accounted for on the basis that the only effective redox reagents remaining are ferrocytochrome c and molecular oxygen in the presence of trace amounts of metal ions. The slow progressive oxidation in the final stage is illustrated in the last four points in Fig. 1. The logarithmic scale conceals the fact that these four points represent some further 16 h of elapsed time. These values represent oxidation of cytochrome c and not evaporation of solvent from the (capped) cuvettes, nor loss of

functional integrity of the cytochrome c. This was confirmed by repeatedly scanning from 600 to 450 nm, and by reducing the final samples with ascorbate and re-scanning to ensure that the decrease in absorbance at 550 nm was not due to denaturation of cytochrome c. Based on thermodynamic criteria, this last oxidative phase would presumably continue to completion, but several days of observation would be necessary under the prevailing conditions of pH and ionic strength [19]. The effects of scavengers on the various phases of the oscillation are summarized in Figs. 9 and 10.

DISCUSSION

REDUCTION AND OXIDATION OF CYTOCHROME C

Under the usual reaction conditions, less than half of the initial reduction of cytochrome c is due to the direct action of 6-OHDA. Since the rate of reduction of cytochrome c was 63% inhibited by a mixture of SOD and catalase, at most the remaining 37% of the reduction was from direct action of 6-OHDA and/or its semiquinone. The 50% inhibition of the reduction by SOD alone indicates that up to half of the total reduction is directly or indirectly attributable to O_2^-. Anaerobically the rate of reduction of cytochrome c was decreased to that in the presence of Chelex 100 treated buffer, or slightly less than that in the presence of a mixture of SOD and catalase.

From the above considerations, the use of inhibition by SOD as a criterion for the involvement of O_2^- leads to overestimates, since in this system SOD inhibits the reduction of cytochrome c, not only by scavenging O_2^- but even more so by inhibiting the autoxidation of 6-OHDA and thus formation of O_2^- and semiquinone. Moreover the extent of reduction of cytochrome c underestimates the yield of reducing free radicals, since it reflects the steady state resulting from the net effect of both reduction and a substantial rate of re-oxidation.

IDENTIFICATION OF THE MAJOR OXIDIZING AND REDUCING SPECIES

The extent to which oxidizing and reducing reactions alternately dominate the steady state at various times is evident in the observed sequence of oscillations in the redox state of cytochrome c. The main reducing and oxidizing species involved can be assigned (at least qualitatively) by a quantitative examination of the effects of SOD, catalase, and mannitol. In the initial reductive phase, the main reducing species present are 6-OHDA itself, its semiquinone, and O_2^-.

The first oxidative phase commenced when the 6-OHDA and its main reducing products were largely exhausted. Since this phase was completely inhibited by catalase it reflects the predominant action of H_2O_2. The second reductive phase begins as H_2O_2 becomes exhausted, and reflects some reductive contribution by O_2^-. However the fact that about half of the reduction resisted inhibition by SOD in the 6-OHDA containing system (after the disappearance of any detectible residual 6-OHDA) implies the participation of some other reducing radical related to 6-OHDA (most plausibly its semiquinone) since in the simple two component system shown in Fig. 7 the same concentration of SOD inhibited reduction of cytochrome c virtually completely. SOD reportedly converts O_2^- to HO_4^- which is thought to be less effective in the

reduction of cytochrome c [18]. In view of the inhibitory actions of SOD in the first three phases of the reaction, this postulated dimeric species is unlikely to participate significantly in the processes described here.

A significant but smaller oxidizing role for $^{\bullet}OH$ in both the re-oxidation and re-reduction of the cytochrome c can be inferred from the inhibition of the re-oxidation by mannitol, and the acceleration of the re-reduction (Fig. 10). In the final stages of the reaction, the residue of oxygen is the major reactive species present, and the slow, progressive autoxidation of ferrocytochrome c is observed.

OXIDIZING AND REDUCING ACTIONS OF HYDROGEN PEROXIDE

In some conditions H_2O_2 causes net oxidation of cytochrome c (Fig. 4 and [12]), while under others it causes net reduction (Figs. 5 and 6; [18]). The explanation of the apparently contradictory effects of H_2O_2 upon the redox state of cytochrome c lies in the metal catalysed disproportionation of H_2O_2 and the concomitant generation of O_2^-. The extent of the role of O_2^- in the reductive actions of H_2O_2 is reflected in the inhibitory effects of SOD upon this process, shown in Figs. 6 and 7.

The ability of cytochrome c to be either reduced by O_2^- or to be oxidized by H_2O_2 means that in the simultaneous presence of any given stationary concentration of both, the redox state of cytochrome c tends toward a certain steady state. Thus when the cytochrome c is largely oxidized, the net effect of the H_2O_2 in moving it toward that steady state will be that of reduction (Fig. 6). In contrast when the cytochrome c is initially largely reduced, movement toward the same steady state will be oxidative. While the 6-OHDA/O_2 reaction is proceeding, the actual position of the steady state is not stationary, but varies as the instantaneous concentrations of O_2^-, $^{\bullet}OH$ and H_2O_2 change with time. The changes in the redox state indicated in Fig. 1 represent the net effect of these thermodynamic effects, as modified by the kinetics of the individual redox processes.

ROLES OF TRANSITION METAL IONS

Transition metal ions present in the buffers salts (rather than the distilled deionized water) played a crucial role in the oscillations. This was reflected in the substantial inhibitory effects of substituting buffers which had been treated with Chelex 100 resins, or of adding desferrioxamine (Fig. 10) in several phases of the oscillation. In contrast desferrioxamine inhibited re-reduction in the H_2O_2/cytochrome c system. Addition of cupric acetate to this simpler system led to stimulation of reduction of

ferricytochrome c at some concentrations and inhibition at others. In some reaction mixtures the presence of metal ions was essential if autoxidation of 6-OHDA was to occur at all.

The problems concerning the participation of metal ions in biologically significant redox systems are at the same time fundamentally important and experimentally forbidding. Despite extremely low concentrations of transition metal ions in our Chelex treated buffer solutions, addition of desferrioxamine had residual effects, suggesting that even the smallest traces of metal ions may have decisive effects. On the other hand, running all reactions in the presence of metal chelating agents carries its own problems. The proper solution to these questions will require that known amounts of transition metal ions be added to all systems in which they may participate. Iron and copper salts are obvious candidates, but dare one neglect manganese? Clearly graded mixtures of metal ions should be studied and a great deal of experimental work will be necessary before adequate answers are available.

The ability of cytochrome c either to receive an electron from O_2 or to donate one to H_2O_2, together with its relatively high concentration renders it an effective intracellular substitute for free inorganic transition metal ions in free radical reactions. However the substantial inhibition resulting from the use of

Chelex 100 treated buffers, together with the almost complete inhibition by desferrioxamine rule out such a role for cytochrome c in the current system.

ROLES OF HYDROXYL RADICALS

Despite the preponderance of the roles of O_2^- and H_2O_2, a significant role for $^{\bullet}OH$ is evident from the effects of mannitol. The 25% inhibition of the initial re-oxidation of cytochrome c by mannitol indicates some participation of $^{\bullet}OH$ as an oxidant of cytochrome c in this phase of the reaction. Predictably however, $^{\bullet}OH$ do not participate significantly in the reductive phase, except perhaps to retard it slightly, as indicated by the 7% acceleration of this phase induced by the addition of mannitol.

REASONS FOR THE OSCILLATIONS

The unusual succession of alterations observed in the redox state of cytochrome c, results from the marked differences in the reactivity of oxygen in different stages of reduction, toward cytochrome c. Superimposed on these are successive fluctuations in the concentration of reaction intermediates. Thus, the transfer of a series of electrons from 6-OHDA to oxygen causes successive reversals of the effective redox activity of subsequent intermediates, with respect to cytochrome c. The first product of

reduction of O_2, namely O_2^-, is a REDUCING agent with respect to ferricytochrome c whereas the second product, H_2O_2 (despite the greater electron saturation of its molecular orbitals), is a net OXIDIZING agent.

