VANADIUM -CATALYZED OXIDATIONS OF l-ASCORBIC ACID AND CATECHOLAMINES: ROLES OF OXYGEN AND HYDROGEN PEROXIDE

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VANADIUM-CATALYZED OXIDATIONS OF ASCORBATE AND CATECHOLAMINES: ROLES OF OXYGEN AND HYDROGEN PEROXIDE

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ABSTRACT

Progress in understanding and treatment of free radical-mediated pathology is contingent upon a deeper and more systematic understanding of the roles of metals in endogenous free radical reactions. Vanadium is a particularly convenient focus for research of this type since the redox state of vanadium is an essential determinant of its biological activity, and this metal has diverse and unique biological effects, at least some of which are mediated by free radicals. We report the results of three series of experiments in which reactions of vanadium compounds with molecular oxygen, reduced oxygen species and reducing agents were studied. Oxygen consumption by V(III) or V(IV) was sensitive to modulation by pH, and ligands. Intracellular vanadium exists mainly as V(IV)-ATP complexes, and ATP inhibits O_2 consumption by both V(III) and V(IV) in the pH range 6.0 - 7.4. Thus, under the conditions which prevail intracellularly, reduced vanadium is protected from oxidation by O_2. Reactions of V(IV) or V(V) with H_2O_2 are also strongly affected by ligands. Hydrogen peroxide increases the rate at which V(IV) is oxidized to V(V), but in HEPES buffer, the rate of O_2 consumption is increased (at least initially) whereas in phosphate buffer, O_2 evolution is increased. Hydroxyl radical ($OH\cdot$) formation has previously been reported from reactions of V(IV) with H_2O_2. Nevertheless, the $OH\cdot$-mediated oxidation of deoxyribose to thiobarbituric acid-reactive products was not accelerated by the presence of either V(IV) or V(V). Instead, V(V) decomposed H_2O_2 via a catalase-like mechanism which is inhibitable by superoxide dismutase. Thus, $OH\cdot$ generation by V(IV) may be quantitatively unimportant. Further, the catalytic oxidation of reducing agents by vanadium may be more dependent upon reactions with reduced oxygen species than those of other transition metals. Vanadium(V)-mediated NADH oxidation is enhanced by exogenous H_2O_2. Vanadium-catalyzed oxidations of 6-OHDA were also highly dependent upon reactions involving reduced oxygen species. Anaerobically, V(V) oxidized twice as much 6-OHDA as V(IV) or Cu(II), although all three metal ions oxidized approximately one half the amount expected stoichiometrically Aerobically, V(V) and V(IV) were less effective than Fe(II) or Cu(II). In contrast to these other metal ions, superoxide dismutase inhibited strongly. In

addition all of the H_2O_2 produced during the reactions involving V(IV) or V(V) was decomposed via catalase-like mechanisms. In the oxidation of ascorbate, V(IV) and V(V) were virtually ineffective anaerobically or aerobically, being far less effective than Cu(II) or Hg(II) in both cases. However in the presence of exogenous H_2O_2, both V(IV) and V(V) catalyzed rapid ascorbate oxidation via mechanisms involving $O_2^{\cdot-}$. Vanadium is fundamentally different from other metals of physiological and or toxicological significance in its participation in free radical reactions. These differences depend upon pH, the nature of available ligands, and presence of reducing agents.

DEDICATION

For Toby,

Thanks buddy.

ACKNOWLEDGEMENTS

Very special thanks to all the people who helped when it counted, and just because they cared.

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CHAPTER 1

GENERAL INTRODUCTION

A. PURPOSE OF THE CURRENT THESIS

The purpose of this thesis is to provide new perspectives on the roles of metal ions in biologically relevant free radical reactions. Free radicals have been implicated in an ever growing number of disease and toxic phenomena. Although there has been a tremendous increase in the amount of applied research, there has been no parallel development in our understanding of the basic chemistry and biochemistry of free radical reactions, particularly as they may occur *in vitro* or *in vivo*. Interactions between metal ions, oxygen, reduced oxygen species and or biological reducing agents are fundamental to all free radical pathology. We have undertaken to reestablish and extend this essential area of research by examining and comparing the behavior of vanadium with that of a variety of physiologically and or toxicologically significant metal ions in biologically relevant free radical reactions.

1.0.0 TRANSITION METALS CATALYZE ENDOGENOUS FREE RADICAL REACTIONS

Transition metal ions may serve as both efficient initiating and propagating agents in endogenous free radical reactions. In fact, these metals and O_2 are highly regulated in biological systems in large part because of their inherent tendencies to interact together with autoxidizable organic compounds, generating toxic free radical species, since in the absence of metal ions and reducing agents, O_2 is not cytotoxic. The ease with which different transition metal ions participate in free radical reactions is not well documented. Although the vast majority of research has focussed on a few transition metals, in particular iron and copper, in principle, many other metal ions may undergo univalent oxidation or reduction in the presence of sufficiently reactive species (Venugopal and Luckey,1977), and so participate in endogenous free radical reactions. Three fundamental types of reactions may be identified: a) reactions with molecular oxygen, b) reactions with reduced oxygen species ($O_2^{\cdot-}$, H_2O_2) and c) reactions with reducing agents (either biological reducing agents or xenobiotics).

1.1.0 Reactions of metal ions with oxygen or activated species of oxygen are chain phenomena

Molecular oxygen in its ground state (triplet) is a stable biradical which is, by virtue of kinetic barriers, chemically inert in the absence of at least trace amounts of metal ions . Since the molecular orbitals of lowest available energy contain two unpaired electrons with parallel spin, electron transfer to molecular oxygen must necessarily occur by a series of univalent electron transfers to overcome spin restrictions in accordance with Pauli's principle. Metal ions are ubiquitous in biological systems, often serving as the primary catalysts for the activation of molecular oxygen. In particular, iron and copper have partly-filled d or f orbitals, exhibit variable oxidation states and at least some paramagnetic properties (Venugopal and Luckey,1977). Interactions with O_2 species are facilitated then, since the spin restrictions which limit the chemical reactivity of O_2 are circumvented. Reactions of metal ions with molecular oxygen cause univalent reduction of oxygen to superoxide (O_2^-) or its conjugate acid, the perhydroxyl radical ($HO_2\cdot$) (Sawyer et al, 1980) and the concomitant oxidation of the metal ion. Superoxide itself is a moderate reducing agent toward most metals (Youngman,19841), but is also basic, serving as a strong nucleophile towards a variety of organic compounds (Ingraham and Meyer,1985). As a radical, O_2^- can abtract hydrogen atoms (Ingraham and Meyer,1985). With regard to metal ions, O_2^- (serving as a reductant) can promoting redox cycling between reduced and oxidized forms. Similarly, reactions of metal ions with hydrogen peroxide (H_2O_2) (the two electron reduction product of O_2) results in rapid oxidation of reduced metal ions, and the production of a further-reduced species of O_2, the hydroxyl radical ($OH\cdot$). Thus reactions of metal ions with oxygen or activated species of oxygen are chain processes which may continue for undetermined periods of time. Redox cycling of metal ions may well be involved where the reducing action of O_2^- is sufficient to counter the oxidizing effects of O_2 and H_2O_2.

1.2.0 The Haber-Weiss and Fenton reactions describe reactions of transition metals with O_2^- and H_2O_2

As a result of the rapid spontaneous disproportionation of O_2^-, reduction of oxygen to O_2^- will yield at least some H_2O_2. In its original formulation, the Haber-Weiss reaction postulated interactions between O_2^- and H_2O_2 (Haber and Weiss,1934; Fig.1.0.0). These interactions were deduced on the basis of the formation of a strong oxidizing agent - the hydroxyl radical (OH·) (Beauchamp and Fridovich, 1970; Babior et al. ,1975; Cohen,1977). Further research clearly demonstrated that this reaction does not occur in 'pure' chemical system, but requires the presence of at least catalytic amounts of transition metal ions (McCord and Day, 1978; Halliwell,1978; Halliwell,1981b; Halliwell,1982). More importantly, the Haber-Weiss reaction may seldom if ever occur under physiological conditions. The necessity for the presence of metal ions capable of acting as catalytic agents suggests that the Haber-Weiss reaction may in fact be more accurately represented as the sum of two separate reactions (Fridovich,1983) (Fig.1.0). The simplest Fenton system requires the presence of only a reduced metal ion such as Fe(II) or Cu(I) and H_2O_2. Superoxide generated in the course of these reactions acting (Symonyan et al.,1983) as a reductant, allows some redox cycling of reactive metal ions. Although the physiological relevance of Fenton reactions in general has been debated in the past, it is now widely accepted (Winterbourn,1981; Halliwell,1982; Halliwell and Gutteridge,1984). When metals become "decompartmentalized", all the other required components are available in vivo as intermediates formed for example in the reduction of molecular oxygen during the course of normal cellular respiration (Fridovich,1975; Boveris,1984).

1.3.0 Metal-catalyzed oxidations of biological reducing agents serve as useful model systems

The inclusion of a reducing agent in Fenton-type systems allows redox cyclng of the metal ion and so prolongation and amplification of the reaction. A number of molecules, including O_2^-, ascorbate, thiols (Bucher et al.,1983; Searle and Willson,1983) or various xenobiotics including herbicides like

paraquat may also fulfill this role. These agents may themselves undergo redox cycling and may also form relatively long-lived complexes with free metal ions. Electron transfer reportedly occurs within a ternary metal-reductant-oxygen species complex (Gee and Davison,1984). This feature of O_2 reduction has several important implications. The nature of the reducing agent, metal and ligand may influence both the rate of reaction, the chain length and the steady state yield of various oxygen radical species. The formation of secondary radical species may increase the toxicity of a given metal/reductant complex. For example, l-ascorbic acid (Khan and Martell,1967; Martell,1982), 6-OHDA (Gee and Davison,1984; Bandy and Davison,1988; Steele et al,1988) and certain antitumor drugs (Favaudon,1982; Muindi et al.,1984; Zweier et al,1986) form complexes with metal ions. Mechanisms of this type may be more general than previously supposed.

Interactions between metal ions, O_2, reduced oxygen species and or biological reductants have usually been discussed in terms of steric accessibility and the standard potentials of the individual reactants. Recent research reports, however, have indicated a number of general lines of evidence which argue effectively against a reliance on purely thermodynamic approaches - emphasizing instead kinetic factors. These lines of evidence include the following - (1) The abilities of various metals and organic molecules to reduce O_2 and or H_2O_2 (and thus redox cycle) are not well documented; (2) Much recent evidence has emphasized the fact that the reactivity of radical reaction chain intermediates may not be reflected accurately by either steric (entropic) or energetic (enthalpic) properties of reactants and products, since the rapidity of reactions between coreactants may ultimately determine their reactivity; (3) time-dependent changes in the relative presence of intermediates may also alter the nature of radical reactions, leading to complicated effects not observed over short periods of time (Davison and Hewlett,1971; Davison and Gee,1984). Clearly then, free radical reactions may be characterized accurately, only if the modulations in reaction components occurring as a result of the formation of complexes of intermediates over time are known.

For these reasons, the choice of appropriate test systems is problematic. Since under normal physiological conditions, concentrations of reductants far exceed those of metal ions, one useful way to model these processes is in terms of metal-catalyzed oxidations of reductants. Reactions of particular interest are shown in Fig.2.0. The most straightforward approach involves the use of simple systems which can be characterized as completely as possible. Determination of the ability of metal ions to oxidize reductants directly (in the absence of O_2) provides information regarding the nature of different metal-reductant chelates or complexes.

On the other hand, the ability of metal ions to oxidize reductants in the presence of O_2 provides an indication of the ability of a metal ion to activate O_2, perhaps within metal-O_2 or metal-reductant-O_2 complexes and the ability of the metal to redox cycle in the presence of O_2). Similarly, the rate and extent of O_2 consumption should also reflect the ability of a metal to activate O_2 and redox cycle. Further insights may also be gained by comparing measures of reductant oxidation and O_2 consumption. Superoxide production (and the extent to which it acts as an initiating and or propagating agent) may be determined by observing the inhibitory effects of superoxide dismutase on the progress of the reaction. Endogenous H_2O_2 production and decomposition may be assessed through observiing the inhibitory effects of catalase on the progress of the reaction, the emphasis in this case being on the possible participation of a given metal ion in Fenton reactions and or the formation of reactive metal peroxo complexes, as well as the ability of the metal to redox cycle under conditions where H_2O_2 acts as the ultimate electron acceptor. These phenomena should be exaggerated in the presence of added H_2O_2. Generation of OH· or OH·-like species may also be assessed by means of the deoxyribose-thiobarbituric acid assay for example, or through observing the effects of OH· scavengers such as mannitol or ethanol. Finally, useful information may be gained by observing the effects of ligands on the reactivity of a metal ion towards reductants, O_2 and or reduced O_2 species.

B. FOCUS AND CONTENT OF THE CURRENT THESIS

Vanadium provides a particularly convenient focus for research of this type since there is currently tremendous interest in the biological effects of this metal, at least some of these apparently being mediated by free radicals. Further, vanadium provides a particularly convenient focus for research of this type since: (1) although present at only ultratrace concentrations in mammalian tissues, vanadium exerts powerful biological effects, (2) the redox state of vanadium is an essential determinant of its biological activity since for example V(V) is much more toxic than V(IV), and while vanadium in plasma exists primarily as V(V), intracellularly V(IV) predominates, (3) the redox chemistry of vanadium is at the same time one of the most complex and least characterized of any biologically relevant metal, and (4) reactions of V with O_2, reduced O_2 species and or reductants are not well characterized.

Thus, the major questions we sought to address in the present thesis included the following: 1) how does the catalytic effectiveness of vanadium compounds compare with that of other physiologically and or toxicologically relevant metal ions?; 2) to what extent are the mechanisms of action of vanadium species similar to those of these other metal ions?; 3) are the specific roles of O_2 and H_2O_2 in the catalytic mechanisms of action of vanadium unique?; 4) to what extent do ligands and alterations in pH modulate the redox activity of vanadium in the presence of l-ascorbic acid or catecholamines?; 5) are the catalatic actions of vanadium apparent in either the V(IV)- or V(V)-stimulated oxidations of l-ascorbic acid or 6-hydroxydopamine (6-OHDA)?; 6) since vanadate is apparently not an effective catalyst of ascorbate oxidation, yet vanadium interactions with ascorbate are biologically significant, are there conditions under which vanadium is a good catalyst?; and 7) can the greater catalytic activity of vanadium as compared with other metals towards catecholamines be explained?

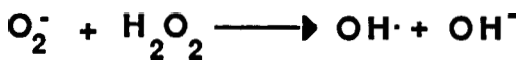
In Chapter 1 of this thesis, we review basic information concerning interactions between metals, molecular oxygen or reduced oxygen species and reducing agents. In Chapter 2 available information

concerning the variety of chemical species which comprise biochemically active vanadium, the relative ability of vanadium species to activate molecular oxygen and form strongly oxidizing species such as $OH\cdot$ in reactions with O_2^- and H_2O_2 and vanadium-catalyzed oxidations of l-ascorbic acid and catecholamines, as well as the involvement of the latter in vanadium-mediated tissue damage is presented. Original data concerning the ability of vanadium to decompose H_2O_2 by Fenton or catalase-like mechanisms is also presented since these reactions may be important in determining the biological effects of vanadium. Subsequently in Chapter 3, we consider the mechanisms underlying the vanadium-catalyzed oxidations of the synthetic catecholamine analog 6-OHDA, comparing the catalytic properties of vanadium with those of iron and copper in order to determine those aspects of vanadium-catalyzed reactions which are unique. The results of a similar, but more detailed study in which the catalytic properties of V(V) and V(IV) towards l-ascorbic acid were compared with those of a variety of other transition and non-transition metals are presented in Chapter 4. Finally, in Chapter 5, the major conclusions to be drawn from this thesis are summarized and discussed, and possible directions for future research presented.

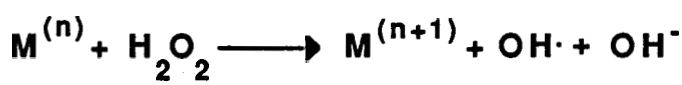
Figure 1.0

The Haber-Weiss reaction, classical and non-classical Fenton reaction systems

Haber-Weiss reaction



Classical Fenton reaction



Classical and non-classical Fenton reaction systems

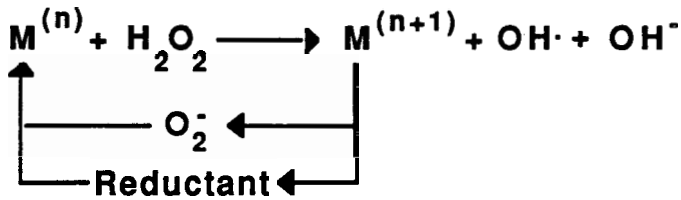
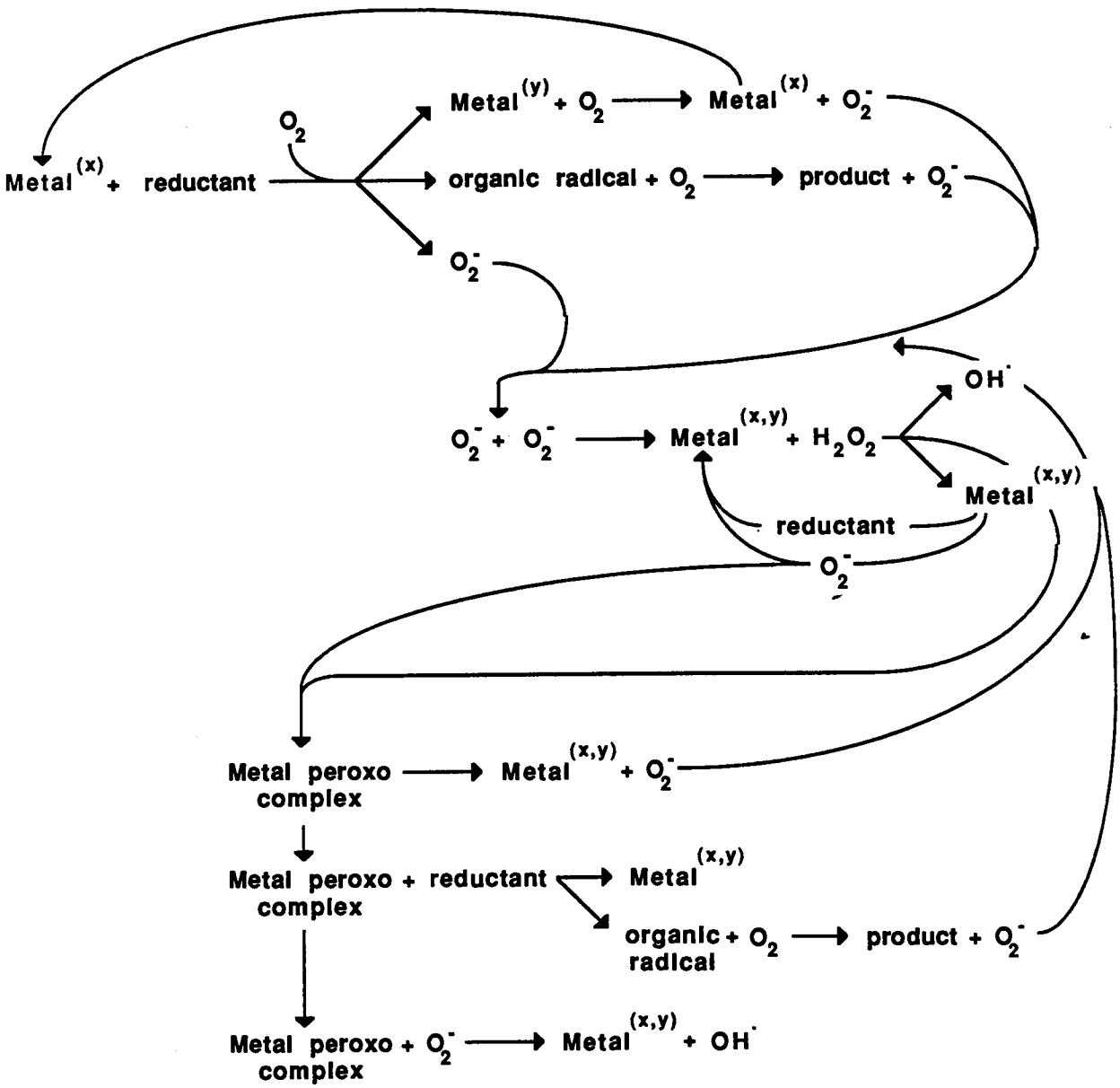


Figure 2.0

Biologically relevant interactions between metal ions, oxygen, reduced oxygen species and or reducing agents



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CHAPTER 2

BIOLOGICALLY RELEVANT REDOX REACTIONS OF VANADIUM: A CRITICAL REVIEW AND COMMENTARY

ABSTRACT

Submicromolar amounts of vanadium have a variety of effects on living cells both *in vitro* and *in vivo*. These reflect the actions of an intracellular pool of vanadium existing in a dynamic steady state among a variety of oxidation states. Cellular oxidations and reductions of vanadium occur constantly and simultaneously. These redox reactions of vanadium determine its biological activity, amplifying some cellular responses and inhibiting others. The nature of the ionic species of vanadium which occur intracellularly determines their redox potentials and reactivity. These involve at least three oxidation states, a myriad of ionic and oligomeric forms, as well as complexes with oxygen in various states of reduction, oxygenated and nitrogenous ligands. The relative concentrations of the different redox states of vanadium change as the dynamic steady state shifts under the influence of changing conditions. Important determinants include vanadium concentration, pH, ligands, ionic strength, and cellular concentrations of oxidants and reductants.

Oxidations of V(III) or V(IV) by molecular oxygen are accelerated by high pH, phosphate buffer over HEPES, histamine and desferrioxamine, but slowed by ATP and serum albumin. Thermodynamic considerations determine many of the redox reactions of vanadium. Some however, are explicable only by reference to kinetic effects. Oxidations of V(III) or V(IV) by O_2^- or H_2O_2 involve formation of incompletely characterized complexes, and may result in generation of either free or "site-specific" hydroxyl radicals. Vanadium (V) is also subject to oxidations by O_2^- or H_2O_2 in which partial transfer of electrons to bound oxygen occurs. Such complexes represent conversion to a higher formal oxidation state. Their potential biological relevance lies in their tendency to decompose, yielding reactive species. Reactions of V(V) with H_2O_2 include a (superoxide-dependent) ability to accelerate disproportionation of H_2O_2 to O_2 and H_2O in a catalase-like reaction. In general, the predominantly reduced state of vanadium intracellularly is determined in part by the lower concentrations of O_2. In addition, the lower pH intracellularly, and complexation by adenine nucleotides alter the oxidation potential of vanadium in favour of the reduced form.

The extent of reduction of cellular vanadium can be crucial to its biological actions. For example, oxidants can increase the insulin-like ability of V(V) to increase IGF-II binding to adipocytes of rats (Kadota et al, 1987). In the steady state, oxidations of vanadium are balanced by reductions at an equivalent rate. Cellular reductants of V(IV) and V(V) include ascorbate, catecholamines, NADH and cytochromes. Reductants may either decrease or increase vanadium toxicity. For example, ascorbate decreases the lethality of vanadium in rats injected with vanadium, decreasing renal lipid peroxidation. Ascorbate at 10^{-4} M increases the inhibitory effect of V(V) on Na^+/K^+-ATPase, while 2×10^{-3} M ascorbate partially reversed inactivation. Similarly, catecholamines reverse the V(V)-mediated inhibition of Na^+/K^+-ATPase. Some of the effects of vanadium on heart and brain result from its ability to oxidize and interfere with transport of catecholamines. A detailed and quantitative knowledge of the redox activity of vanadium is clearly prerequisite to a full understanding of its biological actions.

A. REDOX REACTIONS OF VANADIUM DETERMINE ITS BIOLOGICAL ACTIONS

1.0.0 VANADIUM HAS DIVERSE AND UNIQUE BIOLOGICAL EFFECTS

The biological effects of vanadium are diverse and unique. With increasing awareness of the biological potency of vanadium, the rate of appearance of research articles dealing with the biological effects of this metal has increased exponentially (by about 15% per year) over the past 5 years. At the cellular level, vanadium inhibits enzymes including ATPases (Cantley et al,1977, North and Post,1984), glucose 6-phosphatase (Singh et al, 1981) acid and alkaline phosphatases including phosphotyrosine phosphatases (Chasteen,1983; Swarup et al,1982a; Swarup et al,1982b, Nechay,1984). Conversely, vanadium stimulates adenylate cyclase (Schwabe,1979; Hackbarth et al,1980; Krawietz et al,1980; Krawietz et al,1982), stimulates esterification of protein bound tyrosine residues (Tracy and Gresser,1986) and exerts insulin mimetic effects (Heyliger et al,1985; Ramasarma and Crane,1981; Tamura et al,1984; Dubyak and Kleinzeller,1980; Kadota et al,1987). It also inhibits cellular protein degradation (Seglen and Gordon,1981), modulates the activity of enzymes involved in nucleic acid metabolism (Sabbioni et al,1983), blocks cell differentiation (English et al,1983), and promotes cell proliferation (Hori and Oka,1980; Carpenter,1981; Smith,1983; Kinksnorth et al,1986; Marini et al,1987), induces neoplastic transformation (English et al,1983), accelerates animal growth (Schwarz and Milne,1971; Strasia,1971) and inhibits tumor colony growth *in vitro* (Hanauske et al,1987). Each of these biological effects involves some amplification mechanism, without which the submicromolar concentrations of vanadium in tissues would be ineffective.

Amplification of the effects of vanadium may occur as a result of: (1) binding of the divalent cation VO^{2+} to regulatory binding sites for divalent cations such as Ca(II) or Mg(II), (2) non-enzymatic esterifications of targets of protein kinases by V(V), leading to an autocatalytic cascade of phosphorylations (Tracey and Gresser,1986), (3) stimulation of adenylate cyclase (Schwabe,1979; Hackbarth et al,1980; Krawietz et al,1980; Krawietz et al,1982), or (4) redox cycling of the metal ion leading to the generation of activated species of oxygen or organic radicals.

The redox state of vanadium is crucial to its involvement in each of these mechanisms. Numerous studies emphasize the need to understand the unique redox chemistry of vanadium under physiological conditions. However, no comprehensive overview of the factors governing the redox state of vanadium in biological systems, reactions of V(III), V(IV) and V(V) with biological oxidants and reductants, or the involvement of redox reactions in the biological effects of vanadium has previously appeared.

The present work addresses the need for such a review, being a compilation of currently available information concerning the redox chemistry of vanadium, and the involvement of redox reactions in its biological effects. The general questions posed in the following sections are - (1) to what extent does the biological potency of vanadium depend on its redox state?, and (2) which redox reactions of vanadium amplify its biological effects?. Specifically, we review available information concerning the ability of biochemically active vanadium to: (1) reduce molecular oxygen to O_2^-, (2) form free or complexed radical species in reactions with O_2, O_2^- or H_2O_2, (3) undergo reduction anaerobically or aerobically by biological reductants such as cytochrome c, NADH, l-ascorbic acid, catecholamines, and thiols, (4) undergo redox cycling, (5) induce lipid peroxidation *in vitro* or *in vivo*, as well as (6) the involvement of lipid peroxidation in specific biological effects of vanadium. Finally, we consider the relative effectiveness of metal chelating agents as therapeutic agents against vanadium toxicity.

2.0.0 THE CHEMICAL NATURE OF BIOLOGICALLY ACTIVE VANADIUM REQUIRES CLEARER DEFINITION

Prior to considering these topics in detail, it is important to be aware that: (1) the chemical nature of biologically active vanadium is not well defined since vanadium exists in a diverse array of possible redox states, oxygen complexes and ionic species, (2) vanadium in tissues occurs almost exclusively in monomeric form, (3) virtually all intracellular vanadium is bound to physiological ligands, (4) vanadium metabolism involves interconversions between the V(III), V(IV) and V(V)

oxidation states, and (5) factors such as pH and ligands may modulate the redox potentials of vanadium in the complicated environment of the cell. In order to extend these generalizations, the chemical nature of biologically active vanadium requires clearer definition.

2.1.0 Vanadium exists in a diverse array of possible redox states, oxygen complexes and ionic species

The charge, oxidation state, ligands and states of protonation of the vanadium species which occur *in vitro* or *in vivo* are not known with certainty. The redox chemistry of vanadium in aqueous solution is perhaps the most complex and least characterized of any biologically relevant metal. Vanadium has access to at least three redox states (in addition to the metallic state) under the conditions prevailing intracellularly. In any of these oxidation states, vanadium forms complexes with oxygen in any of its four redox states (H_2O, $OH\cdot$, O_2^-, $HO_2\cdot$). Most of the twelve species thus possible can exist in various states of protonation determined by their respective values of K_a and the prevailing pH. Further, although the formal oxidation state can be stated for different vanadium species, as a result of competition for binding between dissolved O_2 and water, the capacity for electron transfer to either cannot be assigned with any confidence.

At the concentrations, and under the conditions found in blood, V(V) may exist predominantly as a mixture of HVO_4^{2-} and $H_2VO_4^-$ (Harris et al,1984; Chasteen et al,1986). The steady state favours V(V) (see section 3.2.0), but to the extent that V(IV) is present, it may be in the form $VOOH^+$ and an unidentified species perhaps of the form $VO(OH)_{2(S)}^+$ may predominate (Chasteen,1983). Intracellular vanadium may exist primarily as V(IV)-phosphate complexes (see section 2.4.0). In the absence of other ligands, the species shown in Fig. 1.0 should be considered in assessing the candidates for participation in vanadium-mediated free radical reactions. The impact of the mixture of ligands (both small molecules and proteins) found intracellularly is also unknown. Pending more precise information, it is perhaps prudent for researchers merely to show vanadium merely as V(III), V(IV) and V(V). Representations of vanadium as VO^{2+}, VO_2^+, VO_4^{3-} or $H_2VO_4^-$

(for example) may misrepresent the species in question and lead to erroneous conclusions concerning their state of charge or state of complexation. This in turn may lead to spurious conclusions regarding availability of the metal to cationic or anionic ligands, to charged sites located on the surface of proteins, or its ability to form esters for example.

2.2.0 Vanadium in tissues is almost exclusively monomeric

In aqueous solution, oligomeric and polymeric vanadium species readily form in the pH range observed physiologically. Nevertheless, vanadium in tissues is overwhelmingly monomeric (Rubinson,1981; Chasteen,1983). In part, this is because vanadium concentrations in biological samples are usually extremely low, so low in fact that accurate measurement is prohibitively difficult. Tissues contain from < 1 to 140 nanograms of vanadium per gram wet tissue range (Nechay et al,1986). Mean concentrations in human serum are between 0.035 to 0.67 ng/ml (Cornelis et al,1981; Versieck and Cornelis,1980; Simonoff et al,1984). The total body burden in humans is approximately 100 μg (Nechay et al,1986). These concentrations are far below the limit for detectable oligomer formation *in vitro* (10^{-6} to 10^{-4} M) (Pope and Dale,1968; Rubinson,1981; Chasteen,1983).

Many studies of the biological effects of vanadium have involved use of vanadium stock solutions containing predominantly polymeric species. The results of such studies must be interpreted with some caution. Despite numerous studies (Sillen and Martell,1964; Souchay,1963; Connor and Ebsworth,1964; Phill et al,1971; Chasteen,1981), the equilibrium constants for oligomerization and protonation of different vanadium species remain uncertain. The matter is further complicated by the fact that oligomer formation is highly dependent upon vanadium concentration (Pope and Dale,1968). Interconversions between polymeric and monomeric forms may occur, but only slowly (Long et al,1979; Druskovitch and Kepert,1975; Clare et al,1973a; Lemerle et al, 1980;

Rubinson,1981; Darr and Fridovich,1985). Further, the redox properties of polymeric species differ from those of monomeric forms.

2.3.0 Virtually all intracellular vanadium is bound to physiological ligands

Cellular reactions of vanadium are those of vanadium complexes rather than the free metal (Rubinson,1981; Nechay et al,1986). Less than 1% of either extra- or intracellular vanadium (<1%) is free (Post et al,1979; Chasteen,1983; Nechay et al,1986). Under these conditions, polymer formation is even further inhibited (Itoh,1975; Zare,1979; Rubinson,1981).

Vanadium (IV) (as VO^{2+}) forms stable complexes with many small molecules in including citrate, phosphates, proteins and free amino acids (Chasteen,1983; Nechay et al,1986). Of these, phosphate residues trap approximately 90% of the total intracellular VO^{2+}, much of this in the form of a 2:1 V(IV)-ATP complex (Post et al,1979; Sakurai et al,1982; Nechay et al,1986). Weaker 1:1 complexes are also formed between VO^{2+} and orthophosphate or pyrophosphate (Kendig and Rieger,1976; Copenhafer et al,1976; Parker et al,1970; Woltermann et al,1974; Imamura et al,1979; Sakurai et al,1982). In general, association constants for VO^{2+} with phosphates are higher than those with most other common biological ligands (pK values reported by Nechay et al (1986) - ATP, 5.9; ADP, 5.5; AMP, 5.1; phosphate, 4.3; creatine phosphate, 3.6; glutathione 2.7; ascorbate, 3.3). Complexation by phosphates may promote intracellular accumumulation of VO^{2+} (Nechay et al,1986). Hydrolysis of ATP and PP_i occurs in the presence of V(V) (Imamura et al,1979). The effects of ligands on the redox potentials of different vanadium species deserves detailed elaboration.

Similarly, additional information is required concerning the binding of vanadium species to proteins such as albumin and transferrin as well as enzymes. The redox kinetics and complexation of V(IV) and V(V) with albumin and transferrin in both buffer and serum were studied by Chasteen et al (1986).

2.4.0 Vanadium metabolism involves interconversions between the V(III), V(IV) and V(V) oxidation states

Discussions of vanadium metabolism and toxicity in mammals are usually restricted to V(IV) and V(V) since in mammals V(IV) predominates intracellularly whereas the vast majority of extracellular vanadium exists as V(V). Measurable concentrations of V(III) are occur only in certain tunicate species (Rezaeva,1964; Swinehart et al,1974; Carlson,1975; Tullius et al,1980; Dingley et al,1982; Dingley et al,1981). However, vanadium metabolism involves interconversion between all three oxidation states. The redox state of intracellular vanadium (like that of other components) is in fact a steady state, representing the net effects of a large number of dynamic phenomena. For example, small but significant steady state amounts of V(III) may exist in the presence of biological reducing agents (Rubinson,1981). ESR spectra of reaction mixtures containing decavanadate and excess NADH exhibit signals characteristic of V(VII), V(III) and V(IV), the intensity of signals increasing with time (Vijaya et al,1984). This accumulation of reduced forms of vanadium occurred only when NADH was present in excess, and when all available dissolved O_2 had been exhausted. Further, even traces of V(III) in the steady state may be important, since their removal in any reaction will tend to displace the equilibrium, resulting in a constant replenishment of the scarce species. Steady state levels of V(IV) are higher than predicted by thermodynamic citeria, since V(III) is oxidized significantly more slowly by molecular O_2 than V(IV) (see section 3.2.0). Moreover, V(III) may be stabilized by ligands. Vanadium (III)-protein complexes have been demonstrated *in vitro* (Chasteen et al,1986). The extent to which V(III) formation may modulate O_2 reduction, redox cycling or yields of toxic radical species is not known. On the other hand, a low steady-state level of V(V) can serve as the source of a much larger amount, through continual displacement of the steady state and replenishment (by redox reactions) as it is consumed.

2.5.0 Uptake and distribution vanadium species is dependent on their redox state

Thus, interconversion of vanadium species in different oxidation states may be an important, yet underestimated factor in experimental studies even where large doses of V(IV) or V(V) are

administered. In particular, data are needed regarding the relative ease of absorption of V(IV) and V(V) from the gastrointestinal tract, and the role of stomach acidity and duodenal alkalinity in changing the fraction absorbed by modulating the redox state (Wiegmann et al,1982). The pH of injected solutions containing vanadium compounds modulates their effect, less toxicity being observed at lower pH, and greater toxicity being observed if sodium bicarbonate is administered prior to the vanadium (Mitchell,1953). Further, the toxic effects of orally administered vanadium are mainly observed in the gastrointestinal tract (Musialowicz,1976) since only a small fraction of the administered dose is absorbed (Curran et al,1959; Comar and Chevallier, 1967; Byrne and Kosta,1978).

Studies of the tissue distribution of vanadium in experimental animals and humans indicate that vanadium (administered as either V(V) or V(IV)) accumulates in liver, kidney, bone, lungs, heart and thyroid (Scott et al,1951; Wilson et al,1953; Tipton and Cook,1963; Schroeder & Balassa,1967b; Hamilton et al,1973; Sabbioni and Marafante,1978; Wiegmann et al,1982; Hansen and Aaseth,1981; Hansen et al,1982; Hansen et al,1986). Vanadium (V) is taken up more rapidly than V(IV) by human erythrocytes and perhaps other cells (Hansen et al,1982). The extent to which the relative toxicity of V(V) and V(IV) depends upon the degree to which they are taken up by cells, and subject to metabolic processing also need clarification, since (as noted more than a decade ago by Sabbioni and Marafante (1978)), insufficient data concerning the metabolism of trace amounts of vanadium at the molecular level exists to allow assessment of the specific biological effects of different vanadium species.

Human erythrocytes incubated with $^{48}VOSO_4$ or $Na^{48}VO_3$ accumulate nearly twice as much V(V) as V(IV) (Hansen et al,1982). Both incubation of V(V) and glutathione (GSH) prior to addition to erythrocyte suspensions, and pretreatment of erythrocytes with diethylmaleate (thus blocking the ability of intracellular GSH to reduce V(V)) reduced uptake of V(V), these effects being interpreted as possible evidence for GSH-dependent uptake mechanisms in

erythrocytes. Further, since V(V) is taken up by erythrocytes more rapidly than V(IV), yet V(IV) induces hemolysis much more rapidly, the mere presence of vanadium intracellularly is not sufficient to induce hemolysis. The active species is likely the V(IV) cation, produced as a result of intracellular reduction by GSH or other reducing agents (Hansen et al,1986). Studies of the intracellular distribution of ^{48}V indicate that vanadium accumulates primarily in the nuclear fractions of cells (Hopkins and Tilton,1966; Sabbioni and Marafante,1978), and to a lesser extent in cytosolic and mitochondrial fractions. These distributions vary over time, with the observed patterns of distribution depending on tissue type (Sabbioni and Marafante, 1978). These kinetic phenomena may underly the biological effects of vanadium in different cells.

2.6.0 Intracellular metabolism of vanadium determines its biological effects

For example, whereas addition of 2.5 or 5.0 mM V(V) causes cessation of*Saccharomyces cerevisiae* growth (but not cell death since washing of the cells allows growth to resume), addition of 2.5 mM V(IV) has no effect on cell growth and 5.0 mM V(IV) stimulates growth (Willsky et al,1984). The different effects of V(V) and V(IV) were not due to differences in the ability of the cell to accumulate each metal ion. The results of experiments using ^{48}V indicated that V (IV) actually entered the cells more quickly. Intracellular reduction of V(V) to V(IV) was demonstrated by means of ESR techniques, the shapes of the V(IV) ESR spectra indicating binding to low molecular weight compounds. The possibility that alterations in the metabolic processing of vanadium underly the resistance of vanadate-resistant *S. Cerevisiae* mutant was suggested, along with the hypothesis that vanadium metabolism may be an important element in metabolic regulation in general. This view is further supported by more recent evidence concerning of the mechanisms of vanadate resistance of certain *Saccharomyces cerevisiae* mutants Willsky et al,1985). In contrast to certain *Neurospora crassa* mutants (Bowman et al,1979; Bowman,1983), vanadate resistance could not be attributed to the inability to transport V(V) or V(IV) into the cell,

but rather to differences in vanadium metabolism which prevent intracellular accumulation of toxic vanadium species (Willsky et al,1985).

2.7.0 pH and ligands modulate the redox potentials of vanadium

Details of the thermodynamics of redox reactions of vanadium with biological redox agents which would explain biologically relevant redox reactions are needed. Thus, elucidation of those factors such as pH and ligands which modulate the redox potentials of vanadium in the complicated environment of the cell (and the extent to which vanadium may modify intracellular redox processes) is essential. Such a detailed understanding of the thermodynamics of redox reactions of free and bound vanadium with O_2, reduced O_2 species and or biological reducing agents needed to understand its participation in endogenous redox processes has proved elusive. The complications involved in calculating redox potentials for free and bound vanadium species under biological conditions have been emphasized by Rubinson (1981), while the electrochemistry of vanadium has been reviewed both extensively (Israel and Meites,1976), and more selectively (Hill et al 1971). The following standard reduction potentials are reported for the V(V)/V(IV) and V(IV)/V(III) redox couples (Latimer 1952; Hill et al 1971; Rubinson 1981).

$$VO_2^+ (aq) + 2H^+(aq) + e^- \longrightarrow VO^{2+} (aq) + H_2O (liq) \quad E^0 = 1.000\ V$$

$$VO^{2+} (aq) + 2H^+(aq) + e^- \longrightarrow V^{3+}(aq) + H_2O(liq) \quad E^0 = 0.361\ V$$

Thus, the standard potential for the two electron reduction of VO_2^+ to V^{3+} (the latter perhaps existing as $H_2VO_3^-$ or VO^+) is 1.36 V (Weast et al 1983). The $E^{0'}$ for VO_2^+/VO^{2+} at pH 7.4 can be calculated to be 0.127 V (Rubinson 1981). However, since biological ligands bind more strongly to V(IV) than V(V), the reduction potential at pH 7.4 will be significantly higher in biological systems (Rubinson 1981). Although an explanation of the electrochemical potentials in the presence

of ligands has also been presented (Wilson et al,1974), potentials for different vanadium species bound to physiological ligands are not readily accessible in the literature. In the absence of this information, analysis of relevant reaction mechanisms must rely on a variety of other types of relevant information. The most fundamental of these concerns the ability of vanadium to reduce O_2, and to form reactive radical species in reactions with O_2^- or H_2O_2.