The two reductive phases are separated from each other because the accumulation of effective concentrations of O_2^- is delayed until after the substantial disappearance of both the 6-OHDA reactant and the subsequent H_2O_2 product/intermediate. In this regard it is significant that either of these substances is capable of reacting with and destroying O_2^-.

Somewhat different arguments account for the separation of the two oxidative phases. Although the final reaction of ferrocytochrome c with molecular oxygen is highly favored thermodynamically, such oxidation is not manifested earlier in the reaction sequence because this process is very slow, being kinetically hindered by electron orbital spin considerations. In contrast, the reaction with H_2O_2, which is less favored on thermodynamic grounds, proceeds much more rapidly, and is thus observed early in the sequence of events. Detection of the autoxidation of ferrocytochrome c must await virtual disappearance of all other redox reagents. Thus the two oxidative phases occur in succession rather than simultaneously. The final product, H_2O, is of course inert with respect to cytochrome c. Although $^{\bullet}OH$ can either

reduce ferricytochrome c (by electron tunnelling [20]) or oxidize ferrocytochrome c [5], the procedures we used reveal only a oxidizing role for $^{\bullet}OH$. This role was substantial in the first re-oxidation, and of only minor importance in the re-reduction.

A number of additional studies deserve to be carried out. In particular, the addition of scavengers and scavenger mixtures at different stages of the reaction should yield information not obtainable when the initial presence of the scavenger terminates the oscillation. Also the addition of the cytochrome c at various stages of the reaction will indicate the extent to which the 6-OHDA/O_2 reaction is being influenced by the presence of cytochrome c. Finally dose/response curves of the effects of transition metal ions will allow a more quantitative analysis of the factors involved.

CONCLUSIONS

The spectrum of redox relationships, together with the kinetic constraints inherent in the chemical nature of the species involved provide necessary but not sufficient conditions for the oscillations. Oscillations were reproducibly observed only after appropriate reaction conditions were defined. Even the initial phase, the oxidation of 6-OHDA by molecular oxygen was not always observed. In fact it reproducibly failed to occur if metal ions,

O_2^- and H_2O_2 were rigorously excluded by the simultaneous presence of SOD, catalase, and diethylenetriaminepentaacetate [21]. The occurrence of oscillations is somewhat more demanding, being more pronounced at pH 7 or above, low ionic strength, and in oxygen- rather than air. The roles for H_2O_2 and O_2^- inferred from the actions of SOD and catalase in the current system provide consistent explanations for previously reported oscillations [12, 22].

In summary, a complex set of interactions between intermediates of the 6-OHDA/O_2 couple, and the redox reactivities of cytochrome c lead to an unusual series of fluctuations in the redox steady state of cytochrome c. The oscillations are understandable in terms of the nature of the reaction intermediates which are kinetically dominant at each stage.

REFERENCES

1. Heikkila, R.E. and Cohen, G. (1974) J. Biol. Chem. 249, 2447-2452.
2. Floyd, R.A. and Wiseman, B.B. (1979) Biochim. Biophys. Acta 586, 196-207.
3. Heikkila, R.E. and Cohen, G. (1973) Science 181, 456-457.
4. Liang, Y.O., Wightman, R.M. and Adams, R.N. (1976) Eur. J. Pharm. 36, 455-458.
5. McCord, J.M. and Fridovich, I. (1973) Photochem. Photobiol. 17, 115-121.
6. Gee, P. (1984) Ph.D. thesis, Simon Fraser University, British Columbia, Canada.
7. Gee, P. and Davison, A.J. (1984) Arch. Biochem. Biophys. 231, in press.
8. Gee, P. and Davison, A.J. (1984) IRCS Med. Sci. Biochem. 12, 127.
9. Wagner, K. and Tredelenburg, U. (1971) Naunyn-Schmiedelbergs Arch. Pharmakol. 269, 112-116.
10. Ballou, D., Palmer, G. and Massey, V. (1969) Biochem. Biophys. Res. Comm. 36, 898-904.
11. Winterbourn, C.C. (1981) Arch. Biochem. Biophys. 209, 159-167.
12. Davison, A.J. and Hulett, L.G. (1971) Biochim. Biophys. Acta 226, 313-319.

13. Van Gelder, B.F. and Slater, E.C. (1962) Biochim. Biophys. Acta 58, 593-595.

14. Davison, A.J. and Burr, A.H. (1979) in Kinetic Data Analysis (Endrenyi, L. ed.), pp. 59-72, Plenum Press, New York.

15. Halliwell, B. (1973) Biochem. J. 135, 379-381.

16. Schubert, J., Sharma, V., White, E. and Bergelson, L. (1968) J. Am. Chem. Soc., 90, 4476-4478.

17. Singh, A. (1978) Photochem. Photobiol. 28, 429-433.

18. Bernofsky, C. and Wanda, S.-Y.C. (1983) Biochem. Biophys. Res. Comm. 111, 231-238

19. Boeri, E. and Tosi, L. (1953) Biochim. Biophys. Acta 13, 83-92.

20. Van Leeuwen, J.W., Raap, A., Koppenol, W.H. and Nauta, H. (1978) Biochim. Biophys. Acta 503, 1-9.

21. Gee, P. and Davison, A.J. (1983) Fed. Proc. 42, 2935.

22. Kertesz, D. (1968) Biochim. Biophys. Acta 167, 250-256.

LEGENDS TO FIGURES:

FIGURE 1: Oscillations in the Redox State of Cytochrome c During the Autoxidation of 6-OHDA: Reactions were carried out at 25°C, in phosphate buffer pH 7.00 and ionic strength of 0.2. Final concentrations of reactants in the cuvette were 6-OHDA, 254 uM and cytochrome c, 24.6 uM. Zero time represents the time of addition of 6-OHDA. ●—●, log concentration of ferricytochrome c.

FIGURE 2: Initial Reduction of Ferricytochrome c by 6-OHDA - Effects of Catalase or Superoxide Dismuatase: Reaction conditions were as described in Fig. 1, except that prior to addition of cytochrome c or 6-OHDA, SOD and/or catalase was added as indicated to respective final concentrations of 10 and 25 U ml^{-1}. Zero time represents the time of addition of ferricytochrome c. ●—●, Control (No scavengers); O—O, inhibition by catalase; ■—■, inhibition by SOD; □—□, inhibition by both catalase and SOD. Confidence limits for the points are indicated by error bars corresponding to one standard deviation.

FIGURE 3: Rate of Autoxidation of 6-OHDA - Effects of Superoxide Dismutase or Catalase: Reaction conditions were as described in Fig. 2. Zero time represents the time of addition of 6-OHDA. ●—●, control; O—O, inhibition by catalase; ■—■, inhibition by SOD; □—□, inhibition by both catalase and SOD.

FIGURE 4: First Re-oxidation of Ferrocytochrome c by Products of Autoxidation of 6-OHDA - Effects of Superoxide Dismutase or Catalase: Reaction conditions were as described in Fig. 2. Zero time represents the beginning of the re-oxidation phase. ●—●, control; O—O, inhibition by catalase; ■—■, inhibition by SOD; □—□, inhibition by both catalase and SOD.

FIGURE 5: Roles of Metals or Hydroxyl Radicals in the Re-oxidation of Cytochrome c: Effects of Chelex 100 treated buffer, desferrioxamine, iron, and mannitol on rates of re-oxidation of cytochrome c. Reaction conditions were as described in Fig. 1 except buffers were at pH 8.0, ionic strength 0.04. Final concentrations were: iron, 100 uM; desferrioxamine, 500 uM; mannitol, 10 mM. Zero time represents the beginning of the re-oxidation phase. ●—●, Chelex treated buffers; O—O, iron added; ■—■, iron plus desferrioxamine; □—□, iron plus mannitol.

FIGURE 6: Re-reduction of Ferricytochrome c by Products of Autoxidation of 6-OHDA - Effect of Superoxide Dismutase: Reaction conditions were as described in Fig. 1. (Only SOD was added, since in the presence of catalase, this phase was not seen). Zero time represents the beginning of the second reduction phase. ●—●, control; O—O, inhibition by SOD.

FIGURE 7: Reduction of Ferricytochrome c by H_2O_2 - Effect of Superoxide Dismutase: Reactions conditions were the same as in Fig. 1 except that 6-hydroxydopamine was not present. Initial concentrations of cytochrome c and H_2O_2 were 40 uM and 200 uM, respectively. Zero time represents the time of addition of ferricytochrome c. Final concentrations of SOD were: O—O, 0.7 U ml^{-1} and ■—■, 11.5 U ml^{-1}. ●—●, control.

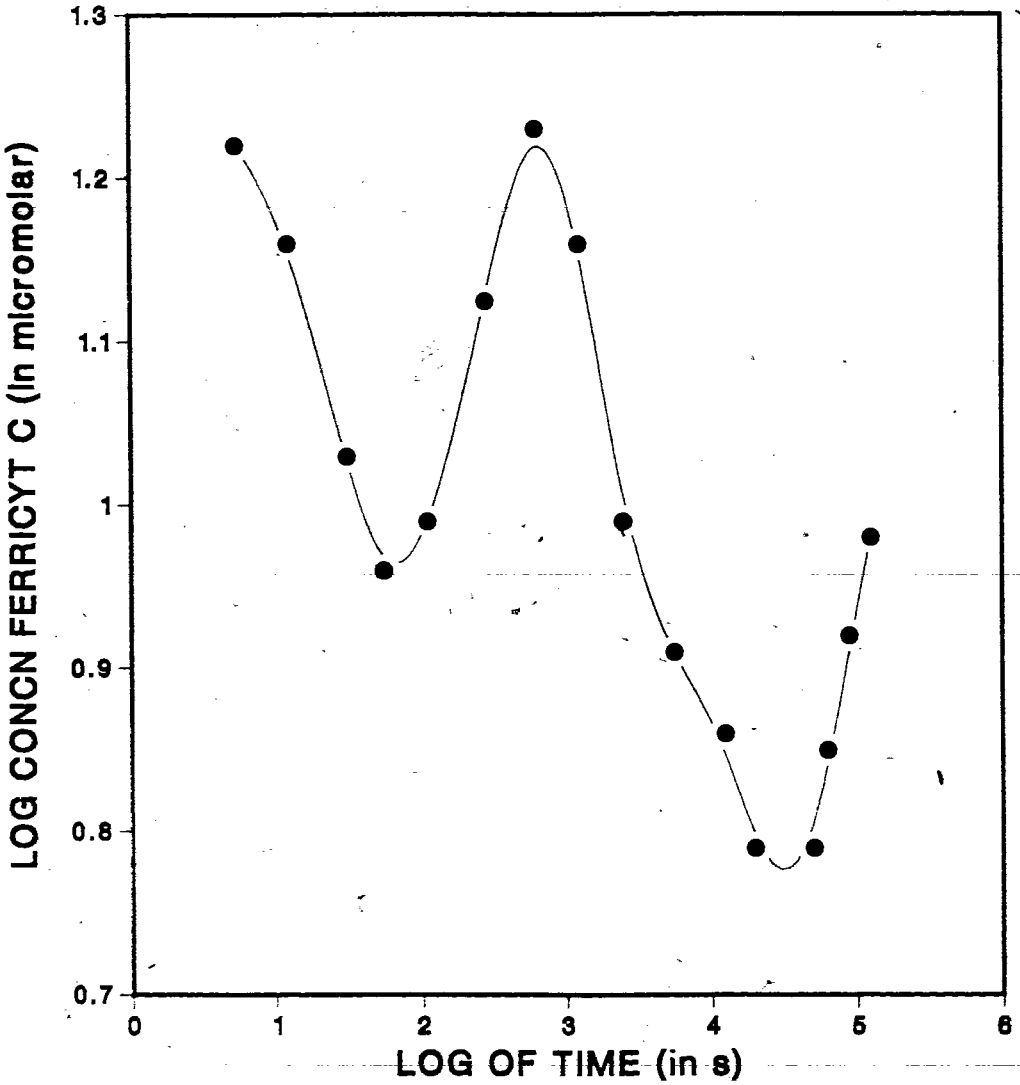
FIGURE 8: Roles of Metals or Hydroxyl Radicals in the Re-reduction of Cytochrome c: Effects of Chelex 100 treated buffer, desferrioxamine, iron, and mannitol on rates of re-reduction of cytochrome c. Reaction conditions were as described in Fig. 1 except buffers were at pH 8.00, ionic strength 0.04. Zero time represents the beginning of the second reduction phase. ●—●, Chelex treated buffers; O—O, iron added; ■—■, iron plus desferrioxamine; □—□, iron plus mannitol.

FIGURE 9: Rates of the Various Phases of Redox Change of Cytochrome c in the Presence of the 6-OHDA/O_2 Redox Couple - Effects of Scavengers: Reaction Conditions were as described in Figs. 1 to 5. The leftmost cluster of bars (6-OHDA autoxidation) represents, for comparison, the effects of the same scavengers on the autoxidation of 6-OHDA. The bars for catalase in the rightmost two clusters (re-oxidation and re-reduction) represent

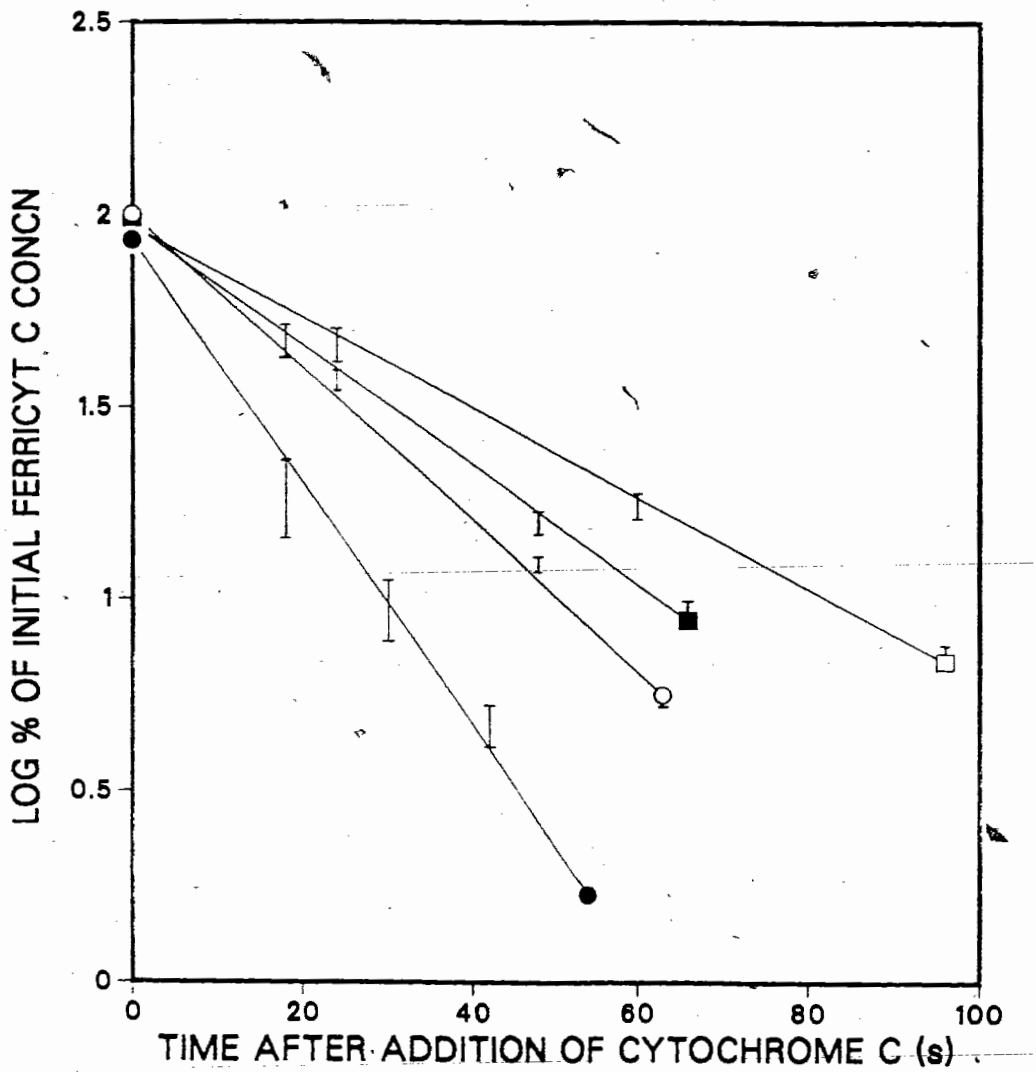
fluctuations around zero, since neither of these two phases were observed in the presence of catalase.