B. REACTIONS OF ALL FORMS OF VANADIUM WITH INTRACELLULAR OXIDANTS RESULT IN FORMATION OF FREE RADICALS

3.0.0 REACTIONS OF VANADIUM WITH O_2

3.1.0 O_2 consumption by Fe(II), V(III) or V(IV): effects of buffer type

Both V(III) and V(IV) readily reduce ambient O_2 , forming V(V) (Martin and Bentley, 1962). Since the air autoxidation of Fe(II) has recently been shown to proceed via different reaction pathways in phosphate as compared to HEPES buffer (Tadolini,1987), and in order to better characterize reactions of V(IV) with O_2, we conducted a series of experiments in which O_2 consumption by 250 μM $FeSO_4$, $VOCl_3$, or $VOSO_4$, in phosphate and HEPES buffers (50 mM, pH 7.4, 25° C) was determined polarographically (Fig. 2.0). Rates of O_2 consumption were highest in phosphate buffer as compared to HEPES buffer for Fe(II) (19.3 vs 4.3 μM s^{-1}), V(III) (13 vs 0.7 μM s^{-1}) and V(IV) (5.6 vs 2.0 μM s^{-1}). Thus, the order of effectiveness in phosphate buffer was Fe(II) > V(III) > V(IV), and in HEPES buffer Fe(II) > V(IV) > V(III). These results are in agreement with those of Tadolini (1987) demonstrating that the air autoxidation of Fe(II) also proceeds more slowly in HEPES buffer than in phosphate buffer, and further suggest that as in the case of Fe(II), the reduction of O_2 by V(III) or V(IV) proceeds by different pathways in phosphate and HEPES buffers (Tadolini,1987). In both cases, reactions of the reduced metal ions with H_2O_2 are important. Notably, none of the three metal ions tested consumed any where near the amount of O_2 expected stoichiometrically. Since no H_2O_2 was apparent on addition of catalase (20 U/ml) to reaction mixtures containing Fe(II), V(III) or V(IV) when reactions were near completion, H_2O_2 is

consumed during (and accelerates) the air autoxidation of both reduced iron (Tadolini,1987) and vanadium (Fig. 2.0).

The total O_2 consumption by V(III) was more than twice that of V(IV). This total O_2 consumption for each metal ion thus reflects competing reactions in which the reduced metal ions are oxidized by O_2 or H_2O_2. With the possible exception of reactions involving V(III), where some redox cycling may occur since the ability of O_2^- to re-reduce the oxidized metal ions is apparently limited since superoxide dismutase inhibited reactions involving Fe(II), V(III) or V(IV) to only a limited extent. Similarly, catalase also caused only minimal inhibition (data not shown). Interestingly, the rates and extent of O_2 consumption by Fe(II), V(III) or V(IV) were also increased on addition of the OH· scavengers mannitol or EtOH (data not shown). These findings demand reconsideration of the often made assumption that hydroxyl radical scavengers are inert in reactions of this type. Mannitol for example is reportedly more effective than a variety of other hydroxyl radical scavengers in inhibiting the air autoxidation of Fe(II) (Tadolini,1987). On the basis of these results, we conclude that while V(IV) reacts more slowly than Fe(II) with O_2, reactions with H_2O_2 may be more important. Further, where even small amounts of V(III) are formed, redox cycling between the V(IV) and V(V) oxidation states may also occur, promoting radical formation. Since phosphate enhanced the ability of vanadium to reduce O_2, it was of interest to examine the effects of a variety of ligands - both physiological ligands and metal chelating agents on O_2 reduction by V(III) or V(IV).

3.2.0 V(IV) and V(III)-mediated O_2 consumption: effects of pH and ligands

Binding of vanadium to phosphate ligands and or certain proteins alters the rate of its oxidation. For example, the tetravalent oxidation state is stabilized by complexation to most biological ligands (Chasteen,1983; Boyd and Kustin,1984). Similarly, VO^{2+} bound to transferrin is protected from oxidation to V(V) (Harris et al,1984). In order to better characterize reactions of free and

bound V(III) or V(IV) with O_2, we studied O_2 consumption by V(III) or V(IV) alone, and in the presence of ATP, histidine, EDTA, DES or BSA in HEPES or MES buffers over the pH range 6.0, 7.0 and 7.4 (Wu, 1987). The results are presented in Figures 3.0 and 4.0.Vanadium (IV) reduced O_2 1.6 to 4 times as rapidly as did V(III). The highest rates of O_2 consumption (and smallest differences between the rates for reactions involving V(III) or V(IV)) were observed at pH 7.4. The rates of O_2 consumption at pH 6.0, 7.0 and 7.4 for V(III) were 2.0×10^{-2} μM s^{-1}, 6.0×10^{-2} μM s^{-1} and 1.0×10^{-1} μM s^{-1}, and for V(IV), 7.9×10^{-2} μM s^{-1},1.3×10^{-1} μM s^{-1} and 1.6×10^{-1} μM s^{-1}. ATP inhibited the reduction of O_2 by V(III) by 52%, 71% and 81% at pH 6.0, 7.0 and 7.4, whereas EDTA inhibited by 31%, 26% and 15% over the same range of pH and other combinations of ligands had no effects which reached statistical significance (at $p< 0.05$). ATP also inhibited O_2 consumption by V(IV) (by 63%, 76% and 80% at pH 6.0, 7.0 and 7.4 respectively). Histidine accelerated O_2 consumption by V(IV) at pH 7.0 and 7.4 (by 130% and 50% respectively), while EDTA produced inhibition of from 80-90% at all three pH. Again, serum albumin had no effect.

3.3.0 Intracellular steady state levels of V(III), V(IV) and V(V) must be explained on the basis of both thermodynamic and kinetic considerations

Clearly, both V(III) and V(IV) are readily oxidized by O_2 at physiological pH, thus accounting for the preponderance of the oxidized V(V) state in plasma. At lower pH, lower pO_2, or in the presence of ATP, these autoxidations are slower, thus accounting for the preponderance of reduced forms intracellularly. The preponderance of V(IV) intracellularly, has hitherto been explained primarily on the basis of thermodynamic considerations (Nechay et al,1986). The argument is that the intracellular presence of phosphate ligands selectively stabilizes the V(IV) which is thus the most stable form intracellularly. The current data together with the recent results of Chasteen et al (1986) require that kinetic considerations be given at least equal weight. On this basis, the intracellular levels of V(IV) reflect not an equilibrium but a steady state. The binding of vanadium by intracellular ligands (Nechay et al,1986), as well as the lower intracellular pH and O_2 concentrations results in a steady state which overwhelmingly favors the V(IV) state. At the same

time, intracellular reducing agents contribute to steady state reduction of vanadium to V(IV) and perhaps V(III) (Rubinson,1981; Chasteen,1983; Chasteen et al,1986), while O_2 and perhaps H_2O_2 contribute to the reoxidation. Since V(III) reacts with O_2 substantially more slowly than does V(IV), bioaccumulation of V(III) may be favored kinetically under conditions where substantial reduction and oxidation of vanadium are occurring simultaneously (thermodynamic considerations notwithstanding). It is interesting to note that the intracellular pH of blood cells and vanadocytes from the tunicate *Ascidia nigra* are not acidic, having pH of approximately 7.4 and 7.2 respectively (Dingley et al,1982).

4.0.0 REACTIONS OF VANADIUM WITH O_2^-

4.1.0 The biological relevance of reactions of vanadium with O_2^- is not established

Although reactions of O_2^- with the oxidized forms of other transition metals such as iron and copper result in facile reduction of the metal ion, reactions of V(V) with O_2^- result instead in the formation of complexed radicals. ESR studies of O_2 adsorbed to vanadium pentoxide which had been partially reduced to V(IV) suggested covalent bonding of O_2 to V(IV), but that spin density on the vanadium was lower than that for tetravalent vanadium compounds. This was interpreted as evidence of adsorbed O_2^- (Shvets et al 1968). However, in a stopped-flow study of the reaction between $HO_2\cdot$ (generated from Ce(IV)/H_2O_2 in 1 M $HClO_4$,) and V(V), no complexed radical was formed between $HO_2\cdot$ and V(V), unless H_2O_2 was first complexed to the V(V) (see section 6.4.0). Complexes between V(V) and O_2^- (Darr and Fridovich 1984,1985) or V(IV) and O_2^- (Khandke et al, 1986) reportedly participate in the vanadate catalyzed oxidation of NADH. However, little direct evidence exists supporting participation of such complexes in biologically relevant reactions. For example, the recent findings of Yoshino et al (1988) that vanadium does not stimulate NAD(P)H oxidation in intact erythrocytes weaken the hypothesis that these phenomena have relevance intracellularly. Similarly, Birnboim (1988) recently reported that sodium orthovanadate enhances DNA strand breakage by O_2^- produced as a result of stimulation of

granulocytes by the tumor promoter PMA (phorbol-13-myristate-12-acetate). The maximum increase observed was 1.5-1.7-fold for V(V) concentrations of up to 1.0 mM, with a plateau in the dose-response curve at 50-100 μM. Further, although the reaction of V(V) with O_2^- is inhibited by superoxide dismutase (Liochev and Fridovich,1985),V(V)-mediated enhancement of DNA strand breakage was not inhibited by 20-100 μg /ml superoxide dismutase, even under conditions where O_2^- production by granulocytes was limited by means of the metabolic inhibitor A23187. In addition, V(V) reversed the inhibition of H_2O_2-induced strand breaks by A293187 in the absence of O_2^-. On the basis of these results it was concluded that V(V) increases DNA strand breakage by activating a specific metabolic DNA strand-break pathway, rather than by reactions with O_2^-.

5.0.0 REACTIONS OF V(IV) WITH H_2O_2

5.1.0 Reactions of V(IV) with H_2O_2 yield OH· or crypto-OH· radicals

Hydrogen peroxide rapidly oxidizes V(IV) (Brooks and Sicilio,1971). Addition of equimolar H_2O_2 and VO^{2+} results in rapid development of an amber color, and then a permanently the bright yellow colour associated (speculatively, in the absence of a more detailed characterization) with the decavanadate ion (Cotton and Wilkinson,1972). Determination of the reactive products formed in this overall reaction deserves a high priority in future research, particularly since intracellular vanadium exists as V(IV). Recently reactions of V(IV) with H_2O_2 have been described in terms of a simple Fenton mechanism (Keller,1987; Liochev and Fridovich,1987), however the reaction pathways are considerably more complicated, involving a variety of complexed radicals. Further, it is not clear that Fenton mechanisms are relevant to either the in vitro or in vivo effects of V(IV) since the relative ability of V(IV) to generate OH· from H_2O_2 is slight as compared to other reduced transition metals such as Fe(II) and Cu(I). Moreover, V(IV) does not appear to redox cycle in the presence of H_2O_2 (section 5.2.0). The possible involvement of O_2 (as in the case of the iron-catalyzed Fenton decomposition of H_2O_2 (Cohen and Ofodile,1983) remains to be determined.

Setaka et al (1969) used ESR to study the reaction between V(IV) and H_2O_2 under acid conditions. At reaction times less than 30 msec ESR spectra characteristic of VO^{2+} were observed, while at longer times a new signal developed which, on the basis of the ESR spectra, was ascribed to a VO_3^+-$HO_2\cdot$ radical complex rather than to an OH· complex. The following overall reaction sequence was suggested -

$$VO^{2+} + H_2O_2 \rightarrow VO^{3+} + OH\cdot + OH^- \quad (1)$$

$$OH\cdot + H_2O_2 \rightarrow H_2O + HO_2\cdot \quad (2)$$

$$VO^{3+} + HO_2\cdot \rightarrow VO^{3+}\text{-}HO_2\cdot \quad (3)$$

The VO^{3+}-HO_2 was described as fairly stable, and likely not reactive toward organic compounds.

In contrast, Brooks and Sicilio (1971) also detected formation of an intermediate radical complex ('I' below) upon reaction of V(IV) with H_2O_2, but ascribed it to a product of the reaction between $VO(OO)^+$ ('VO_3^+' below) and OH·.

$$VO^{2+} + H_2O_2 \leftrightarrow OVOOH^+ + H^+ \quad (1)$$

$$OVOOH^+ \rightarrow VO_2^+ + OH\cdot \quad (2)$$

$$VO_2^+ + H_2O_2 \rightarrow VO_3^+ + H^2O \quad (3)$$

$$VO^{2+} + OH\cdot \rightarrow VO_2^+ + H^+ \quad (4)$$

$$VO_3^+ + OH\cdot \rightarrow I + \text{other products} \quad (5)$$

In the presence of excess H_2O_2, the decay of VO^{2+} ion initially follows pseudo-second order kinetics with respect to VO^{2+} (Brooks and Sicilio,1971). The peroxyvanadium complex VO_3^+ is the predominant product below pH 2.0 (Dean 1962, Brooks and Sicilio 1971), while at pH 4.0 the diperoxyvanadium (V) complex VO_5^- is the predominant product (Brooks and Sicilio 1971). The rates of (3), (4), and (5) were fast relative to (1) and (2). Consistent with this reaction scheme, and

with competition for OH· between reactions (4) and (5), addition of methanol as an OH· scavenger changed the kinetics from pseudo-second order with respect to [VO^{2+}] to pseudo-first order kinetics (at least over very short periods of time). Methanol also decreased the rate of decay of V(IV) by 50%, and prevented formation of intermediate I. Addition of EDTA slowed the rate of VO^{2+} decay 100 fold or more, supporting an inner sphere mechanism for reactions (1) and (2). The intermediate (I) observed by ESR was further characterized (Brooks and Sicilio 1971). If no V(V) was added to the reaction mixture initially, the maximum rate of formation of I was ([I] max) observed when [VO_3^+] = [VO^{2+}], that is when the reaction was approximately half over. Addition of VO_2^+ initially, decreased the time required for achievement of [I] max and also accelerated the rate. The maximum rate of formation of I increased with [VO_2^+], levelling off at very large values of [VO_2^+]. These data suggested that the intermediate was a V(V) complex with a paramagnetic oxygen ligand - possibly $OVOO^{2+}$.

Radical products of reactions between V(IV) and H_2O_2 or O_2^- were also recently studied by ESR using DMPO spin trapping (Keller et al,1987). Signals characteristic of OH·, which increased with increasing V(IV), were detected in solutions of V(IV) plus H_2O_2. Hydroxyl radical adducts were also detected in solutions of V(V) or V(IV) and O_2^- (generated enzymically by xanthine oxidase plus xanthine), but it was noted that dismutation of O_2^- to H_2O_2 and subsequent reduction of H_2O_2 by V(IV) would probably account for the OH· production. On this basis, a Fenton mechanism was suggested, similar to that proposed for other transition metals such as iron and copper (and consistent with reaction schemes for reduction of H_2O_2 by V(IV) presented by Setaka et al (1969) and Brooks and Sicilio (1971):

$$V(IV) + H_2O_2 \rightarrow V(V) + OH^- + OH\cdot$$

Less direct evidence for a V(IV)-catalyzed Fenton reaction has also been presented by Liochev and Fridovich (1987) who reported rapid NADH oxidation in the presence of V(IV) and H_2O_2, ethanol

(1%) being found to inhibit NADH oxidation by V(IV) and H_2O_2 in the presence of superoxide dismutase. Alternately, the possibility that OH· generation from vanadium may involve attack of superoxide on vanadium peroxide complexes is suggested by the earlier work of Samuni and Czapski (1970) as well as the more recent studies of Kawanishi et al (1986) concerning reactions of chromate with H_2O_2 (see section 6.4.0). In additions, spin trapping of reactions involving bleomycin-V(IV) complexes and H_2O_2 gave ESR signals characteristic of OH· (Kuwahara et al,1985). Further, the bleomycin-V(IV) complex is effectively capable of cleaving DNA, demonstrating a somewhat different site specificity than bleomycin-Fe(III) complexes, and being about 50-fold less active than bleomycin-Fe(III) complexes under the same experimental conditions.

5.2.0 Reactions of V(IV) with H_2O_2 or O_2 are buffer-dependent

Given the complicated nature of reactions of vanadium with H_2O_2, ligands might be expected to modulate these reactions to a significant degree. We have recently studied the effects of buffer type on reactions of V(IV) with H_2O_2 by means of polarographic (O_2 consumption) techniques (Fig. 5.0), and by means of the deoxyribose-TBA (thiobarbituric acid assay) for OH· radicals (Fig. 6.0).

In O_2 consumption experiments, induction periods of approximately 30 seconds and 6 seconds preceded the onset of measurable O_2 consumption in HEPES buffer and phosphate buffer respectively. The maximum rate of O_2 consumption in phosphate buffer was almost 4-fold greater than that in HEPES buffer (1.34×10^{-7} M^{-1} s^{-1} vs 7.2×10^{-7} M^{-1} s^{-1}). Total O_2 consumption did not differ in the two buffers. Catalase (25 U/ml) or superoxide dismutase (5 U/ml) inhibited by 36.5% and 28% respectively. Similar amounts of albumin were ineffective. Addition of 250 μM H_2O_2 to reaction mixtures containing an equivalent concentration of V(IV) decreased the lag periods associated with the onset of O_2 consumption by V(IV) alone (to approximately 1.5 and 3 seconds in HEPES and phosphate buffers respectively, while the maximum rates of O_2 consumption were increased 2.4-fold and 1.4-fold over the rates for V(IV) alone. The apparent total O_2 consumption

was decreased by approximately 9.1% in HEPES buffer and 7.9% in phosphate buffer. Superoxide dismutase (5 U/ml) did not inhibit these phenomena. Thus in both buffers, added H_2O_2 increases the rate of oxidation of V(IV) to V(V), but in HEPES buffer, the rate of O_2 consumption is accelerated, whereas in phosphate buffer the rate of O_2 evolution, that is H_2O_2 breakdown to O_2, is accelerated. Thus, for reactions involving 250 μM V(IV) and 2.5 mM H_2O_2, the maximum rates of change in O_2 concentration were further increased as compared to the rates observed for 250 μM V(IV) and equimolar H_2O_2 (4.71-fold in HEPES buffer, 4.97-fold in phosphate buffer), but whereas O_2 was consumed in HEPES buffer, it was evolved in phosphate buffer. The absolute change in O_2 concentration was further diminished in HEPES buffer (-4.6%) and relatively unchanged in phosphate buffer (14%). For reactions in HEPES buffer only, an increase in O_2 concentration was observed approximately 10 minutes after initiation of the reaction.

5.3.0 The TBA and DMSO assays for OH· do not reveal measurable OH· production by V(IV) and H_2O_2

In order to further investigate the nature of ligand effects on reactions of V(IV) with H_2O_2, we employed the deoxyribose-TBA assay (Halliwell and Gutteridge (1981) (Fig. 6.0). Metal ions ($CuSO_4$, $FeSO_4$, $FeCl_3$, $NaVO_3$ or $VOSO_4$) alone (50 or 100 μM final concentration) or with added H_2O_2 (0, 0.25, .5, 1.0, 2.5 or 5.0 mM) were incubated with reaction mixtures containing 0, 0.88 or 1.76 mM deoxyribose. All reactions were conducted in 50 mM HEPES/KOH buffer, pH 7.4 at 25° C in triplicate. Following a 15 minute incubation in a circulating water bath at 37° C, trichloroacetic acid and thiobarbituric acid reagents were added, and the tubes then placed in a second water bath 1 at 100° C for a further 15 minutes. Subsequently, tubes were removed and cooled in ice water, and the absorbance at 532 nm was recorded as soon as possible. There was significant background activity in the absence of added metals, this being most noticeable at higher deoxyribose and H_2O_2 concentrations. Patterns of OH· production for Fe(II), Fe(III) and Cu(II) differed. Since Fe(III) and Cu(II) were added in their oxidized forms, the OH· generated by these metal ions must depend on

redox cycling . In the absence of other reducing agents, O_2^- presumably serves as a reductant of the metal ions. Iron(II) was more effective than Fe(III) or Cu(II) at low metal concentrations (presumably acting via a simple Fenton mechanism), however OH· production levelled off rapidly with increasing H_2O_2 concentrations since Fe(II) does not redox cycle in the presence of excess H_2O_2 (Symonyan and Nalbandyan,1979). In contrast, for reactions obtained with V(IV) and V(V), slight increases over control values were observed at deoxyribose concentrations of 1.76 mM and H_2O_2 concentrations of up to 1.0 mM (particularly for V(IV). However, under all other conditions, the values obtained for both V(IV) and V(V) were equal to or below control values.