FIGURE 10: Rates of Re-oxidation and Re-reduction of Cytochrome c in the presence or absence of iron or mannitol. Reaction conditions were as described in Fig. 8.

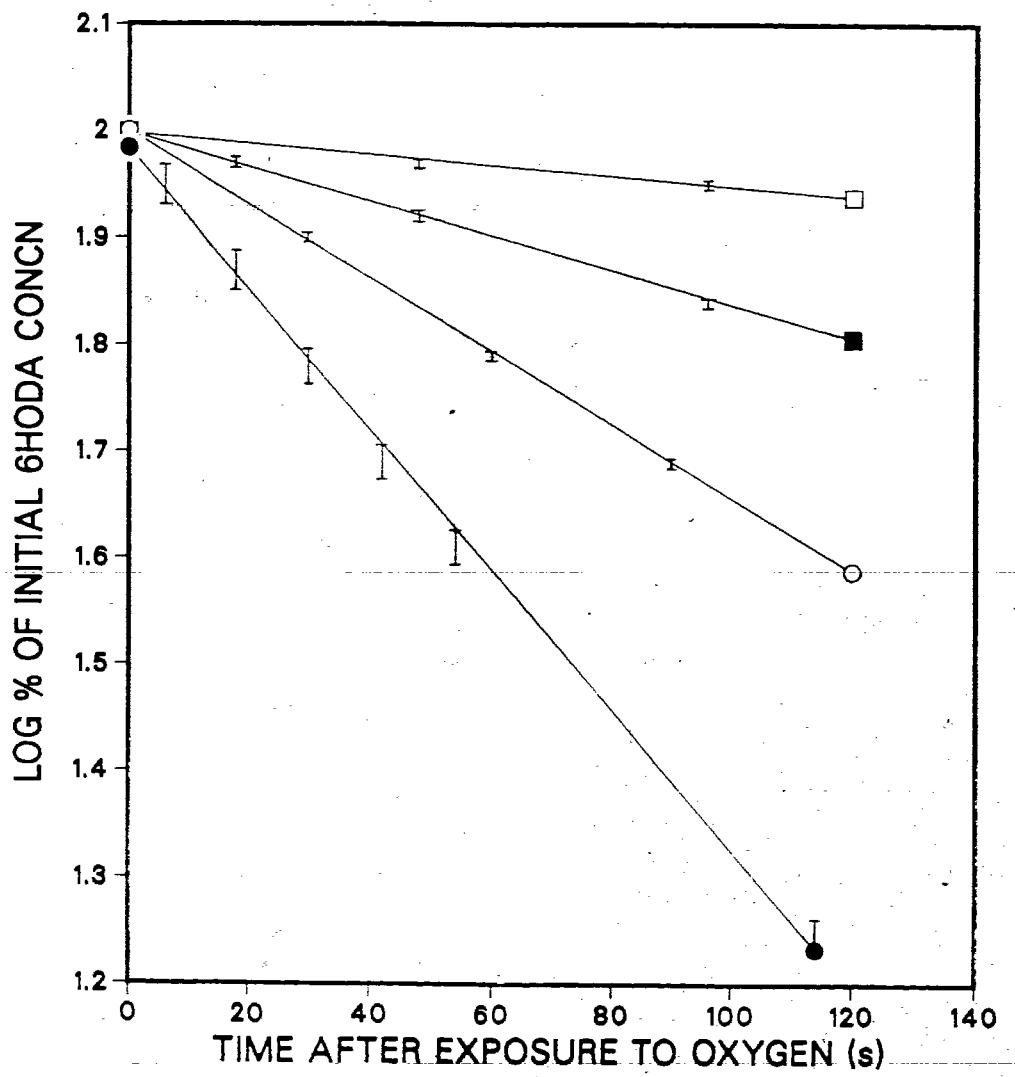
OSCILLATIONS IN REDOX STATE OF CYT C INDUCED BY 6HODA/OXYGEN



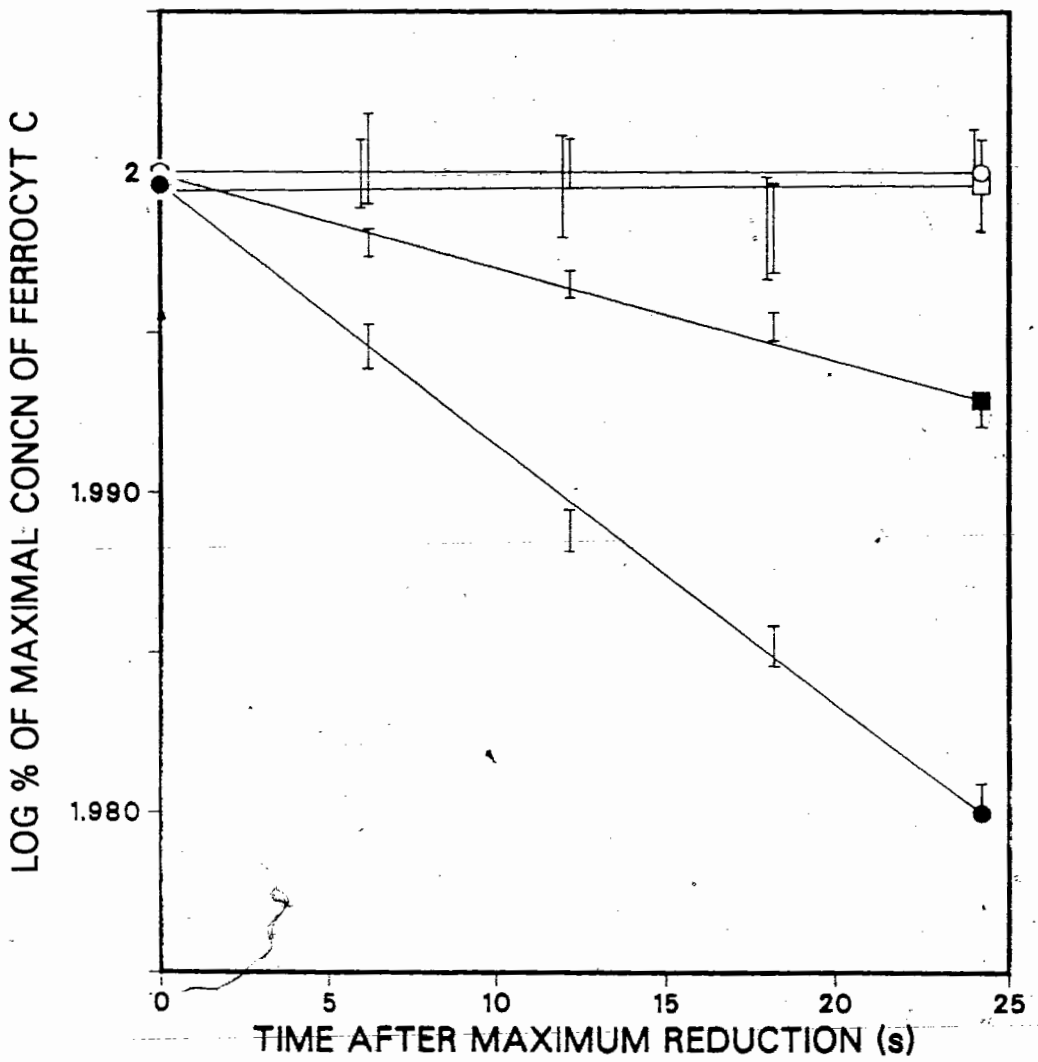
INITIAL REDUCTION OF CYT C BY 6HODA/OXYGEN: EFFECTS OF CATALASE OR SUPEROXIDE DISMUTASE



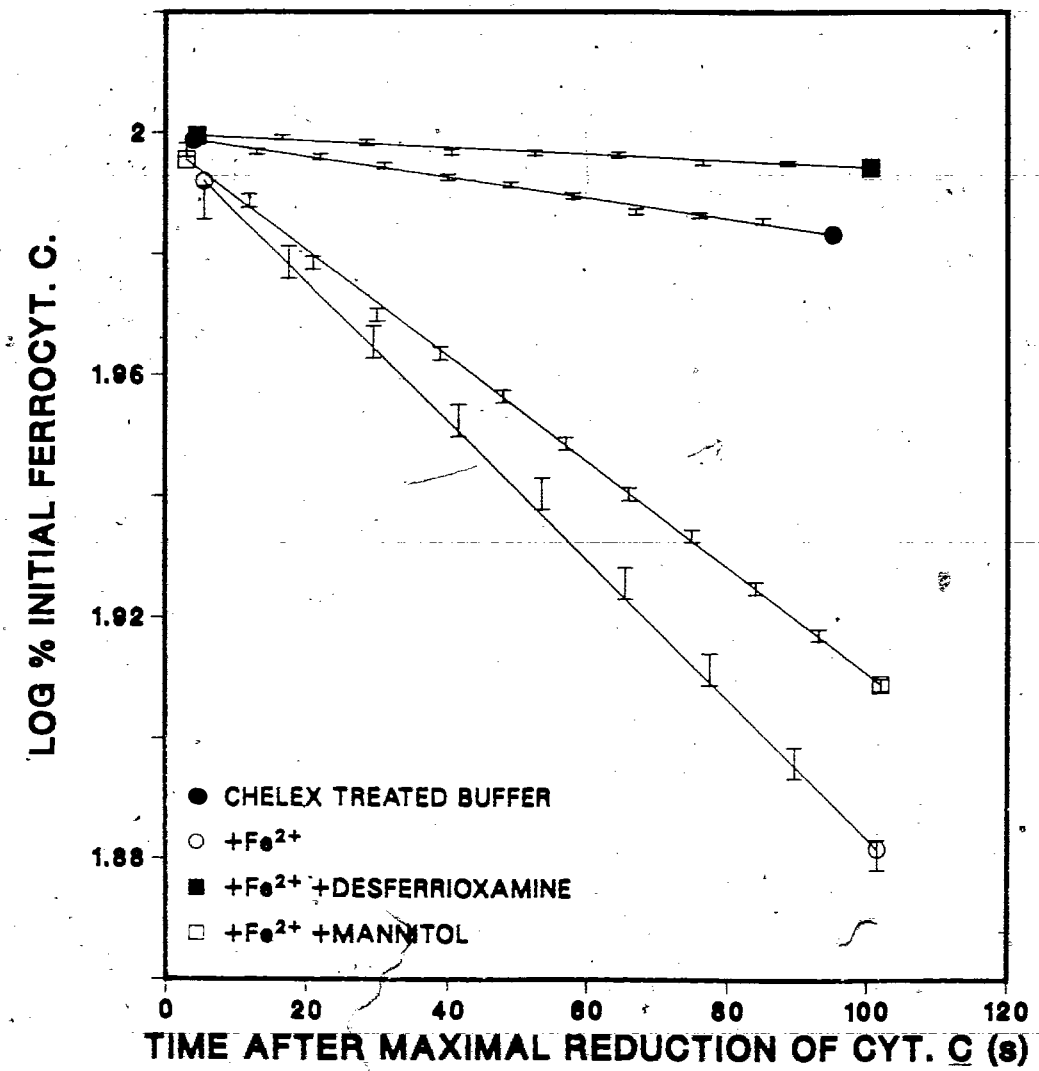
AUTOXIDATION OF 6HODA: EFFECTS OF CATALASE OR SUPEROXIDE DISMUTASE



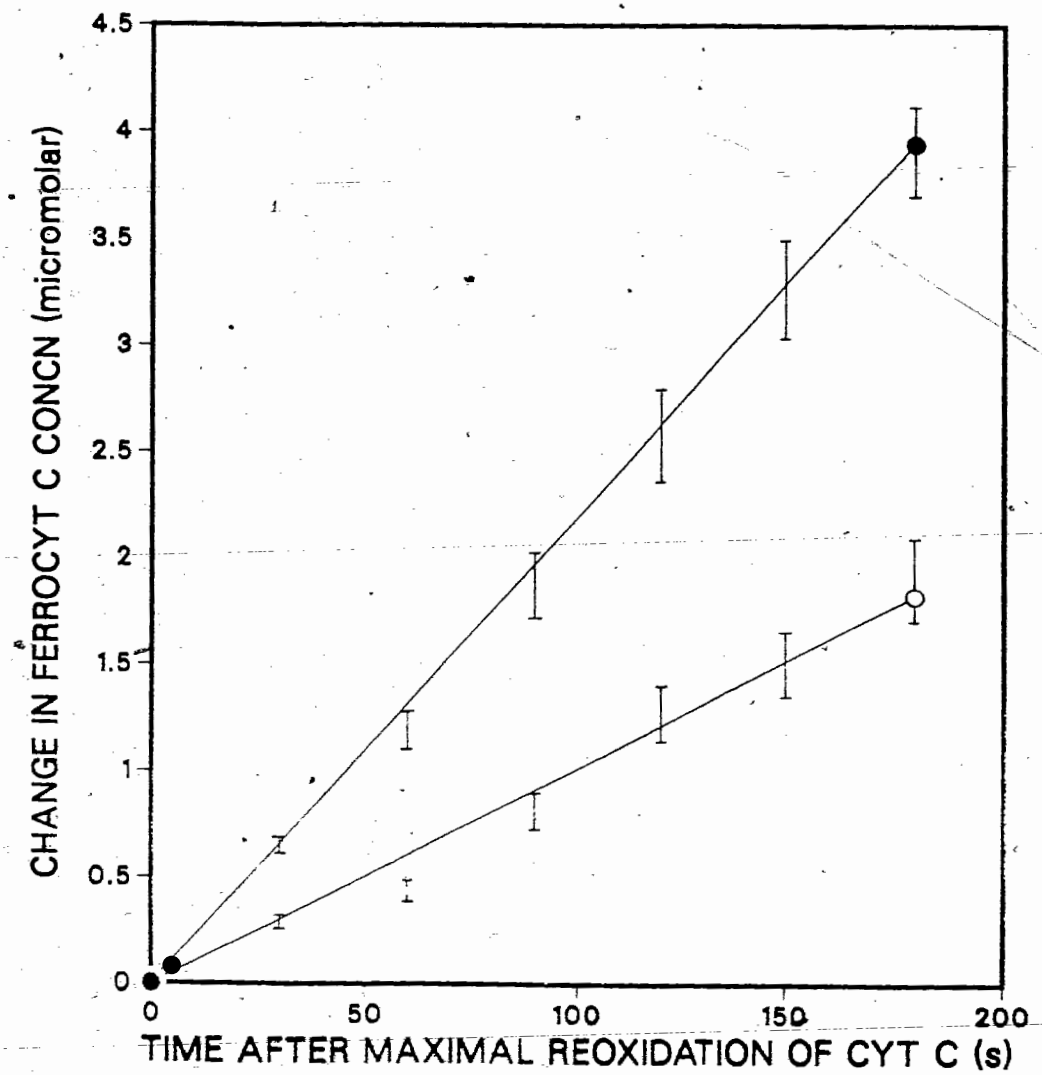
RE-OXIDATION OF CYT C: EFFECTS OF CATALASE OR SUPEROXIDE DISMUTASE



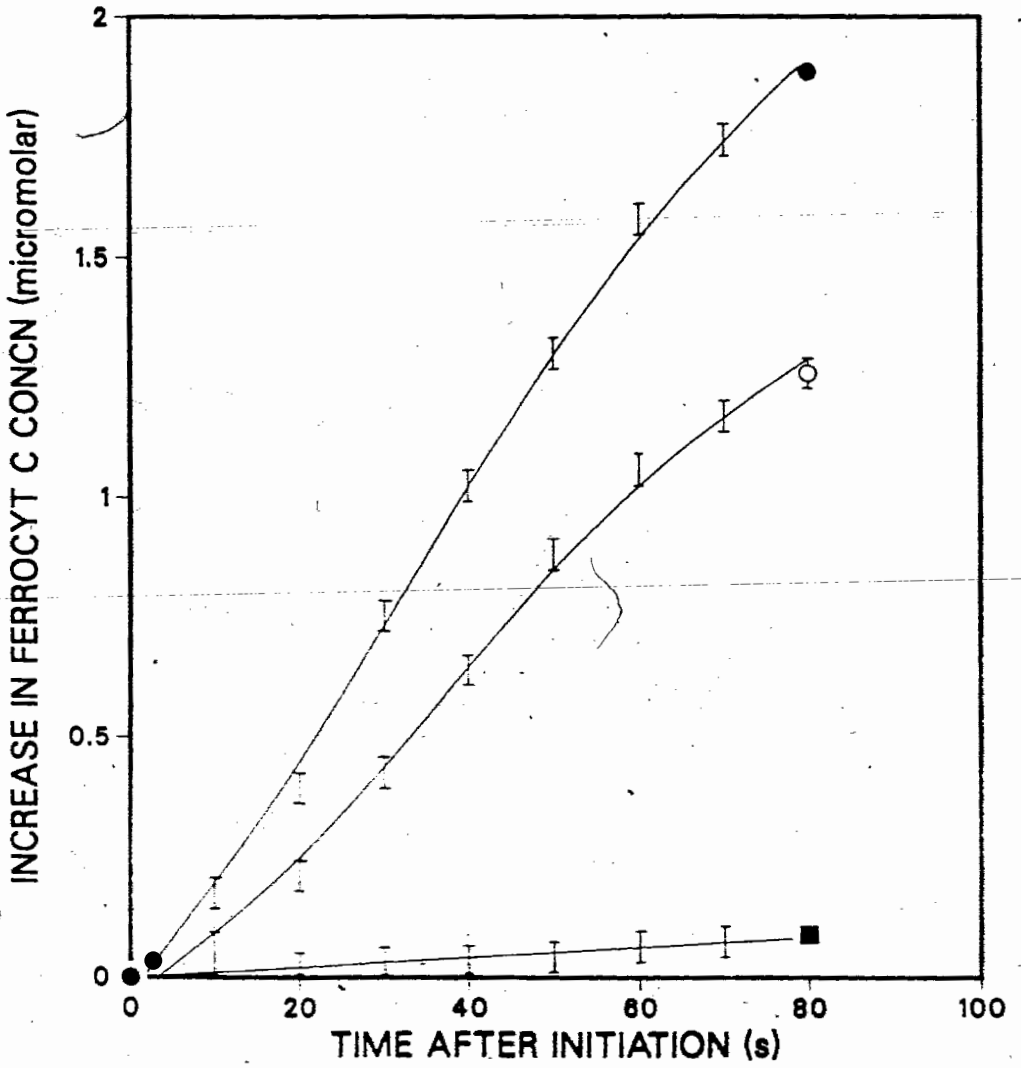
RE-OXIDATION OF CYTOCHROME C: EFFECTS OF CHELEX TREATED BUFFER, DESFERRIOXAMINE, $FeSO_4$, OR MANNITOL