The current results imply that OH· production from V(IV) is quantitatively unimportant, wherease the catalase-like actions of V(V) are. The simplest explanation of the difference is that the occurrence of Fenton-type reactions is contingent upon reaction conditions and reaction concentrations which prolong the lifetime of H_2O_2, beyond that seen in the current studies. As noted by Tadolini (1987) although the ability of HEPES buffer to scavenge OH· is well documented (Bielski and Shine,1979), the rate constant of the reaction of OH· with HEPES is not much higher than that of other compounds that are frequently used in such studies. For example the rate constant of the reaction of OH· with EDTA is 2.76×10^9 M^{-1} s^{-1} (Walling,1975) and that of ADP, 2.5×10^9 M^{-1} s^{-1} (Anbar and Neta,1967). Further, OH· generation from iron and copper was clearly evident under the reaction conditions employed. Although vanadate reportedly interferes with the thiobarbituric acid reaction, and so prevented the demonstration of MDA resulting from peroxidative damage to human erythrocytes in the presence of vanadate, iodoacetate and ferricyanide (Heller et al,1987), other workers have reported no intereference with the assay by vanadium (see section 11.0.0). Further, given the low levels of intracellular vanadium, the possible biological significance of OH· production from reactions of V(IV) with H_2O_2 is not immediately apparent.

5.4.0 Ligands protect V(IV) against oxidation by H_2O_2

It is possible that cellular ligands may promote OH· formation by V(IV) in the presence of H_2O_2. With the exception of ATP, little is known of the possible modulating effects of physiological ligands on the reaction of V(IV) with H_2O_2. ATP protects VO^{2+} against oxidation by H_2O_2 (Woltermann et al,1974). A large excess of H_2O_2 added to a VO^{2+}-ATP solution causes it to turn amber (as for mixtures of VO^{2+} and H_2O_2 alone), then pale yellow-green over a period of minutes. ESR spectra indicate that some V(IV) is still present at this point. When [V(IV)]=[H_2O_2], and ATP was present, only ~50% of the V(IV) was oxidized, as shown by the intensity of ESR spectra (Woltermann et al,1974). Brooks and Sicilio (1971) observed similar stoichiometry, and suggested that some of the H_2O_2 undergoes vanadium catalyzed decomposition to O_2 and H_2O. Intracellular vanadium, existing mainly as V(IV)-ATP complexes should thus be protected from oxidation by both O_2 and H_2O_2. Di- and triphosphate nucleotides of adenosine, cytidine and guanosine have been reported to slow the oxidation of Fe(II), maintaining it in a form in which in can generate OH· from H_2O_2 in Fenton reactions (Floyd,1983; Floyd and Lewis,1983). Interestingly, the amount of free radical trapped increases as a function of ADP concentration, perhaps as a result of cooperativity of the nucleotides in forming catalytically-active complexes (Floyd,1983). The possible implications of these findings as regards biologically significant reactions of vanadium require further study.

6.0.0 REACTIONS OF V(V) WITH H_2O_2

6.1.0 Vanadium(V) peroxo complex formation as an amplification mechanism

Reactions of V(V) with H_2O_2 may yield significant amounts of toxic radical species. Reactions of V(V) with H_2O_2 are of particular interest since - (1) vanadium-peroxo complexes are relatively stable (Dean 1961), perhaps stable enough to persist within the cellular environment long enough to interact with vulnerable tissue components, (2) at the same time, they are reactive enough to yield a variety of oxidant species upon decomposition, these species being capable of modifying tissue components, possibly in a site specific manner (Brooks and Sicilio,1971; Mimoun et al,1983;

Djordjevic and Wampler,1985) and (3) the presence of H_2O_2 may promote redox cycling by further increasing the oxidant properties of V(V) through enhancement of its ability to accept electrons from marginal donors. The key questions to be answered center on the mechanisms by which V(V) reacts with H_2O_2, and the nature of the radical species formed on decomposition of V(V) peroxo complexes.

6.2.0 Monomeric monoperoxo or diperoxo complexes predominate under physiological conditions

Relatively few articles have dealt with the chemistry of V(V) peroxo complexes (Dean,1961; Connor and Ebsworth,1965; Howarth and Hunt,1979). Surprisingly, although reactions between V(V) and H_2O_2 have been studied since at least the early part of this century (Cain and Hostetter,1912; Auger,1921; Meyer and Pawletta,1927; Wright and Mellon,1937; Dean,1961), absorption maxima and extinction coefficients of these complexes have not yet been reported. Spectrophotometric studies have indicated that in acid solution two separate V(V)-peroxo complexes absorbing in the short wavelength visible and near UV regions exist, a red, cationic, monoperoxo complex (absorbing maximally at approximately 450 nm), and a yellow, anionic, diperoxo complex (absorbing maximally in the UV region) (Dean,1961). Addition of excess H_2O_2 to a neutral solution of metavanadate (mainly $H_2VO_4^-$), the species thought to account for the majority of V(V) biologically, produces a pale yellow solution containing a number of different V(V)-peroxo species (Dean,1961; Howarth and Hunt,1979). The decrease in pH observed upon mixing of V(V) and H_2O_2 suggests formation of a V(V)-peroxo species with the same number of protons as metavanadate, but a lower pKa (Howarth and Hunt,1979). Polymerization of V(V)-H_2O_2 complexes may occur, polymerization being favored at lower pH, higher vanadium concentrations, and lower H_2O_2 concentrations (Connor and Ebsworth 1964).

The relative proportions of different V(V) peroxo species is also determined by the pH, and

the relative concentrations of V(V) and H_2O_2, with diperoxy complexes favored at higher pH (above 7.0) and higher H_2O_2 concentration (Howarth and Hunt,1979). As with vanadium alone, at low V(V) concentrations the principal V(V)-H_2O_2 species are likely monomeric (Orhanovic and Wilkins,1967). The monomeric V(V)-peroxo complexes may dominate even at higher vanadium concentrations since $(OO)_2^-$, unlike O_2^-, is not available for catenation (Howarth and Hunt 1979) or (OOV) is not as susceptible to catenation as O-V (Howarth and Hunt,1979).

Formation constants for the reactions -

$$VO_2^+ + H_2O_2 \underset{}{\overset{k_5}{<-->}} VOO_2^+ + H_2O \qquad (1)$$

$$VO(O_2)^+ + H_2O_2 \underset{}{\overset{k_6}{<-->}} VO(O_2)_2^- + 2H^+ \qquad (2)$$

have been reported as $k_5 = 3.5 \times 10^4$ and $k_6 = 1.3$ at I=1 and t=25° C (Orhanovic and Wilkins 1967). Thus, when [V(V)]>[H_2O_2], virtually all the added H_2O_2 is associated with V(V) as monoperoxy complexes. Where [H_2O_2]>>[V(V)], most V(V) will exist as diperoxy complexes (Orhanovic and Wilkins 1967; Howarth and Hunt,1979). At very high relative H_2O_2 concentrations, triperoxo-complexes (eg. $HVOOO_3^{2-}$) are formed , but they are apparently thermodynamically unstable since even in the presence of excess H_2O_2 some diperoxo species exist (Howarth and Hunt,1979). Depending on the concentration of H_2O_2, the major species present at neutral pH include $HVO_2OO_2^{2-}$, $HVO(OO_2)_2O^{3-}$ and $HVO(OO)_3^{2-}$ (Howarth and Hunt 1979). Above this pH HVO_3OO^{2-} and, below pH 8.0, the thermo-dynamically unstable $H_2VO_3OO^-$ are also formed. In contrast to the EDTA complex which forms with VO_2^+, EDTA does not readily complex $VO(OO)^+$ (Howarth and Hunt,1979).

6.3.0 Decomposition of V(V) peroxo complexes yields a variety of radical species

The stability of V(V) peroxo complexes depends upon both the solvent and the nature of available ligands, as well as on general reaction conditions, including temperature and pH (Dean,1961; Mimoun et al,1983). Decomposition is autocatalytic (Minoun et al,1983). Neither V(V) nor its peroxy-complexes exhibit an ESR absorption spectrum as a result of their 'Argon-like' closed shell configurations ($1s^2\ 2s^2\ 2p^6\ 3s^2\ 3p^6\ 4s^\circ\ 3d^\circ$) (Samuni and Czapski, 1970). However, species possessing radical characteristics (as indicated by their ESR spectra) may be formed from V(V) peroxo complexes via - a) spontaneous decomposition (Mimoun et al, 1983), b) O_2^- attack (Samuni and Czapski 1970) and c) modulation of stability by heteroligands (Djodjevic and Wampler,1983). Decomposition of V(V)-peroxo complexes (upon heating of an acid solution containing V(V) and excess H_2O_2) produces a mixture of V(V) and V(IV) (Dean,1961). At extremely low or high pH, almost quantitative recovery of V(V) and V(IV) respectively were obtained. However, in the absence of heating and at neutral pH, dissociation of the V(V) peroxo complex is slow and rate limiting (Dean,1961). Two possible mechanisms for decomposition of a V(V) peroxy complex ($perV^+$) were assessed - a) initial dissociation followed by subsequent reaction -

$$perV^+ \leftrightarrow VO_2^+ + H_2O_2 \qquad (1)$$

$$2VO_2^+ + H_2O_2 + 2H^+ \rightarrow 2VO^{2+} + 2H_2O + O_2 \qquad (2)$$

$$2VO^{2+} + H_2O_2 \rightarrow 2VO_2^+ + 2H^+ \qquad (3)$$

or b) direct decomposition (suggested by kinetic experiments, although no detailed mechanism was presented). Homolytic cleavage of V(V) peroxo complexes has also been suggested to result in formation of a radical V(IV)-O-O· species (Mimoun et al,1983). Importantly, although, V(V) peroxo complexes (like Mo complexes) hydroxylate alkane and aromatic hydrocarbons (Mimoun et al 1983), they apparently react by a different mechanism, decomposing by homolytic rather than heterolytic mechanisms to yield reactive V(IV)-OO· species.

6.4.0 Attack by O_2^- on V(V) peroxo complexes yields free and complexed radicals

An important (and hitherto relatively ignored) mechanism which may occur under conditions in which both H_2O_2 and O_2^- are present involves the attack of O_2^- on V(V)-peroxo complexes. As a result of the rapid spontaneous disproportionation of O_2^-, where O_2^- is formed at least some H_2O_2 will be present. Since V(V) reacts rapidly with H_2O_2, and V(V) peroxo complexes are relatively stable, these complexes must be considered as important intermediates, particularly in experimental systems where significant amounts of O_2^- are generated, for example in the presence of enzymatic O_2^- generating systems.

As in the case of mixtures of V(V) and excess H_2O_2 (Dean,1961), Vanadium (V) is reduced to VO^{2+} in solutions containing NH_4VO_3, H_2O_2, and Fe(II) or Ce(IV) (Bains et al 1969). The degree of reduction of V(V) was greater with Fe(II)/H_2O_2 than Ce(IV)/H_2O_2, while Fe(II) alone, but not Ce(IV) or H_2O_2 alone, was also effective. However, the ESR spectra obtained were different from that of oxyvanadium (IV) ions, indicating the presence of a species formed by reaction of V(V) with other radicals present in the redox systems (largely $HO_2\cdot$). The nature of this complexed radical species was studied in more detail by Samuni and Czapski (1970), who also used a Ce(IV)/H_2O_2 stopped-flow system to generate $HO_2\cdot$, and examined the subsequent reaction of $HO_2\cdot$ with peroxy complexes $VO(O_2)^+$ and $VO(O_2)_2^-$. Again, an ESR spectrum different from that of V(IV) was obtained, indicating formation of a complexed radical. Premixing of V(V) with H_2O_2 was required for generation of the complexed radical. The signal of the complexed radical was linearly dependent on [H_2O_2] if and only if [HO_2]>[H_2O_2] and [V(V)]>[H_2O_2], that is, under conditions where virtually all of the H_2O_2 would be complexed to V(V). Both monoperoxy- and diperoxy-complexes reacted with $HO_2\cdot$, the resulting complexed radical in both cases exhibiting a spectrum attributable to a single electron located on a peroxy group associated with a V(V) ion. Decay of the complexed radical, whether monoperoxy-V(V) or diperoxy-V(V), was first-order, with a rate

constant of 4.0 sec^{-1}. Changes in [V(V)] or [H_2O_2] had no effect on this decay rate. Analysis of the kinetics of formation for the complexed radical-

$$VO(O_2)^+ + HO_2\cdot \xrightarrow{k_7} \text{complexed radical} \qquad (1)$$

or

$$VO(O_2)_2^- + HO_2\cdot \xrightarrow{k_8} \text{complexed radical} \qquad (2)$$

gave a value for k_7 of 9.4×10^4 M^{-1} sec^{-1}. Vanadium(V)-peroxo complexes were thus noted to compete for $HO_2\cdot$ (spontaneous dismutation of $HO_2\cdot$ occurs at a rate of 0.9×10^{-6} M^{-1} sec^{-1} (Czapski and Samuni 1969)), and at high enough concentrations of the V(V)-peroxy complex, practically all of the $HO_2\cdot$ radicals would be quenched by V(V)-peroxo complexes (Samuni and Czapski,1970).

6.5.0 Catalase-like activity of vanadium may involve redox cycling of the metal ion

The mechanisms underlying the (autocatalytic) decomposition of V(V) peroxo complexes, and the nature of the free radical species formed are not known in detail, however useful information may be obtained in considering the decomposition of analagous Cr peroxo complexes. Singlet O_2 is generated during the decomposition of crystalline potassium tetraperoxochromate(V) in (Peters et al,1972) and potassium tetraperoxochromate (V) (Hodgson and Fridovich,1974) in aqueous solution. More importantly, O_2^- is also formed during the decomposition of $Cr(V)(O_2)_4^{3-}$ to CrO_4^{2-} (Hodgson and Fridovich,1974; Peters et al,1975). Further, in reactions of Cr(VI) with H_2O_2, it has been suggested that O_2^- may also be formed as a result of decomposition of Cr(V) peroxo complexes. This O_2^- may subsequently react with $Cr^V(O_2)_4^{3-}$ to produce OH· (Reaction 1), or a Cr(V)-peroxo complexe may decompose into a Cr(VI) complex and OH· as in the Fenton reaction (reaction 2). As in the case of V(V) peroxo complexes, it is not clear how many of the bound O_2 may participate in the reaction with O_2^- (Kawanishi et al,1986).

$$Cr^{V}(O_2)_4^{3-} + n\,O_2 + n\,H^+ \rightarrow Cr^{V}(O_2)_{4-n}(O)_n^{3-} + n\,OH\cdot + n\,O_2 \quad (n=1-4) \qquad (1)$$

$$Cr^{V}(O_2)_4^{3-} + H^+ \rightarrow Cr^{VI}(O_2)_3(O)^{2-} + OH\cdot \qquad (2)$$

More importantly, since O_2^- may be formed by decomposing metal peroxo complexes and promote their decomposition, it is possible that chain decomposition of H_2O_2 may occur, involving redox cycling of the metal ion.

Thus, the reported ability of vanadium compounds to effect H_2O_2 decomposition via catalase-like mechanisms (Dean,1961; Brooks and Sicilio,1971) may involve redox cycling of the metal and generation of free radicals, both possibly serving to amplify the effects of vanadium. Dean (1961) reported that on heating an acid solution of pervanadium containing an excess of H_2O_2, O_2 was evolved, and initially excess peroxide is decomposed with the red "pervanadium" colour remaining unchanged (any decomposed pervanadium being regenerated by the excess peroxide), then when the excess peroxide has been consumed, the red color fades rapidly as the pervanadium itself undergoes decomposition. Interestingly, both Dean (1961) and Brooks and Sicilio (1971) employed V(IV) as the initial form of vanadium, however V(V) is apparently the active species. Brooks and Sicilio (1971) suggested a simple oxidation of VO^{2+} by H_2O_2 (reactions 1, 2, 3),

$$VO^{2+} + H_2O_2 \leftrightarrow OVOOH^+ + H \qquad (1)$$

$$OVOOH^+ \rightarrow VO_2^+ + OH\cdot \qquad (2)$$

$$VO^{2+} + OH\cdot \rightarrow VO_2^+ + H^+ \qquad (3)$$

the overall reaction being -

$$2VO^{2+} + H_2O_2 \rightarrow 2VO_2^+ + H^+ \qquad (4)$$

However, H_2O_2 consumption beyond this (ie. a chain reaction) was explained by a net autoxidation-reduction reaction.

$$2H_2O_2 \rightarrow O_2 + H_2O \qquad (5)$$

Stoichiometric data for H_2O_2:VO^{2+} consumption and O_2 evolution supported participation of a chain reaction involving disproportionation (5) (Brooks and Sicilio,1974). Addition of V(IV) to the initial solution accelerated O_2 evolution as well as the steady state concentration of a radical intermediate (presumably $VO(OO\cdot)^+$). This suggests involvment of the intermediate in a chain mechanism such as reactions (6)-(8) (where reaction (4) serves as a termination step). In addition, increasing initial peroxide concentration lowered the ratio of H_2O_2 consumed:V(IV) consumed, presumably by diverting H_2O_2 to reactions (such as decomposition) which do not consume V(IV). Moreover, slow addition of V(IV) such that radical intermediate concentrations remained low increased the ratio, presumably by increasing the fraction of H_2O_2 which reacts with V(IV) and decreasing the occurrence of diversionary side reactions (eg. disproportionation).

$$I + H_2O_2 \rightarrow O_2 + VO_2^+ + OH\cdot + H^+ \qquad (6)$$

$$I + OH\cdot + \rightarrow VO^{2+} + O_2 + H^+ \qquad (7)$$

$$2I \rightarrow VO_3^+ + O_2 + VO^{3+} \qquad (8)$$

A series of polarographic (O_2 consumption) extend these earlier findings concerning the nature of the chain decomposition of H_2O_2 by V(V) (Fig.7.0). In the presence of equimolar H_2O_2, V(V) produced an initial increase in O_2 concentration of 1-2% with respect to the baseline value, followed by a decrease of similar magnitude in both HEPES and phosphate buffers (data not shown). Both effects were slightly more pronounced in phosphate buffer. Superoxide dismutase (5 U/ml) or catalase (25 U/ml) inhibited these phenomena partially, but neither in a specific manner since equivalent amounts of serum albumin caused similar inhibition. These phenomena

were interpreted in terms of the catalatic action of V(V) - that is the formation of V(V) peroxo complexes, decomposition to V(IV) and subsequently reduction of O_2 by V(IV). Addition of 10-fold excess H_2O_2 to reaction mixtures containing V(V) produced a steady linear increase in O_2 concentration, rates of 3.4×10^{-8} M^{-1} s^{-1} and 1.37×10^{-8} M^{-1} s^{-1} being observed in HEPES and phosphate buffers respectively. In both buffers, superoxide dismutase (5 U/ml) completely abolished the increase in O_2 concentration, whereas serum albumin actually enhanced it.

6.6.0 Effects of ligands on the reactivity of V(V) peroxo complexes

Thus, as in the case of reactions of V(IV) and H_2O_2, ligands modulate reactions of V(V) with H_2O_2. In the presence of H_2O_2 and some polydentate ligands, V(V) also forms peroxo heteroligand complexes which are relatively stable towards decomposition and exist in aqueous solutions (Vuletic and Djordjevic,1973; Djordjevic, 1982). Heteroligands may shift the redox potential of the V(V)/V(IV) couple, intramolecular electron transfer within the V(V)-peroxo moiety resulting in concomitant reduction of V(V) to V(IV) and oxidation of the peroxo group to O_2^-, which is then released as a result of weakening of the V-O bonds to the coordinated dioxygen ligand (Djordjevic and Wampler, 1985). However, direct evidence for ligand-induced release of O_2^- from V(V) peroxo complexes has not been presented. Physiological ligands which may modulate the reactivity of peroxo-heteroligand vanadates include citrate or malate (Djordjevic and Wampler,1985).

6.7.0 Biological effects of V(V) peroxo complexes

The biological effects of V(V) peroxo complexes have been explored to only a limited extent. Vanadium (V) and H_2O_2 alone both mimick insulin in increase IGF-II binding to isolated rat adipocytes (Dubyak and Kleinzeller,1980; Degani et al,1981; Kadota et al,1987), however in combination, V(V) and H_2O_2 act synergistically (Kadota et al,1987). IGF-II binding was increased at least 2-fold over the level for insulin alone, and that expected if the effects of V(V) and H_2O_2

were additive. The patterns of IGF-II receptor binding and tyrosine kinase activation were parallel for insulin, V(V), H_2O_2 and their combinations. In a later series of experiments, it was found that V(V) peroxo complexes are the active agents with regards to both IGF-II binding and tyrosine kinase activation. With regard to IGF-II binding, it was found that although addition of catalase simultaneously with mixing of V(V) and H_2O_2 resulted in a mixture having the same potency as V(V) alone, when the enzyme was added > 10 minutes after mixing, the synergistic effects of V(V) and H_2O_2 were still apparent. The potency of mixtures of V(V) and H_2O_2 was retained for periods of up to 24 hours, however in the presence of catalase, this time period was decreased to from 2-3 hours. Interestingly, DNA strand breakage by H_2O_2 is slightly inhibited by V(V) (Birnboim,1988).

C. REDUCING AGENTS MAY AMELIORATE OR AUGMENT THE BIOLOGICAL EFFECTS OF VANADIUM

7.0.0 BIOLOGICAL ACTIONS OF VANADIUM ARE MODULATED BY REDUCING AGENTS

7.1.0 Vanadium in its various redox states can either reduce or be reduced by cytochrome c

The inhibitory actions of V(V) and to a lesser extent V(IV) towards a variety of different enzymes are well documented. In contrast, redox reactions of vanadium compounds with metalloenzymes have not been studied to any degree. In order to further characterize the ability of V(V) to participate in electron transfer reactions with metalloenzymes, we investigated the ability of V(V) to oxidize or reduce cytochrome c. Initial experiments were conducted in the pH range 2.6 - 4.0 since V(V)-mediated oxidations of NADH and ascorbate for example are enhanced at acid pH (refs; Taqui Khan and Martell,1967a, Buettner,1986). Vanadium (V) oxidized ferrocytochhrome c more effectively than the other metal ions tested (Cu(II), Fe(II), Al(III), Mn(II)) at all values of pH (Wu,1987). The unique properties of V(V) are emphasized by a number of observations. At pH 2.6, Cu, Fe, Al, and Mn were totally ineffective, while vanadium accelerated oxidation by 2000%. Similarly, at pH 3.2 where the effects of ligands were

investigated, the effects of vanadium (500% V.aq, 1000% V.EDTA, V.desferrioxamine, or V.histidine) far exceeded those of copper (10% acceleration Cu.aq, 0% Cu.EDTA, Cu.desferrioxamine) or iron (15% Fe.EDTA, 0% Fe.aq, Fe.desferrioxamine). Notably, ligands EDTA, desferrioxamine, and histidine enhanced the oxidizing ability of V(V) by 200% in comparison with controls or ADP. At pH 4.0, vanadium remained the most effective metal, although the other metals tested were also effective.

Vanadium(V) oxidized ferrocytochrome c progressively less effectively as pH increased. Interestingly, the catalytic effectiveness of vanadium as a fraction of control rates increased as pH increased from 2.6 to 4.0 although the rates of both catalysed and uncatalysed oxidation decreased. At pH 7.0 it was effective only in the presence of desferrioxamine. Surprisingly, by pH 7.4 V(V).desferrioxamine could not oxidize ferrocytochrome c at all. On the contrary it was a reductant of ferricytochrome c. However, most ligands accelerated reduction of V(V) by ferrocytochrome c. For example, at pH 6.0 V(V) complexes with desferrioxamine, histidine, ATP, EDTA, and albumin accelerated the initial rate of oxidation of ferrocytochrome c by 160-fold, 23-fold, 27-fold, 8-fold, and 1.4-fold respectively.

These results indicate that directions of the effects of pH and ligands upon the oxidative reactions of vanadium with cytochrome c are the reverse of their effects upon reductions of vanadium. There was one notable exception, namely desferrioxamine which accelerated oxidations and reductions with equal facility. Reactions of V(IV) with cytochrome c were also studied. Anaerobically ferrocytochrome c could not reduce V(IV) to V(III). Instead V(IV) or V(III) reduced ferricytochrome c, V(IV) being (surprisingly) the more effective. However, in the presence of oxygen, V (IV) could oxidize ferrocytochrome c even at pH 7.0, presumably because it reacted in the form of a V(IV).O_2 complex. Positive vanadyl and negative vanadite ions are oxidized by mitochondria or purified cytochrome oxidase, but not by a pathway involving cytochrome a_3 (Crane,1975).

7.2.0 Vanadium-catalyzed oxidations of reducing agents are not well characterized

Vanadium readily forms complexes with and subsequently oxidizes a variety of cellular reductants including NADH, catecholamines ascorbate, and thiols. Intracellularly, the reducing and antioxidant properties of these compounds should normally dominate their reactions with vanadium, since extremely low concentrations of the metal exist in the presence of much higher concentrations of reducing agents. Similarly, ascorbate and thiols are among the most effective antidotes for vanadium toxicity (Jones and Basinger,1983; Domingo et al, 1985). However, reducing agents may also exert prooxidant effects, increasing vanadium toxicity.

Since the primary focus of this review is redox phenomena which could amplify the biological effects of vanadium, the reactions of greatest interest are those in which vanadium acts catalytically. In contrast to iron- or copper- catalyzed oxidations of biological reductants, with the exception of NADH, considerably less is known of the properties of the catalytic properties of vanadium (Cavallini et al,1968; Misra,1974; Trotta et al,1974; Cantley and Kustin,1978; Nyberg et al,1979; Zwart et al,1981; Rowley and Halliwell,1982; Saez et al,1982; Tien et al,1982; Forni et al,1983; Harman et al,1984). Relevant information concerns:
(1) the abilities of different reductants to reduce vanadium, (2) the reactivity of monomeric as opposed to polymeric vanadium species, (3) the ability of vanadium to redox cycle in the presence of different reductants, and under conditions where O_2 or H_2O_2 serve as the ultimate electron acceptor, (4) the identity of those species which serve to initiate and propagate the overall reaction, in particular oxidant species by reactions of vanadium with O_2^- or H_2O_2, (6) the modulatory effects of ligands (in particular physiological ligands such as phosphates), and (7) the catalytic effectiveness of vanadium as compared to other transition and nontransition metals. The problems associated with determining which mechanisms may be of importance *in vitro* or *in vivo* are complicated in that kinetic as well as thermodynamic factors must be considered.

7.3.0 Oxygen consumption by V(IV) or V(V) in combination with l-ascorbic acid, NADH, l-cysteine, reduced glutathione or norepinephrine

In order to further characterize the reactivity of V(IV) and V(V) towards biologically relevant reductants, O_2 consumption by 250 μM V(IV) or V(V) alone and in combination with equimolar NADH, l-ascorbic acid, l-cysteine, reduced glutathione or l-norepinephrine was measured polarographically (Fig. 9.0). Information of interest included: 1) the order of effectiveness for different reductants in the absence and presence of added V(IV) or V(V) in different buffers (individual reactions were conducted in both potassium phosphate and HEPES buffers (50 mM, pH 7.0, air saturated, 20° C), 2) the relative effectiveness of V(IV) as opposed to V(V) in stimulating O_2 consumption by reductants, 3) the relative rates and extent of O_2 consumption by V(IV) in the absence and presence of reductants, and 4) evidence for the catalatic activity of vanadium.

Oxygen consumption in the absence and presence of added vanadium was buffer-dependent. For reactions involving reductants alone, in HEPES buffer rates of O_2 consumption increased in the order NADH (0.17 μM s^{-1}) < norepinephrine (2-fold increase) < ascorbate (5.4-fold increase) < glutathione (8.6-fold increase) < cysteine (18.7-fold increase), and in phosphate buffer NADH (3-fold increase) < glutathione (5.4-fold increase) < ascorbate (9.8-fold increase) < norepinephrine (12.5-fold increase) < cysteine (22.5-fold increase). Thus, reactions in HEPES buffer were slower than in phosphate buffer for ascorbate (4.2-fold), NADH (3.5-fold), l-cysteine (1.1-fold) and norepinephrine (1.2-fold) whereas the opposite was true for glutathione (3.1-fold). For reactions involving V(IV), the orders of effectiveness were glutathione (2.5 μM s^{-1}) < ascorbate (1.3-fold) = NADH < cysteine (1.6-fold) < norepinephrine (2.1-fold) and glutathione (0.89 μM s^{-1}) < cysteine (4.8-fold) < norepinephrine (7.3-fold) < ascorbate (12.1-fold) < NADH (21.7-fold) in HEPES and phosphate buffer respectively. Reactions in phosphate buffer were more rapid than those in HEPES buffer for ascorbate (3.3-fold), NADH (6-fold) and cysteine (1.1-fold) while the reverse

was true for glutathione (2.8-fold) and norepinephrine (1.1-fold). For V(V), in HEPES buffer NADH (0.1 $\mu M\ s^{-1}$) < norepinephrine (6.9-fold) < glutathione (10.4-fold) < ascorbate (14.2-fold) < cysteine (19.7-fold) while in phosphate buffer NADH (0.26 $\mu M\ s^{-1}$) < glutathione (2.3-fold) < norepinephrine (2.5-fold) < cysteine (9.5-fold) < ascorbate (14.4-fold). Reactions in phosphate buffer were more rapid than those in HEPES buffer for ascorbate (2.5-fold), NADH (2.3-fold), cysteine (1.2-fold), while the reverse was true for norepinephrine (1.1-fold) and glutathione (1.8-fold). Without exception, V (IV) (1.2 - 18-fold) accelerated O_2 consumption to a greater extent than V(V) (1.3 - 3.6-fold). V(IV) accelerated O_2 consumption by all reductants in both HEPES and phosphate buffers (ascorbate; 3.5, 2.8; NADH, 18-fold, ?; cysteine, 1.2-fold, 1.2-fold; glutathione, 10.9-fold, 1.9-fold; norepinephrine, 14-fold, 14.5-fold). V(V) accelerated O_2 consumption in HEPES buffer for reactions involving ascorbate (1.6-fold), glutathione (3.6-fold) and norepinephrine (1.95-fold), but not NADH (-40%), cysteine (-36%), and in phosphate buffer for reactions involving NADH (?), glutathione (1.3-fold) and norepinephrine (1.45-fold), but not ascorbate (-5%) or cysteine (-34%).

It is important to note that in reactions of V(IV) or V(V) with each of the reductants tested, since all the H_2O_2 produced was consumed in reactions involving V(IV) or V(V) (as indicated by the addition of 20 U/ml catalase when reactions were near completion), the data reflects both the ability of vanadium to accelerate O_2 consumption and to decompose H_2O_2 via catalase-like mechanisms (see section 6.5.0). Transient increases in the rate of O_2 consumption were readily apparent for reactions of V(IV) with all reductants except cysteine where the initial rate of O_2 consumption was linear for the major part of the first 100 seconds of the overall reaction, and glutathione, where the rate of O_2 consumption for V(IV) plus glutathione was actually below that for V(IV) alone in each buffer. The duration of these initial rapid increases in O_2 consumption. Over the time for which reactions were followed (approximately 1200 seconds), for reactions involving V(IV), the apparent total O_2 consumption was increased over reductants alone for ascorbate, NADH and norepinephrine in both phosphate and HEPES buffer, and cysteine in

HEPES buffer, but decreased for glutathione in both buffers and for cysteine in phosphate buffer. In contrast, in both buffers, the general patterns of O_2 consumption for reactions involving V(V) and reductants were extremely similar to that for reductants alone. The apparent total O_2 consumption was increased for reactions involving V(V) and ascorbate, NADH or norepinephrine in both buffers, and decreased for reactions involving the metal ion and cysteine or glutathione in both buffers.

These results demonstrate that: 1) reactions of vanadium with reducing agents (ie. the order of effectiveness for V(IV) or V(V)) are strongly influenced by ligands (in this case buffer ions), 2) both V(IV) and to a lesser extent V(V) accelerate O_2 consumption by the reducing agents tested, 3) reactions of vanadium with both O_2 and H_2O_2 as well as with reducing agents may influence the progress of the overall oxidation reaction (and so the yield of specific radical species), 4) with the exceptions of GSH in both buffers and norepinephrine in phospohate buffer, all reducing agents accelerate O_2 consumption by V(IV) or V(V), 5) with the exception of reactions involving glutathione and V(IV) or V(V), or norepinephrine and V(V), reactions of V(IV) or V(V) with reducing agents are more rapid in phosphate buffer. Further study is required to determine: 1) the relationships between O_2 consumption and reductant oxidation *per se*, 2) the activity of vanadium bound to physiological ligands and proteins, and 3) the relative yields of specific radical species including $O_2^{\cdot-}$, H_2O_2 and $OH\cdot$ capable of damaging tissue components.

8.0.0 VANADIUM -STIMULATED OXIDATIONS OF NADH

Since the first reports that V(V) accelerates enzymatic (Erdmann et al,1979) and nonenzymatic (Vyskocil et al,1980) oxidations of NADH, a small but complicated literature on these reactions has appeared. The significance of these reactions intracellularly is debatable, particularly in view of recent finding that vanadium does not stimulate NAD(P)H oxidation in intact erythrocytes

(Sullivan et al,1988; Yoshino et al,1988). Further there is little agreement concerning either the relative effectiveness of different vanadium species, or the underlying reaction mechanisms. A detailed discussion of these topics is beyond the scope of the present work. Thus, here we selectively review those aspects which are most clearly relevant to redox reactions of vanadium *in vivo*.

8.1.0 V(V) stimulates oxidation of NADH by biomembranes and enzymes

Many different animal tissues possess NADH oxidase activity (DeMaster et al,1973; Rubinson,1981; Hansen,1981). The stimulatory effects of V(V) on enzymatic oxidations of NADH were first reported by Erdmann et al (1979). Since then, NADH oxidations by membrane preparations from a variety of different tissues and organelles have been demonstrated to be more or less sensitive to the stimulatory effects of both monomeric and polymeric V(V) (Erdmann et al,1979; Crane et al,1980; Menon et al,1980; Erdmann et al,1980; Vyskocil et al,1980; Ramasaram et al,1981; Vijaya et al,1984; Khandke et al,1986; Liochev and Fridovich,1986; Coulombe et al,1987; Patole et al,1987; Rau et al,1987; Patole et al,1988). Tentative explanations for these phenomena have invoked a NADH-V(V) oxidoreductase (Erdmann et al,1979,1980), a V(V)-stimulated NADH oxidase (Menon et al,1980; Coulombe et al,1987) or a membrane-associated NADH dehydrogenase converted to an oxidase as a result of binding by V(V) (Vijaya et al,1984) have been proposed. Polymeric V (V) also stimulates NADH oxidation by enzymes in solution including malate dehydrogenase (Vyskocil et al,1980), xanthine oxidase (Khandke et al,1980), and lactate dehydrogenase (Khandke et al,1986). In these instances the enzymes facilitating electron transfer from NADH, through polymeric V(V), to O_2 (Khandke et al,1986; Patole et al,1988). Vanadium can also react with cytochromes. Cytochrome c inhibits V(V)-mediated NADH oxidation in the presence of mouse liver plasma membranes at neutral pH (Ramasarma et al,1981). On the other hand, cytochrome c-depleted mitochondria preparations depleted of cytochrome c do not display

NADH oxidizing activity in the presence of V(V) (Rau et al,1987). Cytochrome c inhibits NADH oxidation by V(V) and a variety of membrane preparations, but not rat liver microsomes (Rau et al,1987). NADH oxidation also occurs as a result of redox reactions with cytochrome b_5 (Patole et al,1987).

These enzymatic oxidations may exhibit: (1) variable substrate specificity (eg. NADH, NADPH, NMNH) (Liochev and Fridovich,1986; Coulombe et al,1987), (2) varying specificity for vanadium as compared to other metals such as Mo, and for monomeric as compared to polymeric V(V) species (Menon et al,1980; Coulombe et al,1987), (3) a requirement for phosphate (Ramasarma et al,1981; Vijaya et al,1984; Patole et al,1987), (4) pH optima which vary depending on the particular membrane preparation employed, and may also differ significantly from the pH optima for nonenzymatic reactions, (Ramasarma et al,1981; Liochev and Fridovich, 1986; Coulombe et al,1987), (5) differential susceptibilities to metabolic inhibitors (Ramasarma et al,1981; Coulombe et al,1987; Rau et al,1987), and (6) unreasonable stoichiometry and kinetics more typical of free radical reactions than of enzyme catalysis (Liochev and Fridovich,1987). In addition, the extent to which nonenzymatic reactions contribute to the observed rate of NADH oxidation varies (Vyskocil et al,1980; Coulombe et al,1987). Since stimulation of enzymatic NADH oxidation is observed only at V(V) concentrations far in excess of those in tissues (Vyskocil et al,1980), and some systems are reportedly active only in the presence of polymeric V(V) (Vijaya et al,1984; Khandke et al,1986; Patole et al,1987; Patole et al,1988), these nonenzymatic free radical-mediated reactions are of the greatest interest in the present context.

8.2.0 Vanadium alone does not catalyze the nonenzymatic oxidation of NADH

Alone, vanadium species similar to those which predominate biologically are relatively ineffective in promoting nonenzymatic oxidations of NADH. A rapid initial phase of NADH

oxidation occurs on addition of V(IV) to reaction mixtures containing NADH at neutral pH, at completion of the rection, however, less than a stoichiometric amount of NADH is oxidized (Liochev and Fridovich,1987). Monomeric V(V) causes only slow oxidation of smaller than expected amounts of NADH (Vyskocil et al,1981; Vijaya and Ramasarma,1984; Darr and Fridovich,1985; Liochev and Fridovich, 1987; Keller et al,1987; Steele, unpublished observation). Thus in both cases, redox cycling of the metal and chain oxidation are limited. Polymeric V(V) species are reportedly far more effective than monomeric forms in both nonenzymatic (Vijaya and Ramasarma,1984) and enzymatic (Menon et al,1980; Vijaya and Ramasarma, 1984; Patole et al,1987; Rau et al,1987; Patole et al,1988) oxidations of NADH. However detailed explanations for this greater reactivity in thermodynamic or steric terms are lacking.

Some conflicting reports concerning the activity of different vanadium species are explicable in terms of differences in the nature of vanadium stock solutions (Erdmann et al,1980; Vyskocil et al,1981; Darr and Fridovich,1985; Khandke et al,1986), and or experimental conditions. For example, V(IV) is reportedly both less effective (Vijaya and Ramasarma,1984) and more effective than V(V) (Liochev and Fridovich,1987) in promoting nonenzymatic oxidations of NADH, and ineffective in certain enzymatic systems (Menon et al,1980; Vijaya and Ramasarma, 1984; Patole et al,1987; Rau et al,1987; Patole et al,1988). Vanadyl sulphate and orthovanadate caused nonenzymatic oxidation of NADH under the experimental conditions employed by Liochev and Fridovich (1987), and metavanadate (Liochev and Fridovich, 1985) and orthovanadate (Coulombe et al,1987) oxidized NADH in the presence of rat liver microsomes. Yet in other nonenzymatic and enzymatic systems, polymeric V(V) alone is effective (Menon et al,1980; Vijaya and Ramasarma, 1984; Patole et al,1987; Rau et al,1987; Patole et al,1988). Polymerization of vanadium is affected by vanadium concentration, pH, temperature, ionic strength and available ligands (Pope and Dale,1968; Rubinson,1981). It is therefore often difficult to identify the exact form of the metal species present in stock solutions. Further, polymeric V(V) (decavanadate) may depolymerize to monomeric forms (metavanadate, orthovanadate) on standing, or on addition of aliquots of these

solutions to reaction mixtures at neutral pH (Darr and Fridovich,1985). In solution, vanadium distributes itself among a series of varying states of polymerization. Mixtures of orthovanadate and decavanadate oxidized NADH in the rat liver microsome system of Menon et al (1980), while small amounts of polymeric V(V) accelerate NADH oxidation by metavanadate in the similar system of Patole et al (1988). Vanadyl or metavanadate, in contrast, did not.

8.3.0 Vanadium may accelerate NADH oxidation by reduction of O_2

The abilities of different V(IV) or V(V) species to reduce O_2 in the presence of NADH under physiological conditions are largely unknown. This property is important since O_2^- has been assigned key roles as an initiating and propagating agent in reactions involving V(IV), monomeric or polymeric V(V), and in the absence or presence of biomembranes (see section 9.4.0). ESR signals characteristic of V(IV) are produced by mixtures of monomeric V(V) and NADH (Vyskocil et al,1981). Reaction mixtures containing NADH, decavanadate, rat erythrocyte plasma membranes and DMPO reportedly show ESR signals characteristic of V(IV), V(III) and V(II) in the presence of excess NADH (Patole et al,1987). The abilties of these species to reduce O_2 may differ considerably from that of V(IV). However, reduction of polymeric V(V) does not yield the vanadyl cation (VO^{2+}) (Rau et al,1987). Moreover, under some conditions, NADH oxidation by polymeric V(V) and rat liver microsomes reportedly does not involve O_2 consumption and is not inhibited by superoxide dismutase (Patole et al,1987).