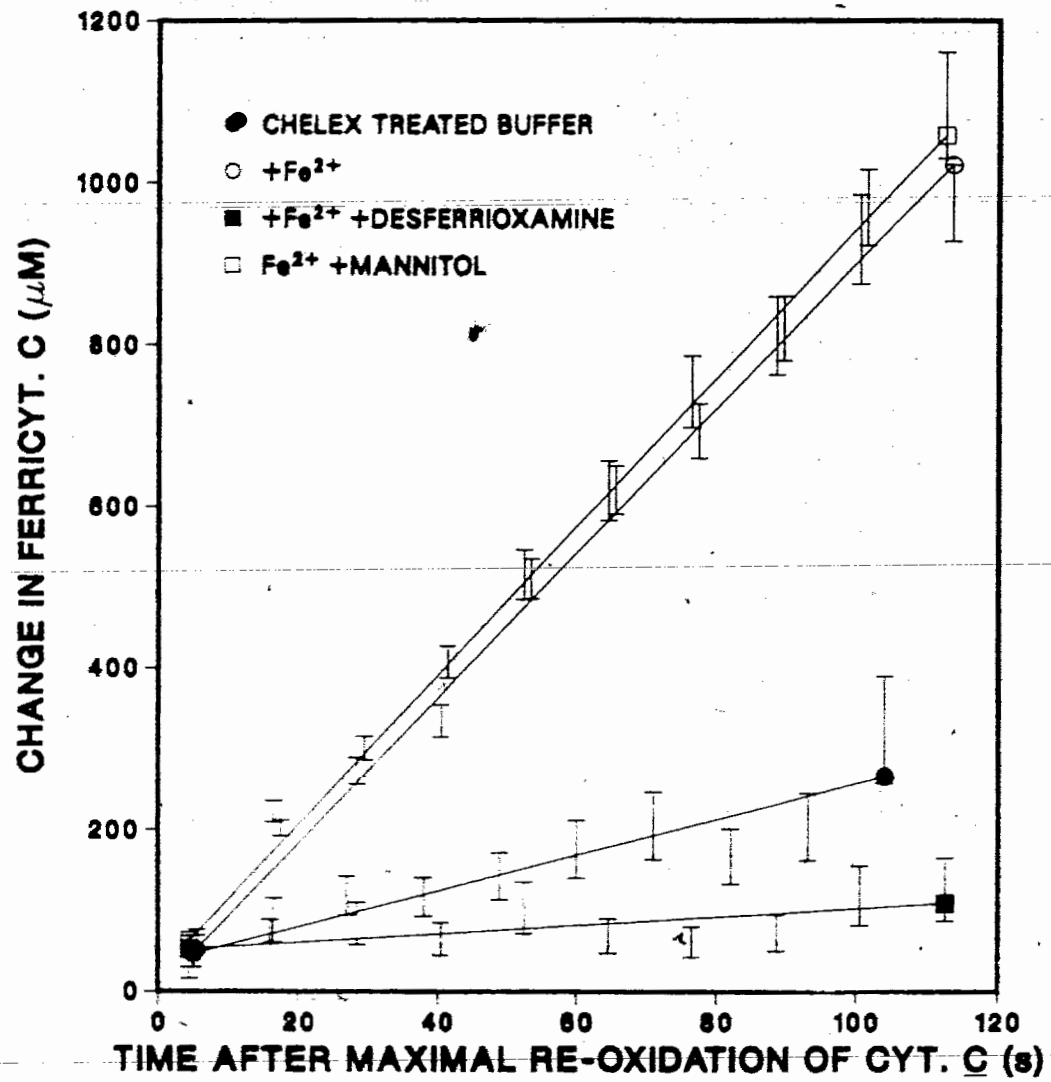
RE-REDUCTION OF CYT C: EFFECT OF SUPEROXIDE DISMUTASE



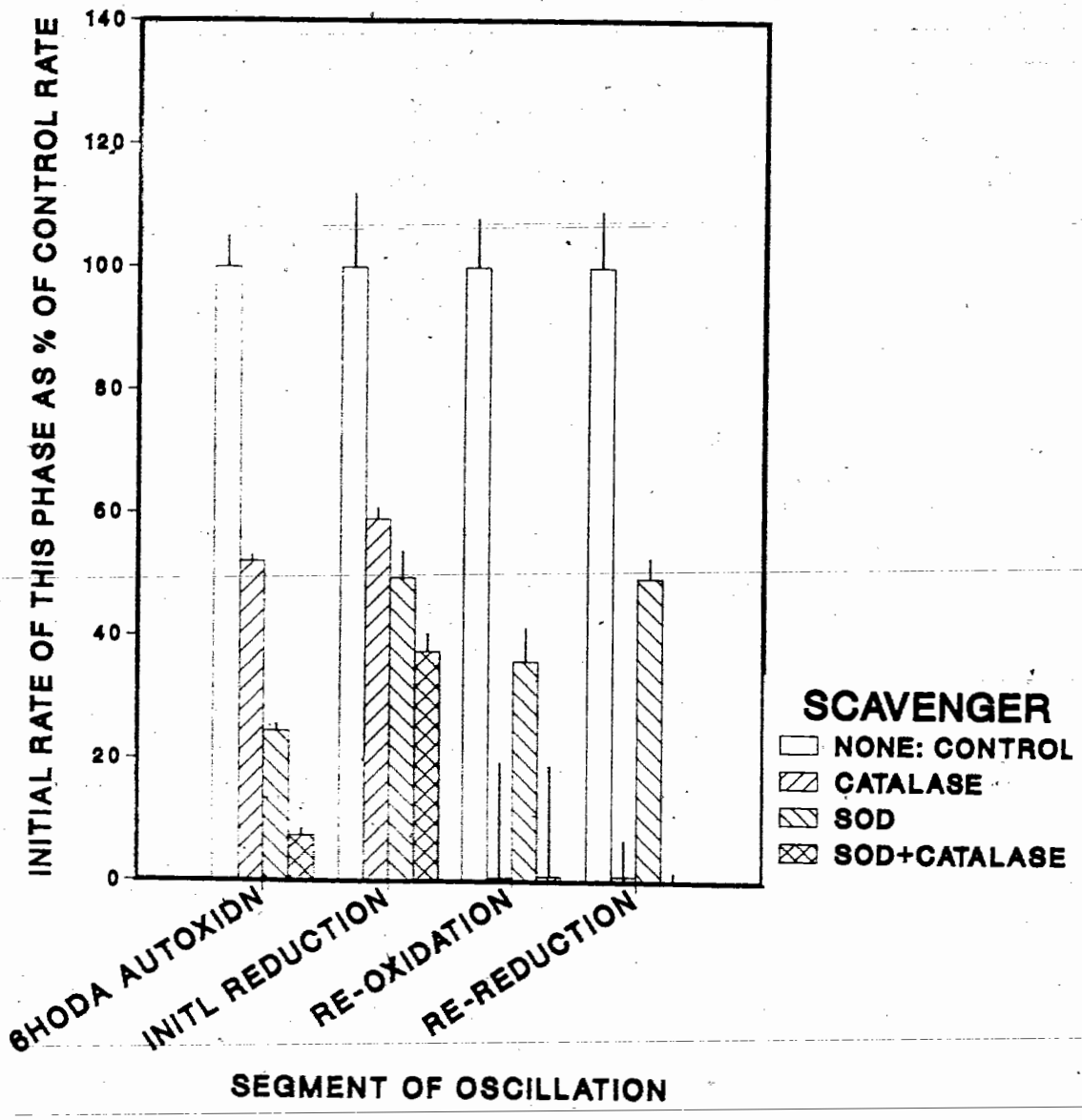
REDUCTION OF CYT C BY HYDROGEN PEROXIDE: INHIBITION BY SUPEROXIDE DISMUTASE



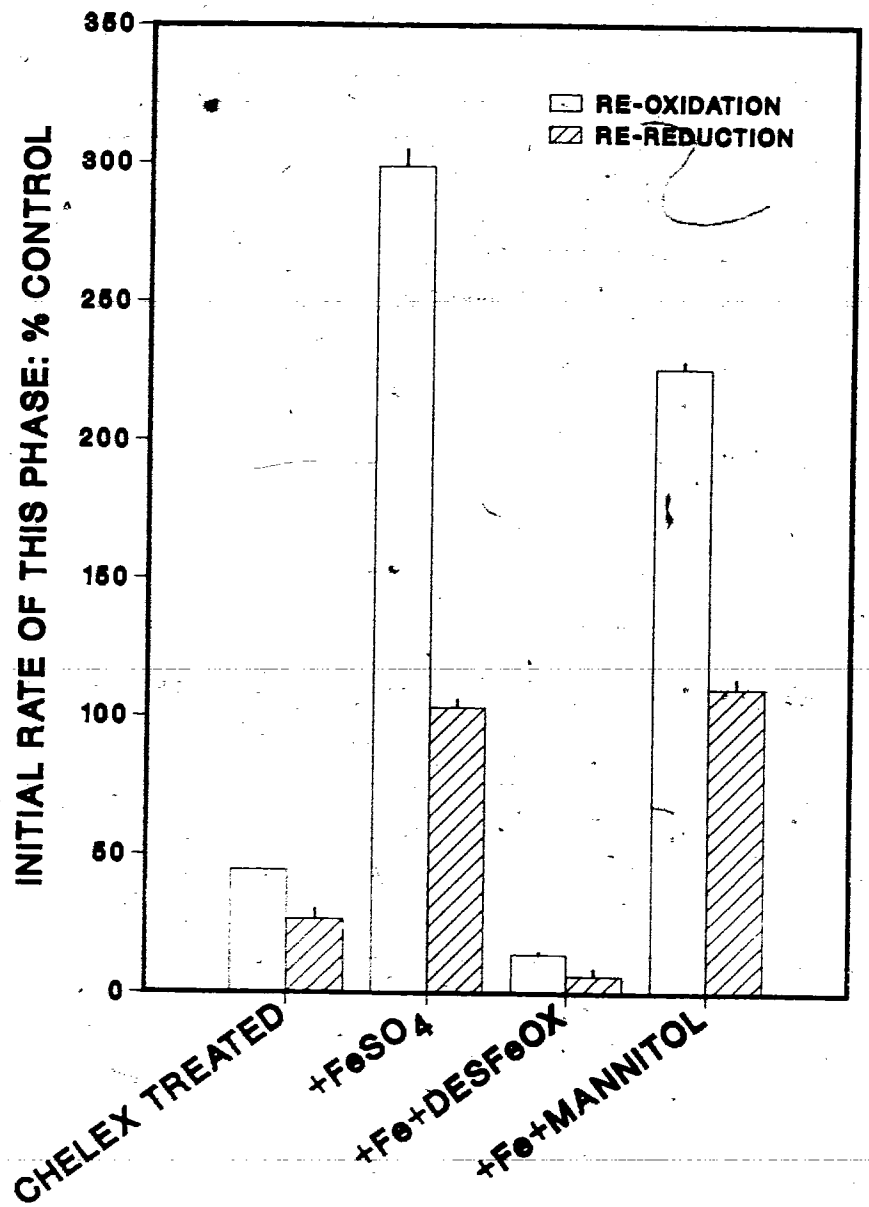
RE-REDUCTION OF CYTOCHROME C: EFFECTS OF CHELEX TREATED BUFFER, $FeSO_4$, DESFERRIOXAMINE, OR MANNITOL



RATES OF REDOX CHANGES OF CYTOCHROME C
IN THE PRESENCE OF 6HODA/OXYGEN:
EFFECTS OF SCAVENGERS



RATES OF REDOX CHANGES OF CYTOCHROME C IN THE PRESENCE OF 6HODA/OXYGEN



OVERVIEW AND PERSPECTIVE

DETAILS OF THE MECHANISM OF 6-OHDA OXIDATION

Some aspects of a mechanism for the oxidation of 6-hydroxydopamine (6-OHDA) can now be considered to be established. The following reactions represent the univalent reductions of O_2 involved in the initiation phase:

$$\text{6-OHDA} + Me^{n+}/O_2 \longrightarrow \text{semiquinone} + Me^{n+}/O_2^- + H^+ \quad (1)$$

$$\text{6-OHDA} + Me^{n+}/O_2^- + H^+ \longrightarrow \text{semiquinone} + Me^{n+} + H_2O_2 \quad (2)$$

The production and later removal of O_2^- in the above two reactions form part of the propagation phase. In contrast, production of the p-quinone from dismutation of the semiquinones represents a chain termination step:

$$2\ \text{semiquinone} \longrightarrow \text{p-quinone} + \text{6-OHDA.} \quad (3)$$