8.4.0 Reactions of vanadium with O_2^- may mediate NADH oxidation

Superoxide is a key intermediate in many vanadium-stimulated oxidations of NADH and similar substrates. Superoxide dismutase, and other O_2^- scavengers ($MnCl_2$, ascorbate, catecholamines, isoproterenol, caffeic acid, adriamycin) inhibit V(V)-stimulated NADH oxidations in both nonenzymatic (Vijaya and Ramasarma,1984; Liochev and Fridovich,1987) and

enzymatic systems (Ramasarma et al,1981; Vijaya and Ramasarma,1984; Rau et al,1987). Information is needed concerning the effects of such scavengers on V(IV)-mediated reactions. Further, the specific mechanisms underlying the stimulatory effects of O_2^- are not known.

Two general mechanisms have been proposed. One one hand, O_2^--dependent chain oxidation of NADH is seen as a fundamental characteristic of both nonenymatic (Darr and Fridovich, 1984,1985; Liochev and Fridovich,1985,1987a,b; Liochev and Ivancheva,1988) and enzymatic (Liochev and Fridovich,19860 oxidations involving V(IV) or V(V). Linear and nonlinear dependencies of NADPH oxidation on the concentration of rat liver microsomes are observed in the absence or presence of monomeric V(V) repsectively. The former is characteristic of enzyme catalyzed reactions, and the latter of a free radical-mediated chain oxidation (Liochev and Fridovich, 1986). Effective O_2^- generating systems include: xanthine/xanthine oxidase (Darr and Fridovich,1984,1985; Liochev and Fridovich,1985), rose bengal, flavin mononucleotide, methylene blue, eosin yellowish and KO_2 (Liochev and Fridovich,1985). In each case the NADH oxidation is inhibited by superoxide dismutase. Vanadium (IV) and biomembranes also serve as sources of O_2^- (Liochev and Fridovich,1986,1987). In this scheme, a peroxy V(IV) species formed by reaction of V(V) with O_2^- is the primary oxidant of NADH. However little is known concerning the structure of this species. Superoxide reacts with V(V) alone rather with V(V)/NADH complexes. This follows from the observation that NADH and NMNH are oxidized at similar rates in the presence of ? V(V) and a xanthine/xanthine oxidase O_2^- generating system (Darr and Fridovich,1985). Moreover, the rates of oxidation of NADH and NMNH respond similarly to changes in substrate concentration (Darr and Fridovich,1985).

A second view argues that V(V) itself is the primary oxidant of NADH, and that O_2^--dependent chain oxidation does not occur. In this scheme, O_2^- serves merely to oxidize V(IV), yielding active V(V) (Vijaya and Ramasarma,1984; Vijaya et al,1984; Khandke et al,1986; Patole et al,1987;

Rau et al,1987; Patole et al,1988). Without exception, support for this general mechanism comes from studies involving polymeric V(V). Superoxide dismutase inhibits NADH oxidation by polymeric V(V). The observation that superoxide dismutase (which catalyzes the dismutation of O_2^- to H_2O_2) inhibits V(V)-stimulated NADH oxidation which produce H_2O_2 is seen as central to this reaction schema (Vijaya and Ramasarma,1984; Vijaya et al,1984). Arguments against an O_2^--dependent chain reaction include: (1) a lack of correlation between initial concentrations of radiolytically-generated O_2^- and chain length (Chan and Bielski,1974; Bielski and Chan,1976), (2) NADH oxidation by NADH oxidation by polymeric V(V) and xanthine oxidase in the absence of xanthine (Khandke et al,1986), (3) a requirement for polymeric V(V) in both nonenzymatic and enzymatic reactions (Vijaya and Ramasarma,1984) and (4) a 1:1:1 NADH:O_2:H_2O_2 stoichiometry for the overall oxidation reaction (Vijaya et al,1984; Khandke et al,1986; Patole et al,1987; Rau et al,1987; Patole et al,1988). Again, experimental evidence for the proposed mechanism is lacking. Superoxide dismutase reportedly inhibits formation of a 240 nm-absorbing vanadium hydroperoxide species formed by reaction reactions of V(IV) with O_2^-. (Khandke et al,1986). Neither superoxide dismutase nor catalase alter absorbance at 240 nm once this species is formed. Peroxyvanadate ($V^{V}OO\cdot$ or $V^{IV}OO$) species may also occur as intermediates (Vijaya and Ramasarma,1984; Khandke et al,1986; Patole et al,1987; Rau et al,1987; Patole et al,1988).

Inhibition by superoxide dismutase does not necessarily imply direct participation of O_2-. Autoxidations and metal-catalyzed oxidations of many organic compounds are mediated by O_2^-, yet are also inhibited by superoxide dismutase. Further, although polymeric V(V) is supposedly required, interconversion of some polymeric V(V) (decavanadate) species to monomeric forms (metavanadate, orthovanadate) may occur, with orthovanadate serving as the active metal species (Darr and Fridovich,1985) (see section x.x.x). In addition, NADH oxidation and O_2 consumption are not tightly coupled (Rau et al,1987). The inhibitory effects of superoxide dismutase on NADH oxidation and O_2 consumption are not always similar (Vijaya et al,1984; Patole et al,

1987; Rau et al,1987). More detailed experiments will reveal the implications of these observations.

8.5.0 Reactions of vanadium with H_2O_2 may mediate NADH oxidation

Radical species other than O_2^- may also be significant in vanadium-stimulated oxidations of NADH. For example, H_2O_2 also stimulates NADH oxidation by V(IV) and V(V). ESR studies show OH· formation from V(IV) and H_2O_2 (Keller et al,1987). NADH oxidation by V(IV) is extreemely sensitive to the effects of added H_2O_2. Thus 40 μM H_2O_2 significantly accelerated NADH oxidation by 0.5 mM V(IV) at neutral pH (Liochev and Fridovich,1987). The rapid initial oxidation was mediated by OH·. There followed a second, slower phase dependent upon O_2^- (Liochev and Fridovich,1987). However, as in the case of reactions involving V(IV) alone, less than the stoichiometrically exxpected amount of NADH was oxidized. Since H_2O_2 accelerates the autoxidation of V(IV) (Steele,1988), reactions of V(IV) with H_2O_2 may contribute to NADH oxidation by V(IV) alone. Effects of added H_2O_2 on oxidations involving V(V) are not well established. Catalase and OH· scavengers did not inhibit NADH oxidation by polymeric V(V) in the study of Vijaya and Ramasarma (1984). Exogenous H_2O_2 did not stimulate nonenzymatic NADH oxidation by metavanadate in the study of Liochev and Fridovich (1987). On the other hand, catalase and mannitol partially inhibited NADH oxidation by metavanadate at pH 7.0 in the study of Liochev and Ivancheva (1988).

Biomembranes reportedly stimulate H_2O_2 production by V(V) and NADH (Vijaya et al,1984). As in the case of nonenzymatic oxidations, enzyme-mediated oxidations of NADH mediated by polymeric V(V) are variably inhibited by catalase and OH· scavengers. This may reflect inhibition of catalase by V(V) (Khandke et al,1986), an inhibition which is increased in the presence of NADH (Rau et al,1987). Catalase partially inhibited NADH oxidation by polymeric V(V) and mouse liver plasma membranes (Ramasarma et al,1981). Anaerobically

(argon atmosphere), addition of excess H_2O_2 to reaction mixtures containing NADH, polymeric V(V) and mouse liver plasma membranes significantly accelerated NADH oxidation (Ramasarma et al,1981). Although O_2 generation resulting from H_2O_2 decomposition was invoked to explain this phenomenon, radical generation from V(V) peroxo complexes is a more likely explanation for the stimulatory effects of H_2O_2 (Steele,1988). At concentrations of 0.01 and 0.025 M, mannitol at concentrations of 0.01 and 0.025 M *increased* the rate of NADH oxidation by polymeric V(V) in the presence of mouse liver plasma membranes by up to 20%. At 0.1 M mannitol, oxidation was inhibited by 50% (Ramasarma et al,1981), while formate (0.1 M) caused a slight activation. Howerver, neither Tris, mannitol, benzoate nor thiourea inhibited NADH oxidation by decavanadate and rat erythrocyte plasma membranes (Vijaya et al,1984). On the other hand, in the system employed by Rau et al (1987), Tris and benzoate had little effect on NADH oxidation by polymeric V(V) and rat liver microsomes, but mannitol and histidine inhibited strongly. Information concerning the relative affinities of H_2O_2 for monomeric as compared to polymeric V(V) for H_2O_2 is needed. Reports that approximately one half of the total O_2 consumed exists as H_2O_2 when reactions are at or near completion (Vijaya et al,1984; Khandke et al,1986; Rau et al,1987) suggest that polymeric V(V) may react differently with H_2O_2 than monomeric forms, since at the V(V) concentrations used, all the H_2O_2 would be expected to be in the form of V(V) peroxo complexes (see section 6.2.0).

8.6.0 Phosphate may stimulate oxidation of NADH by vanadium

Relatively little is known of the effects of physiological ligands and metal-binding proteins on vanadium-stimulated NADH oxidations. Nonenzymatic and enzymatic oxidations involving monomeric or polymeric V(V) proceed more rapidly in phosphate than in other commonly used buffers (Tris, HEPES, histidine, imidazole, acetate, citrate, tricine) (Vyskocil et al,1980; Vijaya and Ramasarma,1984; Darr and Friddovich,1985; Coulombe et al,1987; Rau et al,1987).

The stimulatory effects of increasing phosphate concentration are characterized by well-defined optima (Ramasarma et al,1981; Vijaya and Ramasarma,1984; Patole et al,1987; Rau et al,1987), and are not duplicated by other anions (Ramasarma et al,1981). The stimulatory effects of phosphate may result from modulation of the redox potential of V(V), steric factors governing electron transfer within V(V)-NADH complexes (Darr and Fridovich,1984), binding of V(V) to enzymes (Coulombe et al,1987), and/or modulation of the equilibria between monomeric and polymeric V(V) (Darr and Fridovich,1985). Yet, in the presence of a xanthine oxidase O_2^- generating system, phosphate is not required , and (like some other buffer ions) *decreases* the reactivity of V(V) in comparison with media adjusted to the same pH in the absence of buffer salts (Darr and Fridovich,1985). Phosphate may also modulate electron transfer between polymeric V(V) and NADH, perhaps within heteropolynuclear phosphovanadate complexes with NADH (Rau et al,1987; Patole et al,1988). Phosphate is required for NADH oxidation by polymeric V(V) in certain nonenzymatic (Vijaya and Ramasarma,1984) and enzymatic (Vijaya et al,1984; Rau et al,1987; Patole et al,1987; Patole et al,1988) systems. Notably, in each case, polymeric V(V) is effective whereas V(IV) and monomeric V(V) are not. Recently, the possibility that phosphate promotes O_2^- formation by preventing formation of V(IV)/V(V) complexes and so promoting the air autoxidation of V(V) has also been raised (Liochev and Ivancheva,1988). Phosphate also accelerates O_2 reduction (and so O_2^- formation) by V(IV), and alters the reactivity of monomeric vanadium towards H_2O_2 (see section 6.6.0), however the significance of these reactions in terms of vanadium-stimulated oxidations of NADH are unknown. Similarly, no information is available concerning the effects of phosphate on the reactivity of polymeric V(V) with O_2^- or of V(V) species in general with H_2O_2.

8.7.0 The biological significance of vanadium-stimulated oxidations of NADH is not clearly established

It is important to learn the extent to which each of these factors modulate the biological activity of vanadium. In particular, are vanadium-stimulated oxidations of NADH or similar

substrates of any biological significance? Although V(V)-mediated enhancement of enzymatic NADH oxidation may occur, intracellular V(V) concentrations are far lower than those at which NADH oxidase activity occurs with biomembranes *in vitro*. Similarly, redox reactions of vanadium with cytochrome c (Wu,1987), or cytochrome b_5 are seen only at vanadium concentrations much greater than can be attained intracellularly. Of course, compartmentalization may result in local extremes of vanadium concentrations. Binding to fixed ligands is also a form of compartmentalization. Moreover, proton and electron gradients may result in local sequestration of specific redox states in mitochondria or in sites of microsomal redox enzymes. Rapid nonenzymatic NADH oxidation has been demonstrated by V(IV) alone (Liochev and Fridovich,1987), mixtures of V(IV) and V(V) (Liochev and Fridovich,1987; Liochev and Ivancheva,1988), V(IV) and H_2O_2 (Liochev and Fridovich,1987) or V(V) and sugars (Liochev and Fridovich,1987b). However, neither these, nor other studies of vanadium-mediated nonenzymatic NADH oxidations reported in the literature have employed vanadium at catalytic concentrations. Further, reactions of vanadium with O_2 are limited intracellularly, and O_2^- is prone to rapid dismutation to H_2O_2 which in turn is rapidly removed. Reactions of with V(IV) or V(V) may sequester H_2O_2 in a state where it is inaccessible to catalase or glutathione peroxidase. Interactions of vanadium with sugars (Liochev and Fridovich,1987b) and perhaps other intracellular reductants contribute nonenzymatically to oxidations of NADH and other compounds. A multitude of possible targets exist intracellularly which are at least as susceptible to radical type oxidations as is NADH. Thus, the oxidation of NADH in the presence of vanadium may be a prototype for other, more significant cellular mechanisms of NADH toxicity. Loss of some of these constitutes a more serious threat to normal cellular metabolism. Vanadium (as $NaVO_3$) at 0.5 mM does not alter the redox state of NADH in erythrocytes *in vitro* (Yoshino et al,1988). On the basis of these observations, we conclude that vanadium-stimulated NADH oxidations are of only limited significance *in vivo*. However, much further research is required to obtain a definitive answer to this question.

9.0.0 MODULATION OF VANADIUM TOXICITY THROUGH REDUCTION BY l-ASCORBIC ACID

9.1.0 Ascorbate reduces vanadium at acid, but not physiological pH

Both V(IV) and V(V) reportedly oxidize l-ascorbic acid (ascorbate) at acid pH. As shown by dehydroascorbate formation, V (IV) catalyzes the oxidation of ascorbate in the pH range 1.75-2.85, although less effectively than Cu(II) or Fe(III) (Khan and Martell,1967). Over sufficient periods of time, V(IV) is apparently reduced to V(II) both in the absence and presence of O_2 (Khan and Martell,1967). In the presence of O_2, the reaction proceeds by formation of two preequilibrium steps, the first of which involves formation of a V(IV)-ascorbate complex, and the second, binding of O_2 to the V(IV)-ascorbate complex. Rate-determining electron transfer then occurs through from ascorbate, throught the metal ion to O_2. The semidehydroascorbate radical is oxidized to dehydroascorbate as a result of intramolecular electron transfer or reactions with O_2 or $HO_2\cdot$. Interestingly, whereas the Cu(II)- and Fe(III)-catalyzed oxidations of monoionic ascorbate proceeds via inverse hydrogen ion dependent reaction pathways, the V(IV)-catalyzed reaction involves a reaction pathway which is first order in $[H^+]$, perhaps because of the requirement of oxo groups for protons during the rate-determining electron transfer step (Khan and Martell,1967). Similarly, in 1.0 M $HClO_4$, mixing of V(V) and ascorbate results in V(V) reduction as indicated by the appearance of the blue color characteristic of V(IV) (V(IV) absorbs maximally at 750 nm), measured by stopped flow spectrophotometry (Kustin and Toppen,1973). As in the case of V(IV)-catalyzed oxidations of ascorbate, V(V)-ascorbate complex formation occurs initially, followed by univalent inner-sphere electron transfer resulting in formation of V(IV) and semidehydroascorbate. However, on the basis of the observed stoichiometry, it was suggested that the semidehydro-ascorbate formed reacted with V(V) rather than O_2 to produce V(IV) and dehydroascorbate. Yet in the range of physiological pH, neither V(IV) nor V(V) accelerate ascorbate oxidation (Buettner,1986; Adam-Vizi et al,1981; Donaldson and LaBella,1983; Steele,1988). Thus, conflicting reports concerning the effects of ascorbate on vanadium-mediated tissue damage exist in the literature.

Ascorbate administered by intraperitoneal injection attentuates the lethal effects of vanadium (Mitchell and Floyd,1954), perhaps by reduction of V(V) to V(IV) or V(III) (Venugopal and Luckey,1978). ESR techniques showed partial (42%) reduction of V(V) (10^{-4} M) by equimolar ascorbate at neutral pH in rat brain synaptosomal preparations (Adam-Vizi et al,1981). No reduction was observed in the presence of V(V) and ascorbate alone, this being attributed to rapid reoxidation of V(IV) by O_2. Vanadium (V) also had no significant effect on ascorbate oxidation (0.2 M phosphate buffer; pH 8.0, 100 μM ascorbate, 3 - 33 μM V(V)) (Donaldson and LaBella,1983). Nevertheless, in spite of the relative lack of catalytic activity of vanadium in the aerobic oxidation of ascorbate, redox reactions between vanadium and ascorbate are toxicologically significant.

9.2.0 Vanadium toxicity *in vivo* is ameliorated by l-ascorbic acid

Vanadium at toxic levels (in common with other toxicants) depletes endogenous stores of ascorbate (Roshcin,1967). Mortality in mice following intraperitoneal administration of $NaVO_3$ (as doses of 10.9 and 14.6 mg vanadium/kg) is alleviated by ascorbate (also administered intraperitoneally) 20 minutes before exposure to vanadium at a dosage of 1.0 g/kg (Mitchel and Floyd,1954a). In rats and dogs, V (V) toxicity at LD_{70} and LD_{95} levels is decreased by subcutaneous injections of ascorbate (1.0 g/kg), while 125.0 g/kg ascorbate is required to protect mice against toxicity at the LD_{75} level (Mitchel and Floyd, 1954b). Conversely, an 80% decrease in serum ascorbate levels was observed following administration of V_2O_3 in aerosol form to rabbits (Roschin,1967). This finding has been confirmed in rats treated chronically with V_2O_5 (Chakraborty et al,1977). Ascorbate is at least as effective as the most potent metal chelating agents available in countering vanadium toxicity and considerably less toxic (Jones and Basinger,1983; Domingo et al,1985) (Fig. 7.0). Pretreatment with ascorbate also decreases $NaVO_3$-enhanced lipid peroxidation (as measured by malondialdehyde formation) in the kidneys of mice at 24 hours post-injection (Donaldson et al,1985). Symptoms of vanadium toxicity such as central

nervous system depression and paralysis of hindquarters were absent or ameliorated. Urinary protein levels, which were increased by $NaVO_3$ injection were decreased by ascorbate. Injection of ascorbate alone did not influence the level of lipid peroxidation in kidney compared with saline-treated mice. However, in the absence of definitive experimental data, the exact mechanisms underlying the protective effects of ascorbate remain unknown.

9.3.0 Prooxidant effects of vanadium are increased in the presence of l-ascorbic acid

The extent to which ascorbate may enhance the prooxidant effects of vanadium (see section 11.4.0 and Fig. 7.0) intracellularly has been discussed primarily in terms of vanadium-mediated inhibition of cellular Na^+-K^+-ATPase activity. At concentrations of less than 10^{-3} M, ascorbate alone inhibits microsomal and synaptosomal membrane ATPase from brain through induction of lipid peroxidation (Schaefer et al,1974; 1975). Na^+/K^+-ATPase activity is more sensitive to the effects of lipid peroxidation than Mg-ATPase activity (Biushaye and Balasubrananian,1971; Svoboda et al,1984). Ascorbate-induced Na^+/K^+-ATPase inhibition in brain microsome preparations is directly related to the extent to which lipid peroxidation is induced (Boxal and Phizackerly, 1973; Svoboda and Mosinger,1981; Svoboda et al,1984). In the absence of added metals, the prooxidant effects of ascorbate may be attributed to the catalytic actions of trace amounts of transition metal ions, and subsequently formation of activated oxygen or semidehydroascorbate radicals (Goto and Tanaka,1981) which may initiate lipid peroxidation (Boxal and Phizackerly,1973; Schaeffer et al,1975; Svoboda and Mosinger,1981a; Svoboda et al,1984). At higher concentrations, the antioxidant effects of ascorbate predominate, with lipid peroxidation being diminished (Barber,1963; Biushaye and Balasubrananian,1971; Svobida et al,1984) and simultaneous protection of Na^+/K^+-ATPase activity being observed (Boxal and Phizackerly,1973; Schaefer et al,1974; Adam Vizi et al,1981; Svoboda and Mosinger,1981; Svoboda et al,1984). Interestingly, EDTA does not inhibit ascorbate-mediated lipid peroxidation in rat brain microsomes, perhaps as

a result of the inaccessibility of catalytic metal ions or the actions of semidehydroascorbate radicals (Goto and Tanaka,1981; Svoboda et al,1984).