The sum of Reactions 1, 2 and 3 describes the two electron oxidation of 6-OHDA by O_2:

$$\text{6-OHDA} + O_2 \longrightarrow \text{p-quinone} + H_2O_2 \quad (4)$$

6-OHDA also reduces the product, H_2O_2 in a univalent pathway which may be represented by the following:

$$6\text{-OHDA} + Me^{n+}/H_2O_2 \longrightarrow \text{semiquinone} + Me^{n+}/{}^{\bullet}OH + H_2O \quad (5)$$

$$6\text{-OHDA} + Me^{n+}/{}^{\bullet}OH \longrightarrow \text{semiquinone} + Me^{n+} + H_2O \quad (6)$$

The overall rates of the above two reactions are slow enough that the dismutation of the p-quinone is no longer rate limiting:

$$2 \text{ semiquinones} \longrightarrow \text{p-quinone} + 6\text{-OHDA}. \quad (7)$$

Finally, the two electron reduction of H_2O_2 to water can be expressed as the sum of Reactions 5, 6 and 7.

$$6\text{-OHDA} + H_2O_2 \longrightarrow \text{p-quinone} + 2\ H_2O. \quad (8)$$

Although water is the final product of the reduction of O_2, the p-quinone will undergo intramolecular cyclisation to its aminochrome which has in turn been shown to participate in further redox reactions.

GENERAL FEATURES OF THE OXIDATION OF 6-OHDA

Simultaneous removal of metal ions, H_2O_2 and O_2^- completely inhibits the reaction even when molecular oxygen is in excess. If any of the three components was present, the oxidation proceeds since each can act independently as a co-oxidant of 6-OHDA.

"Removal" of the catalytic action of metal ions by chelating agents decreases the aerobic oxidation (by 20 to 67%) without changing the kinetics, while the removal of O_2^- induces sigmoidal kinetics. In the absence of O_2^-, the rates of p-quinone formation and O_2 utilisation are decreased by 96%. Thus O_2^- plays a major role in the formation of a ternary complex consisting of reductant-metal-superoxide which constitutes the rate limiting step in the aerobic oxidation of 6-OHDA. Fe^{2+}, at greater than catalytic concentrations, can substitute for O_2^- to the extent that the oxidation is not inhibitable by superoxide dismutase (SOD).

H_2O_2 contributes very little to the aerobic oxidation since its reduction by 6-OHDA is three orders of magnitude slower than reduction of O_2. Thus removal of H_2O_2 does not inhibit the reactions which constitute the initiation phase of the oxidation 6-OHDA. Consequently, catalase had no inhibitory effects, but was found to increase the oxidation. Although this stimulation was barely discernible, it was observed consistently over a variety of conditions containing other scavengers. This acceleration is not due simply to regeneration of oxygen by catalase from H_2O_2 since it was observed under conditions where oxygen was present in excess relative to 6-OHDA concentrations. It is more likely that catalase removes some inhibition by H_2O_2 or some intermediate produced from it. However H_2O_2, itself can act as oxidant in the absence of O_2. Therefore, H_2O_2 in the aerobic oxidation, competes for O_2^- which has an autocatalytic role in oxidising 6-OHDA.

In the absence of O_2, reduction of H_2O_2 by 6-OHDA requires metal ions. The most likely role for metal ion catalysis is the generation of $^{\bullet}OH$, since the 160% stimulation of the reaction by diethylenetriaminepentaacetate (DTPA) is decreased by hydroxyl scavengers. DTPA when chelated with iron generates $^{\bullet}OH$ from H_2O_2 in Fenton-type reactions. Since the hydroxyl radical does not oxidise 6-OHDA in an autocatalytic fashion, its role in the aerobic oxidation is negligible. In this latter reaction consequently, the hydroxyl scavengers (or catalase) have minimal effects.

Many if not all of the interactions observed in these studies involved inhibition of or catalysis by metal ions. We have characterised much of the general nature of their collective contribution as well as that specific to iron. However clarification of the synergistic and moderating interactions of the various transition metal ions is required if we are to understand the underlying properties which are responsible for their activities. Since their roles depend so greatly on the nature of the ligands which bind metal ions, it is important to determine the factors which change their redox properties or decrease their kinetic accessibility. For example, ethylenediaminetetraacetate (EDTA) increases the activity of iron in generating free radicals and concomitantly the cytotoxicity of the pro-oxidants. DTPA, also an "amino acid", has one more ligand for iron than EDTA. While both form stable chelates with the ferrous ion, Fe^{2+} only DTPA exists in a stable complex with iron in the ferric state. This difference results

in major differences in reactivity, depending on the nature of the targets and systems tested. DTPA retards reduction of bound iron, without preventing its reoxidation. It is also thought to retard redox reactions of metals other than iron. While desferrioxamine (which is a specific chelating agent for iron) has not yet been found to allow redox participation of the chelated iron, EDTA, like DTPA is effective in blocking copper accelerated oxidations.

BIOLOGICAL TOXICITY OF FREE RADICALS

While the above represents the skeleton upon which a mechanism of 6-OHDA autoxidation may be built, much work remains to be done regarding the interactions which are involved in its cytotoxicity. That the hydroxyl scavengers themselves participate in the propagation (as opposed to termination) of the free radical chain, has implications beyond the scope of the studies presented here. Of several examples of activation of by hydroxyl scavengers the most important agent is glucose, a ubiquitous substance found throughout biological systems. Glucose reactivated the aerobic oxidation of 6-OHDA after it had been completely blocked by the scavenger combination DTPA+catalase+SOD. In contrast glucose was moderately inhibitory in the anaerobic oxidation by H_2O_2. Thus glucose may play a significant role in modulating the cytotoxicity of 6-OHDA.

Many cellular metabolites can chelate metal ions so as to either enhance or inhibit toxicity of pro-oxidants. Those metalloproteins

which are redox enzymes have evolved to transfer electrons either in single or two electron steps with minimal side reactions. Many of these are also reactive toward intermediates generated by pro-oxidants and therefore can be recruited to mediate cellular damage. For example, we found that cytochrome c did not inhibit the autoxidation of 6-OHDA, although it competes with 6-OHDA for the catalytic species, O_2^-; instead it acted as a reversible transfer agent to shuttle electrons from reducing equivalents such as the semiquinone or 6-OHDA to intermediates of oxygen reduction.

Thus simple cellular components such as glucose or immensely complex macromolecules such as cytochrome c participate in the intracellular redox reactions of naturally occurring or xenobiotic pro-oxidants. Even macromolecules which do not contain metal ions can bind transition metal ions with various degrees of firmness by co-ordinate covalent or electrostatic interactions. Depending on these interactions, a pro-oxidant such as 6-OHDA may promote damage to specific to the macromolecule. Certainly proteins with free sulphydryl groups have been polymerised during the oxidation of 6-OHDA.

As a final paradox, SOD, an enzyme clincally used in treating pro-oxidant damage was found to accelerate the anaerobic oxidation of 6-OHDA by H_2O_2. Although the effect could be attributed to the known ability of superoxide to remove hydroxyl radicals, it was more probably due to free copper released from a minimal percentage of

denatured enzyme or copper which is no longer restricted by the structure of the native enzyme. Indeed, SOD denatured by boiling, increased this stimulation by approximately ten times. Thus SOD which inhibits the aerobic oxidation of 6-OHDA by 96%, and is considered as one of the cell's major defenses against free radical damage, becomes a metal ion source to promote generation of free radicals. This has far-reaching implications in the understanding of why pro-oxidant damage is more severe under hypoxic conditions.

While fundamental aspects of free radical reactions have been explored, interactions of the participating species with cellular components remain elusive. Important advances in the aetiology and prevention of a variety of clinical problems: including ischaemic injury to heart and brain; preservation of organs for transplantation; traumatic injury to the spinal cord; and many the problems of advancing age (including Parkinsonism) are in abeyance, pending such understanding. Detailed mechanisms of cellular damage are prequisite to rational strategies for effective prevention or treatment.