The effects of V(IV) or V(V) alone and in combination with ascorbate on lipid peroxidation and Na^+,K^+-ATPase activity were studied by Svoboda et al (1984). Lipid peroxidation in brain microsomes was induced by VO^{2+}, but not VO_3^-. Yet, in the range of concentrations found intracellularly (10^{-6}-10^{-5} M),even VO^{2+} did not stimulate lipid peroxidation. Further, addition of 10^{-4} M ascorbate to brain microsomes stimulated lipid peroxidation to a maximum extent in the absence or presence of added VO^{2+} or VO_3^-. Maximum malondialdehyde (MDA) production and minimum Na^+/K^+-ATPase activity were observed at ascorbate concentrations of 10^{-5} to 10^{-4} M. The extent of Na^+,K^+-ATPase inhibition caused by VO^{2+} or VO_3^- was similar, with the effects of low concentrations of ascorbate (10^{-5}) on VO^{2+}-mediated inhibition being strictly additive, and a slight inhibition being observed at high concentrations (10^{-3} M). Similarly, in the study of Adam Vizi et al (1981), ascorbate at low concentrations (10^{-4} M) increased V(V)-mediated inhibition of Na^+/K^+-ATPase activity of rat synaptosmal membranes whereas high concentrations (2×10^{-3} M) partially reversed the inhibition. EDTA inhibited Na^+,K^+-ATPase inhibition caused by ascorbate, but not VO^{2+}. Vanadium(V)-mediated inhibition of Na^+,K^+-ATPase is also unaffected by EDTA (Josephson and Cantley,1977; Post et al,1979). Although on the basis of these results it would appear that ascorbate does not enhance the prooxidant effects of vanadium (perhaps as a result of the chelating properties of ascorbate, (Svoboda et al,1984), it is possible that under conditions where more reactive vanadium species, for example vanadium peroxo complexes, are formed, redox cycling of the metal may occur, resulting in synergistic effects on lipid peroxidation for example (see section 11.0.0).

10.0.0 MODULATION OF VANADIUM TOXICITY BY THE REDUCING ACTIONS OF CATECHOLAMINES

10.1.0 Vanadium-catalyzed oxidations of catechols differ from those of other transition metals

Although the underlying mechanisms are not well understood, vanadium-catalyzed oxidations of catechols (including catecholamines) differ significantly from those of other transition metals (Kustin et al,1974). Several V(III) or V(IV) complexes, and V(V) alone catalyze reactions of catechols with O_2 (Tatsuno et al,1982; 1984; Kustin et al,1974). Kustin et al (1974) studied the kinetics of V(V)-stimulated oxidations of catechol, pyrogallol, 1,2,3-benzenetriol, l-dopa, epinephrine and gallic acid using stopped flow spectrophotometry in acidic media (0.2 - 1.0 M $HClO_4$). In each case, oxidation proceeded via the formation of an intermediate V(V)-catechol complex, then reduction of V(V) to V(IV) and oxidation of the catechol ligand via outer sphere electron transfer.

Reactions of vanadium with catecholamines are of particular interest, since these compounds are physiologically active. Thus, formation of metal complexes may result oxidation of the catecholamine ligand as well as disruption of a variety of physiological processes (Rajan et al,1976,1977; Singh and Singh,1977; Kappus and Schenkman,1979; Donaldson and LaBella,1983). Metal-catalyzed oxidations of catecholamines yield numerous cytotoxic species, a including quinonoid reaction products, $O_2^{\cdot-}$, H_2O_2 and $OH\cdot$. Both the properties of vanadium as a catalyst and the specific catecholamine species with which it interacts may be of importance, since for example the relative production of quinonoid products and oxygen radical species varies for different catecholamines (Graham et al,1978). Thus the unique behavior of vanadium towards catecholamines may be important in determining its cytotoxic profile (Donaldson et al,1983).

Martin et al (1960) used spectrophotometric and manometric (O_2 consumption) techniques to compare the effectiveness of a variety of metal ions in catalyzing oxidations of catecholamines at pH 6.0 in acetate buffer. In terms of the rapidity and intensity of colour development, V(V) was far

more effective than a variety of other metal ions including , Fe(III), Cu(I), Cu(II) and Mn(II) in catalyzing oxidation of l-3,4-dihydroxyphenylalanine, 5-hydroxytyramine, noradrenaline and d,l-5-hydroxytryptophan, and slightly more effective than Cu(II) with regard to oxidation of adrenaline (Martin et al,1960). Further more, V_2O_5, $NaVO_3$, $VOSO_4$ and $VOCl_2$ were all effective in increasing quinonoid product formation. Experimental data were presented only for experiments in which the effects of $NaVO_3$ and $CuSO_4$ on quinoid product formation and O_2 consumption were studied. In general, V(V)-catalyzed reactions were characterized by greater total O_2 consumption with the exception of 5-hydroxytryptamine and l-adrenaline, for which Cu(II) consumed considerably more and an equal amount of oxygen respectively. In contrast, Cu(II) was more effective in catalyzing quinonoid product formation from l-dopa and to a lesser extent l-adrenaline. Vanadium(V) has also reported to produce dose-dependent increases in aminochrome formation from adrenaline and to a lesser extent dopamine, Mn(II) and Cd(II) being far less effective (Donaldson and LaBella,1983).

On the basis of spectrophotometric titrations of V(V) by noradrenaline Cantley et al (1978) reported that V(V) formed a bis-V(IV) complex (ie. a complex with a formula of $V(IV)L_2$). Subsequent oxidation of the catecholamine ligand supposedly occurs by the mechanism -

$$V(noradrenaline)_2 + noradrenaline \rightarrow V(IV) + Q^{-}\cdot$$

where V(IV) is one or more complexes and $Q^{-}\cdot$ is a semiquinone. EPR studies confirm the reduction of V(V) to V(IV) (Cantley et al,1978a; Kobayashi et al,1978; Adam-Vizi et al,1981), however this reduction is incomplete (Cantley and Kustin,1978; Adam-Vizi et al,1981). For example using ESR techniques, Adam-Vizi et al (1981) reported that 10^{-4} M noradrenaline effected only a 20% reduction of 10^{-4} M V(V). The partial reduction of V(V) may have been due to rapid reoxidation by either O_2 or H_2O_2.

10.2.0 Catecholamines reverse inhibition of Na^+/K^+-ATPase by vanadium

Vanadium(V) inhibits Na^+/K^+-ATPase at concentrations as low as 10^{-8} to 10^{-7} M, catecholamines at concentrations as low as 10^{-4} M reversing this inhibition (Cantley et al,1977; Josephson and Cantley, 1977; Adam-Vizi et al,1981). These processes are best studied in brain tissue. Noradrenaline activates Na^+/K^+-ATPase in different parts of the brain (Schaefer et al,1972; Yoshimura,1973; Godfraind et al,1974; Lee and Phillis,1977; Adam-Vizi et al,1979). Rat cerebral cortex synaptosomal preparations contained 0.16 μM vanadium according to neutron activation analysis (Adam-Vizi et al,1980) - ie. not a sufficient amount to interefere with Na^+/K^+-ATPase activity since the concentration of vanadium required to inhibit Na^+/K^+-ATPase activity by 50% was 4×10^{-6} M for vanadium-free ATP.

Early reports of catecholamine activation of Na^+/K^+-ATPase in vitro (Fagan and Racker,1977; Cheng et al,1977; Hudgins and Bond,1977; Josephson and Cantley,1977; Hudgins and Bond,1978) were explained in terms of a simple reversal of inhibition by vanadium bound to ATP (Josephson and Cantley,1977). More recently, Adam-Vizi et al (1980) suggested that it is possible that the physiological role of noradrenaline is to remove the inhibition of the enzyme by an endogenous inhibitor present in the cytoplasm, an idea suggested by different authors (Hexum,1977; Schaefer et al,1972; Scahefer et al,1973; Schaefer et al,1974). Further support for this suggestion was claimed by Adam-Vizi et al (1980) on the basis of experiments demonstrating that in rat brain homogenates where cytoplasmic factor is present, 10^{-4} M noradrenaline is effective in increasing enzyme activity although contaminating vanadium was not present in the mixture. This stimulation did not occur when synaptosomes where employed. Also, the enzyme acitivity of homogenates was lower than that for synaptosomes, indicating that a cytoplasmic inhibitory factor should be present (Hexum,1977; Schaefer et al,1972; Schaefer et al,1973; Scahefer et al,1974). So noradrenaline even in vanadium-free medium is able to stimulate enzyme activity provided cytoplasmic factor is present. Noradrenaline removes the effect of cytoplasmic factor.

The increase by noradrenaline of enzyme activity in synaptosomal preparations - ie. in the absence of the brain soluble fraction, depends on the presence of an inhibitory contaminant in commercial ATP preparations.

These effects are due to complexation and reduction of V(V) by catecholamines (Cantley and Kustin,1978; Adam-Vizi et al,1981). Thus V(V)-mediated inhibition of the enzyme is reversed by high concentrations of noradrenaline (Josephson and Cantley,1977; Cantley et al,1977; Cantley and Aisen,1979), but significantly, is unaffected by EDTA (Post et al,1979; Josephson and Cantley,1977). Adam-Vizi et al (1983) found noradrenaline alone partly reversed vanadium-mediated inhibition of Na^+/K^+-ATPase activity . The same authors also suggested that noradrenaline was less effective than ascorbate in reducing V(V) to V(IV), and that protection was provided via this reduction. However, V(IV) also inhibits Na^+/K^+-ATPase activity with a similar affinity to V(V), and its effect is also blocked by noradrenaline (Svoboda et al,1984a). Partial reduction of V(V) by noradrenaline not explained by Adam-Vizi et al (1983). Possible interactions between catecholamines and ascorbate have also been explored. Thus the cytoplasmic factor of Adam-Vizi (1980) may be ascorbate, which is also appears to be a physiologically significant inhibitor of brain Na^+/K^+-ATPase (Inagaki,1970; Frey et al,1973; Schaefer et al,1972; Schaefer et al,1974). Catecholamines block lipid peroxidation in brain subcellular fractions and simultaneously protect the Na^+/K^+-ATPase activity against ascorbate-induced inhibition (Shaeffer et al,1975; Svoboda and Mosinger,1981a,b).

10.3.0 Vanadium alters regional levels of catecholamines in brain

The toxic effects of vanadium on the central nervous system are manifest as somnolence, convulsions or respiratory failure (Faulkner-Hudson,1964). Although numerous workers have noted that vanadate is an active oxidant under physiological conditions (Chasteen,1983; Coulombe et al,1985) which may oxidize susceptible biogenic amines, or components of the enzymes involved in the metabolic pathways of these compounds, little or no direct evidence exists in support of this

possibility. Following exposure, levels of vanadate in brain equilibrate slowly with those in blood and concentrations in brain up to half that of blood have been reported 5 days after parenteral V(V) administration (Sharma et al,1980). Subacute and chronic exposure of rats to vanadium results in reduction of brain noradrenaline, and an increase in brain dopamine and 5-hydroxytryptamine levels (Witowska and Brezezinski, 1979). Similarly, dietary V(V) caused a dose-related decrease in noradrenaline and vanillylmandelic acid levels in the hypothalamus (Sharma et al,1986). Hypothalamic dopamine levels also declined significantly, but no effect was observed on dopamine metabolites. Levels of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid were not affected. Levels of dopamine in the corpus striatum (where the highest levels of dopamine are observed) were not affected. Effects of vanadium on various catecholamines and their metabolites in other brain regions (cerebral hemispheres, cerebellum, medulla, midbrain) were marginal. Vanadium has a selective effect on adrenergic pathways, and that effects on other hypothalamic amines appear to be secondary (Sharma et al,1986). The lack of dopamine depletion in striatum (also reported for whole brain by Witowska and Brzezinski,1979) has been interpreted as an indication that the *in vivo* effects of vanadium are selective on noradrenaline only.

10.4.0 Vanadium inhibits catecholamine uptake and release

Vanadium concentrations of 10^{-3} and 10^{-4} M also inhibit [^{3}H] noradrenaline uptake by cerebral cortex slices in vitro (Witowska and Brzezinski,1979). Vanadium (V) causes changes in the uptake and release of [^{3}H] noradrenaline in rat brain cerebral cortex and hypothalamus slices (Witowska and Brzezinski,1983), the inhibition being dependent on both time and vanadium concentration. At low V(V) concentrations (10^{-6} to 10^{-5} M) both processes are stimulated, whereas at higher concentrations (10^{-4} to 10^{-3} M) they are inhibited. Chelation of noradrenaline or autoxidation of noradrenaline by V(V) may contribute to the decrease in [^{3}H]noradrenaline uptake (Witowska and Brzezinski,1983). In the V-mediated inhibition of Na^+/K^+-ATPase, chelation of noradrenaline or V-catalyzed autooxidation of catecholamine might contribute to the

decrease of [^{3}H]noradrenaline uptake (Witowska and Brzezinski,1983). Binding of V(V) to noradrenaline would result in oxidation of the catechol and vanadium binding as vanadyl (VO^{2+}) (Shnaiderman et al,1972; Kustin et al,1974). Vanadium-induced lipid peroxidation may also be important (Donaldson and LaBella,1983). Vanadium enhances lipid peroxidation in rat brain homogenates (Donaldson and LaBella,1983). Lipid peroxidation is an index of cellular deterioration (Tappel,1973), including decreased neurotransmitter receptor binding in rat brain membranes (Muakkassah-Kelly et al,1982). Thus behavioral alterations in animals and humans on exposure to vanadium may reflect disruption of neurotransmission through formation of toxic lipid hydroperoxides (Donaldson and LaBella, 1983). Rates of catecholamine catabolism could also be altered. For example, vanadium also stimulates monoamine oxidase and alters 5-hydroxytryptamine (5-HT) metabolism (Bhagavan and Hom,1983 and references therein).

Nerve terminal Na^+/K^+-ATPase may play a role in the release mechanism of transmitters (Paton et al,1971; Vizi,1972; Gilbert et al,1975; Vizi,1977; Vizi,1978). Experimental conditions known to inhibit membrane ATPase augment transmitter release (Paton et al,1971; Vizi,1972, 1977, 1978; Vizi and Vyskocil,1979; Baker and Crawford,1975; Lorenz et al,1980; Meyer and Cooper,1981; O'Fallon et al,1981; Vyas and Marchbanks,1982), whereas stimulation of the enzyme inhibits release of catecholamines (Vizi,1977). Thus the presynaptic inhibitory effect of catecholamines on transmitter release (Gilbert et al,1975; Vizi,1978) is associated with stimulation of this enzyme (Godfraind et al,1974; Schaefer et al,1972; Schaefer et al,1973; Yoshimura,1973; Schaefer et al,1974; Logan and O'Donovan,1976; Lee and Phillis,1977; Hexum,1977; Wu and Phillis,1978). Exposure of axon terminals to K^+-deficient Krebs solution results in inhibition of membrane ATPase activity and in an increase of transmitter release (Paton et al,1971; Baker and Crawford,1975; Reading and Isbir,1980; Vizi et al,1982). The vanadate inhibition of Na^+/K^+-ATPase is potentiated by K^+ ions acting on extracellular sites (Beauge and Glynn,1978). Intracellular vanadium at a concentration of 10^{-4} M inhibits the uncoupled efflux of Ca(II) (Baker and

Singh,1981, DiPolo and Beauge,1981) and the intracellular accumulation of Ca(II) should also lead to transmitter release (Miledi,1973).

10.5.0 Reactions with catecholamines may underly the cardiotoxicity of vanadium

Vanadium-potentiated adrenaline oxidation may be involved in the cardiotoxic effects of vanadium, these including a general depression of organ function and eventual failure (Venugopal and Luckey,1978). Donaldson and LaBella (1983) suggested that vanadium-potentiated oxidation of adrenaline (as opposed to dopamine) the cardiotoxicity of vanadium could be due at least in part to vanadium actions on adrenergic nerves. Administration of adrenochrome results in cellular damage and contractile failure in isolated perfused rat heart (Yates and Dhalla,1975). Enhancement of adrenochrome formation from epinephrine in the presence of vanadium may thus contribute to cardiotoxicity of vanadium. Effects of vanadium on catecholamine oxidation and potentiation of lipid peroxidation may act together with other effects. Vanadium stimulates adenylate cyclase in heart cells (Grupp et al,1979) and inhibits Na^+/K^+-ATPase (Cantley et al,1977) to produce cardiotoxic effects (Donaldson and LaBella,1983). Such an effect would also complement toxicity arising from other toxic or therapeutic effects of vanadium (Grupp et al,1979). Vanadium(V) also stimulates [^{3}H]noradrenaline release from rabbit pulmonary artery, this effect being inhibited by noradrenaline (Torok et al,1982) and likely related to actions on membrane ATPase (Torok et al,1982). Inhibition of Na^+/K^+-ATPase by removal of K^+ prevented the effects of V(V). The inhibitory effect of noradrenaline on the actions of V(V) was reported to be mediated via alpha$_2$-adrenoreceptors since yohimbine prevented the action, and perhaps ultimately Na^+/K^+-ATPase.

11.0.0 INVOLVEMENT OF LIPID PEROXIDATION IN BIOLOGICAL EFFECTS OF VANADIUM

Perhaps the most obvious index of free radical-mediated damage is peroxidative damage to lipid membranes. The molecular mechanisms by which transition metal ions (including vanadium) initiate and promote lipid peroxidation, and conversely, the degree to which lipid peroxidation contributes to the toxicity of different metals are controversial. It has long been known that vanadium can stimulate lipid oxidation (Bernheim and Bernheim,1939). With regard to the mechanisms underlying vanadium-stimulated lipid peroxidation, useful information may be gained in considering - a) differences in the effectiveness of various vanadium species, b) the effectiveness of vanadium as compared to other metals, c) modulation of vanadium-mediated lipid peroxidation by reducing agents, d) the effects of free radical scavengers and or metal chelating agents, e) the relative susceptibility of different tissues, and f) evidence for or against the involvement of lipid peroxidation in specific biological effects of vanadium.

11.1.0 Susceptibility of different tissues to vanadium-mediated lipid peroxidation

Significant differences exist among different tissues with regard to their susceptibility to vanadium-induced lipid peroxidation. Vanadium (V) (as sodium metavanadate or vanadium acetate) significantly increases O_2 uptake by rat or guinea pig liver suspensions at pH 6.7, while O_2 uptake by kidney suspensions is only slightly increased, and no increase in O_2 uptake is observed for brain suspensions (Bernheim and Bernheim,1939). In an *in vitro* study, Donaldson and LaBella (1983) found that absolute levels of malondialdehyde (MDA) formation from mouse tissue homogenates decreased in the order - brain > heart > lung > kidney > liver, however, the extent to which vanadium increased MDA formation over control reactions decreased in the order liver (2.4-fold), brain (1.58-fold), heart (1.42-fold), kidney (1.39-fold) and lung (1.28-fold). In contrast, in a later *in vivo* study (Donaldson et al,1985), it was reported that while acute intraperitoneal injection of $NaVO_3$ significant increased in kidney MDA levels in both rats and mice, no differences in MDA levels between control and V-treated groups were found in brain, heart, lung or liver.

Similarly, subcutaneous injection (40 mg/kg) and chronic (10 weeks) exposure of rats to $VOSO_4$ initially in maternal milk and later in drinking water (500 μmol/L) resulted in increased MDA formation only in kidney. Following iv administration of $NaVO_3$ (6 mg/kg), measurements of TBA reactive material in liver, kidney, lung and brain tissues 3 hrs after treatment revealed high variability and no large differences between untreated controls and experimental groups (Siegers et al,1986). Incubation of human erythrocytes with either $VOSO_4$ or $NaVO_3$ results in MDA formation (Hansen et al,1986). At the subcellular level, MDA formation in the presence of V_2O_5 or $NaVO_3$ alone was greater in rat lung lipid extracts and liver microsomes than in liver mitochondria (Inouye et al,1980). Vanadium (IV)- and V(V)-induced lipid peroxidation is greater in intact as compared to rat liver mitochondria, perhaps as a result of interactions of vanadium with intramitochondrial components (Liochev et al,1988). Although it is clear that vanadium as V(V) or V(IV) induces lipid peroxidation in a number of different tissues, the question remains whether or not vanadium at the levels normally observed in tissues promotes lipid peroxidation to a significant degree. Differences in the effectiveness of V(V) as oposed to V(IV) in inducing lipid peroxidation are important in this regard.

11.2.0 Differences in the effectiveness of various vanadium species

Most studies of vanadium-mediated lipid peroxidation have examined the effects of V(V). Vanadium (V) as sodium metavanadate or vanadium acetate increases O_2 uptake by rat or guinea pig liver suspensions (Bernheim and Bernheim,1939). Malondialdehyde production from rat lung lipid extracts, liver microsomes and liver mitochondria is increased by $NaVO_3$ (Inouye et al,1980). The formation of TBA-reactive substances is also increased in isolated rat hepatocytes incubated with sodium vanadate (Stacey and Klaasen,1980), in mouse lung, kidney, heart, liver and mouse or rat brain homogenates incubated with Na_3VO_4 (Donaldson and Labella,1983). Expired ethane is increased in rats following intraperitoneal administration of $NaVO_3$ (Harvey and Klaasen,1983). Time- and concentration-dependent formation of TBA-chromogens in liver homogenates of

phenobarbital-treated rats were observed by Younes et al (1984), following addition of sodium vanadate (100 μmol/L). No increase in TBA-reactive compounds was observed by Vijaya et al (1984) following incubation of rate erythrocyte membranes with decavanadate and NADH. Malondialdehyde production is also increased in the kidneys of both mice and rats following subutaneous or intra-peritoneal administration of $NaVO_3$ (Donaldson et al,1985). Similarly, in rats, administration of V(IV) as vanadyl sulfate (initially via maternal milk , then drinking water) results in increased lipid peroxidation in kidney (Donaldson et al,1985). Increased formation of TBA-reactive compounds was also observed in the livers of rat pups from dams fed diets containing $NaVO_3$ (100-1000 ppm), and the livers of adult rats administered $NaVO_3$ (1 or 5 mg/kg) by injection (Elfant and Keen,1985). In mice, intravenous injection of $NaVO_3$ (6 mg/kg) did not increase ethane production or the formation of TBA reactive material in tissues after 3 hours (Siegers et al,1986). Vanadium V(V) also reportedly amplifies peroxidative damage resulting from concomitant exposure of human erythrocytes to iodoacetate and ferricyanide (Heller et al,1987).

Direct comparisons of the effects of V(IV) and V(V) on lipid peroxidation have been made in the studies of Inouye et al (1980), Svoboda et al (1984) and Liochev et al (1988). Vanadium (IV) (as V_2O_5) and V(V) (as $NaVO_3$) induced lipid peroxidation in lung lipid extracts, liver microsomes and liver mitochondria to approximately the same extent (Inouye et al,1980). In contrast, Svoboda et al (1984) found V(IV) ($VOSO_4$) to be 5-6 times more effective than V(V) ($NaVO_3$) in inducing lipid peroxidation in rat brain microsomal preparations. Vanadium (IV) induces MDA formation (and hemolysis) in human erythrocytes much more rapidly than (Hansen et al,1986). Identical amounts of $VOSO_4$ and $NaVO_3$ produced 70% hemolysis over a 3 hour period and 20% over 24 hours respectively. Vanadium (IV) is more effective than V(V) in inducing lipid peroxidation (as measured by formation of TBA-reactive materials) in intact and sonicated mitochondria, as well as in phosphatidylcholine suspensions (Liochev et al,1988). Although V(IV) is clearly much more potent than V(V) in inducing lipid peroxidation when large doses of the metal ions are administered *in vitro* or *in vivo*, important questions arise in considering the effectiveness

of vanadium as compared to other metal ions, and the modulating effects of reducing agents on vanadium-mediated lipid peroxidation.

11.3.0 Vanadium is more effective than most other metals in inducing lipid peroxidation

In considering the relative effectiveness of vanadium as compared to other metals in inducing lipid peroxidation, two questions must be addressed - (1) the effectiveness of the metal alone, and (2) the effectiveness of the metal in the presence of reducing agents. With regard to the effectiveness of vanadium alone, Stacey and Klaassen (1980) examined the abilities of a variety of transition and non-transition metal ions including Cd(II), Cr(VI), Cu(II), Fe(III), Hg(II), Mn(II), Ni(II), Pb(II), Se(II), V(V) and Zn(II) to induce lipid peroxidation in isolated rat hepatocytes. At metal concentrations of from 1 to 1000 μM and for incubation times of 20 and 45 minutes, V(V) was found to be the most effective of the metals tested, followed by Fe(III), Hg(II), Cd(II) and Se(II). A smaller number of metals were compared by Inouye et al (1980) with regard to their capacities to induce lipid peroxidation in rat tissues, specifically lung lipid extracts, liver microsomal and liver mitochondrial preparations. In, lung lipid extracts, the approximate order of effectiveness was Fe(III) > Fe(II) = V_2O_5 = $NaVO_3$ (Ni(II), Zn(II) and Mn(II) increasing MDA formation only slightly in comparison to iron or vanadium), while in liver microsome preparations, Fe(II) > Fe(III) > Zn(II) > V_2O_5 > $NaVO_3$ > Ni(II) = Mn(II), whereas for liver mitochondria Fe(III) > Fe(II) > Zn(II) > V_2O_5 > $NaVO_3$ > Ni(II) > Mn(II). Addition of sulfite increased the effectiveness of iron and vanadium to the greatest extent in lung lipid extract preparations, Fe(III) > V(V) being slightly more effective than Fe(II) = V(IV) and the other metal ions tested demonstrating only small increases. In liver microsomal and mitochondrial preparations, vanadium but not iron increased MDA formation in the presence of sulfite in a more than additive fashion. In the case of microsomal preparations, the order of effectiveness for different metal ions in the presence of sulfite was Fe(II) > Fe(III) > V(IV) > V(V) > Zn(II) > Ni(II), while Mn(II) inhibited as compared to sulfite alone. Similarly, for liver mitochondria

preparations, Fe(III) = Fe(II) > V(IV) > V(V) > Ni(II) > Zn(II) and Mn(II) again inhibited. In rats *in vivo*, administration of Fe(II) or V(V) but not $CdCl_2$ increased levels of expired ethane, with V(V) alone being slightly more effective than Fe(II) alone (Harvey and Klaasen,1983). In mice, ethane production following intravenous administration of $CdCl_2$, $HgCl_2$ or $NaVO_3$ was not significantly altered 3 hours after exposure, and levels of TBA-reactive material in liver, kidney, lung and brain did not differ significantly from controls (Siegers et al,1986).

11.4.0 Reducing agents modulate vanadium-mediated lipid peroxidation

Reducing agents may modulate vanadium-mediated lipid peroxidation in either a positive or negative manner. Sulfite and vanadium act synergistically in accelerating MDA formation from rat tissues, including lung lipid extracts, liver microsomes and liver mitochondria (Inouye et al,1980). Interestingly, very small differences are observed between reactions involving V_2O_5 and $NaVO_3$ for lung lipid extracts and liver microsomes, while for liver mitochondria preparations somewhat larger, (although still small) differences are observed. Little if any additional information is available concerning the effects of reducing agents on V(V)- as opposed to V(IV)-mediated lipid peroxidation. In rats, lipid peroxidation (as measured by expired ethane is increased) resulting from dietary exposure to $NaVO_3$ is significantly increased when administered 30 minutes after 0.1 ml CCl_4/kg (Harvey and Klaasen,1983), presumably as a result of increased redox cycling of the metal in the presence of CCl_4. Although V(V) alone was slightly more effective than Fe(II) alone, in the presence of CCl_4 the reverse was true, while $CdCl_2$ did not potentiate lipid peroxidation in the presence or absence of CCl_4 (Harvey and Klaasen,1983). Interestingly, the effects of Fe(II) in combination with CCl_4 were more than additive, but those of V(V) and CCl_4 (in contrast to the results of Inouye et al (1980)) merely additive. However, the addition of ascorbate to rat brain microsomes reportedly stimulates lipid peroxidation to a maximum extent whether vanadium is present or not (Svoboda et al,1984). On the other hand, ascorbate administration by subcutaneous injection *decreases* MDA formation in the kidneys of mice exposed to Na_3VO_4 by the same route

(Donaldson et al,1984). Ascorbate, NADH and glucose (preincubated with V(V)) all act synergistically to increase V(V)-mediated peroxidation of phosphatidylcholine (Liochev et al,1988).

11.5.0 Effects of free radical scavengers on vanadium-stimulated lipid peroxidation

The effects of nonenzymatic or enzymatic free radical scavengers on vanadium-mediated lipid peroxidation merit exploration. Lipid peroxidation induced by sulfite and vanadium is inhibited by superoxide dismutase (72.5 U/ml), alpha-tocopherol (10 μM), hydroquinone (1 mM), 1% ethanol or 10 μM cepharanthine (Inouye et al,1980). The extent of inhibition was dependent on the tissue preparation employed. Thus, for lung lipid extracts, ethanol protected by only about 27%, whereas the other scavengers protected by approximately 98 - 94%. All the scavengers were effective in reactions containing microsomes (97.5- 83.8%), but alpha-tocopherol, hydroquinone and cepharanthine (a membrane stabilizing agent) were significantly more protective (97.4 - 94.4 %) than either ethanol (64.3%) or superoxide dismutase (41.6%). Unfortunately, since no inactive protein controls were included for reactions involving superoxide dismutase, the nature of the inhibitory effects of superoxide dismutase are difficult to assess. Butylated hydroxyanisole, butylated hydroxytoluene and propyl gallate administered in safflower oil at 200 mg/kg as antidotes for V(IV) (110 mg/kg) and V(V) (50 mg/kg) gave results (in survivors/total) of 1, 2, 0 and 5, 7, 1 respectively (Jones and Basinger, 1983). Pretreatment with ascorbate decreased MDA formation in the kidneys of mice following intraperitoneal administration of Na_3VO_4 (Donaldson et al,1985). Similarly, butylated hydroxytoluene, thiourea or diethyldithiocarbamate decreased lipid peroxidation (as measured by changes in membrane integrity, the loss of membrane polyunsaturated fatty acids and or formation of fluorescent chromolipids) in human erythrocytes exposed to the combination of V(V), iodoacetate and ferricyanide (Heller et al,1987). BHT, but not superoxide dismutase, catalase or ethanol inhibits V(IV)-induced peroxidation of sonicated rat liver mitochondria and phosphatidylcholine suspensions (Liochev et al,1988).

11.6.0 Effects of proteins and metal chelating agents

An important area for future studies is the effects of proteins and metal chelating agents on vanadium-mediated lipid peroxidation. For example, while vanadium alone had no virtually no effect on phospholipid oxidation, mixtures of vanadium and washed liver protein were very effective in catalyzing the reaction (Bernheim and Bernheim,1939). On the basis of these results, it was suggested that vanadium-protein complexes are normally the active agents in vanadium-mediated lipid peroxidation. In addition, pyrophosphate (0.25-0.5%) inhibits the oxidation of soy bean lecithin by mixtures of vanadium and washed liver proteins. Thus, vanadium-phosphate complexes may be much less active than the metal alone in catalyzing lipid peroxidation. Further, vanadium in tissues may not be available to metal chelating agents. In the study of Svoboda et al (1984), EDTA-washed rat brain microsomes were as susceptible as untreated preparations to the peroxidative effects of ascorbate. Microsomes preincubated with 1.0 mM EDTA, then washed once showed lower MDA formation in the presence of ascorbate, but after three washings, no difference in treated and untreated preparations were apparent. Thus the authors suggested that the endogenous metals (iron and vanadium) responsible for ascorbate-mediated lipid peroxidation may be tightly bound and so unavailable for chelation by EDTA (Svoboda et al,1984). DTPA is more effective than desferrioxamine in inhibiting $VOSO_4$-mediated hemolysis of human erythrocytes since V-desferrioxamine complexes apparently still possess hemolytic activity (Hansen et al,1986). In contrast, neither chelating agent demonstrated an effect on hemolysis by $NaVO_3$. Desferrioxamine or EDTA inhibit hemolysis (presumably due at least in part to V-stimulated lipid peroxidation) of human erythrocytes exposed to V(V), iodoacetate and ferricyanide, however this inhibition may also be due to decreased uptake of V(V) by the cells as a result of complex formation between V(V) and desferrioxamine or EDTA (Heller et al,1987).

11.7.0 Involvement of lipid peroxidation in specific biological effects of vanadium

The extent to which lipid peroxidation contributes to specific biological effects of vanadium is controversial. Clearly, this question is central to our understand the involvement of free radicals in the biological actions of vanadium. Although no direct evidence was presented, the increased hemolysis observed in erythrocytes incubated with vanadium, or vanadium and sulfite presumably reflected deterioration of the erythrocyte membrane (Inouye et al,1980). $VOSO_4$ induces hemolysis in human erythrocytes more rapidly than $NaVO_3$ (Hansen et al,1986), lipid peroxidation apparently contributing to hemolysis since MDA formation was observed in both cases. Lipid peroxidation (as measured by changes in membrane integrity and the loss of membrane polyunsaturated fatty acids) was also suggested as underlying damage to the membranes of human erythrocytes exposed to V(V), iodoacetate and ferricyanide (Heller et al,1987). Vanadium-mediated lipid peroxidation may also underly the insulin-like effects of the metal (Liochev et al,1988). Both vanadium and copper induce lipid peroxidation in mitochondria (Ivancheva and Russanov,1981; Liochev et al,1988) and phosphatidylcholine suspensions (Gutteridge,1977; Liochev et al,1988), and exert insulin-like effects (Czech and Fain,1972; Kadota et al,1987).

Erythrocytes might be expected to be relatively resistant to peroxidative damage by virtue of their membrane structure and antioxidant defenses, while other tissues (in particular brain, liver and kidney) would be more susceptible (Donaldson and LaBella,1983). Yet (as noted in section 11.1.0), since significant MDA formation was observed only 0.1 - 1.0 mM V(IV). These concentrations are approximately two orders of magnitude higher than those which effectively inhibit brain microsomal Na^+/K^+-ATPase. Vanadium(V) was as effective as V(IV) with regard to inhibition of brain microsomal Na^+/K^+-ATPase). For these reasons, Svoboda et al (1984) concluded that given the range of vanadium concentrations reported for brain tissue, vanadium-mediated lipid peroxidation in vivo is likely not significant (Svoboda et al,1984). Similarly, while V(V) induces lipid peroxidation in isolated rat hepatocytes *in vitro* (Stacey and Klaassen,1980).

However although ethane production is increased in rats exposed to V(V), no parallel increase in liver damage is observed (Harvey and Klaassen,1983). Although the lack of liver damage may have been due to the experimental conditions employed (ie. the extent of lipid peroxidation may not have been great enough), it was concluded that the cytotoxicity produced by V(V) is not due to lipid peroxidation.

On the other hand, lipid peroxidation in mice exposed to Na_3VO_4 was observed within 1 hr postinjection and was sustained throughout a 24 hr period of observation (Donaldson et al,1985). Since tubular necrosis occcurs within approximately 6 - 7 hours in the kidneys of mice exposed to V(V) (Cheng-i-Wei et al, 1982), and MDA is apparently involved in the pathogenesis of renal failure (Kishore et al (1983), it was suggested that the nephrotoxic effects of vanadium may be due at least in part to lipid peroxidation (Donaldson et al,1985). Yet, following intravenous administration of $NaVO_3$ (6 mg/kg). measurements of TBA-reactive materials in liver, kidney, lung and brain tissues 3 hrs after treatment were highly variable and no large differences between controls and any of the treatment groups were observed. Therefore, Siegers et al (1986) concluded that lipid peroxidation was not a mechanism of vanadate hepatotoxicity *in vivo*.

With the exception of the nephrotoxicity of vandium, it would be premature to conclude that lipid peroxidation is involved in either the toxic or pharmacologic effects of vanadium *in vivo*. Caution is warranted in extrapolating from *in vitro* studies to *in vivo* situations. At the same time, since the processes underlying lipid peroxidation are so complicated and susceptible to modulation by a large number of factors, negative results obtained either in vitro or in vivo do not necessarily rule out the involvement of lipid peroxidation in specific biological effects. Since vanadium has been reported to interfere with the TBA assay (Heller et al,1987), more detailed and systematic studies using more sensitive assay procedures are required in order to resolve these questions.

12.0.0 CHELATING AGENTS ARE RELATIVELY INEFFECTIVE AGAINST VANADIUM TOXICITY

12.1.0 Effects of metal chelating agents on tissue distributions of vanadium

The structure of vanadium complexes with various metal chelating agents deserve more detailed study. The best characterized complexes are those of V(V) with ethylenediaminetetraacetic acid (EDTA) (Post et al,1979; Chasteen,1981), and of V(IV) with diethylenetriaminepentaacetic acid (DTPA), EDTA and ? (EGTA) (Smith et al,1974; Post et al,1979). No information is available on complexes of V(IV) with desferioxamine, but the stability constant of the cationic form of V(V) (*cis*-VO^{2+}) with desferal is 6.09 (Luterotti and Grdinic,1986). In the presence of metal chelators such as EDTA and desferrioxamine, complexes containing the cis-VO^{2+} are also formed (Amos and Sawyer,1972; Luterotti and Grdinic,1986). Early reports of the effects of metal chelating agents on vanadium poisoning involved only one or two chelating agents. For example, Lusky et al (1949) found that dimercaptopropanol (BAL) was ineffective against vanadium toxicity in experimental animals. Rabbits exposed to vanadium, then treated with BAL demonstrated a significantly higher mortality than those exposed to vanadium alone, while the use of BAL in similar human therapeutic trials were inconclusive (Sjoberg ,1950). The use of EDTA (Mitchell,1953) and its comparison with ascorbate (Mitchell and Floyd,1954) showed that both were effective and that ascorbate acted sooner. Disodium catechol sulfonate or Tiron also ameliorated vanadium toxicity in rabbits, rats and pigeons (Braun and Luskey,1959).

More recently, desferrioxamine and DTPA were shown to remove injected V(IV) and to a lesser extent V(V) in rats (Hansen and Aaseth,1981). After a single injection of $^{48}VOSO_4$, the organs with the highest levels of V were kidneys, spleen, bone and liver (Hansen and Asseth,1981). Immediate treatment with DTPA or DES (100 μmol/kg) decreased the kidney and liver levels to approximately 60% of control values. A similar decrease was obtained in testes, lungs, brain and blood plasma. The vanadium levels in erythrocytes were, however, unchanged by chelation treatments. The immediately injected desferrioxamine increased urinary excretion, and DTPA fecal

excretion (Hansen and Asseth,1981). Desferrioxamine (30 μmol/kg) administered 24 hours after $VOSO_4$ decreased levels of the metal in kidneys, liver and blood plasma to about 75% that of the control levels. Desferrioxamine was slightly more effective than DTPA. Significant mobilization of vanadium from spleen, bones or kidney was not observed at a dose of 30 μM/kg desferrioxamine, but at 100 and 300 μmol/kg, desferrioxamine diminished reduced kidney and liver levels to 75 and 60% of control levels. At 300 μmol/kg DTPA was ineffective in reducing renal vanadium, perhaps due to its nephrotoxic effect (Hansen and Asseth,1981). *In vitro*, desferrioxamine also removed VO^{2+} from transferrin.

In a more detailed study (Hansen et al,1982), the effects of desferrioxamine or DTPA on tissue vanadium concentrations in rats given intraperitoneal injections of $Na^{48}VO_3$ administered 24 hours previously were dependent upon the dose of the chelator and the tissue examined. At a dosage level of 30 μmol/kg, DES decreased kidney, liver and lung vanadium concentrations by 17%, 0% and 7%, while DTPA caused decreases of 7%, 15% and 0%. At a dosage of 100 μmol/kg, DES caused decreases of 20%, 26% and 25%, and DTPA decreases of 9%, 18% and 25% in the three organs respectively. Not only were both chelating agents were more effective towards $VOSO_4$ than $NaVO_3$ for all tissues of interest, but vanadium retention by spleen and bone appeared to be greater than the other organs examined. Desferrioxamine, EDTA, DTPA and nitrilotriacetic acid were also shown to inhibit uptake of $Na^{48}VO_3$ and $^{48}VOSO_4$ by human erythrocytes, even producing small decreases in intracellular vanadium concentrations (Hansen et al,1982).

12.2.0 Comparative effectiveness of metal chelating agents as compared to reducing agents in countering vanadium toxicity

The comparative effectiveness of a chelating agents and reducing agents, including ascorbate, desferrioxamine, d-penicillamine, DTPA, EDTA, glutathione, Tiron and ethylenediaminetetra-(methylene phosphonate) against the lethality of intraperitoneally injected V(IV) or V(V) in

adult male mice was studied by Jones and Basinger (1983) (Fig. 10.0). The chelating agents were administered 20 minutes after injection of vanadium. The concentration of V(IV) administered (110 mg/kg) was twice that for V(V) since the LD50 values for V(IV) (as $VOSO_4$) and V(V) (as Na_3VO_4) were found to be 95.4 mg/kg and 36.3 mg/kg respectively. Using a ratio of 5:1 (chelating agent: vanadium), for V(IV) the order of effectiveness as indicated by the number of survivors/total was - EDTA (10), d-penicillamine (9), Tiron (9), glutathione (8), sodium pyrogallosulfonate (8), ascorbateC (7), dimercaptosuccinic acid (1) and dimercaptopropane sulfonate (1). At 400 mg/kg, desferrioxamine and DTPA were equally effective (7).
For V(V) the order was ascorbate (10), desferrioxamine (10), EDTA(9), Tiron (9), dimercaptosuccinic acid (9), dPA (8), glutathione (6), dimercaptopropane sulfonate (6), DTPA (6), sodium pyrogallosulfonate (0). An important distinction was made between the abilities of different compounds to form chelates and act as reductants, the suggestion being made that reductants decrease vanadium toxicity by reducing V(V) to V(IV). Thus since V(IV) was administered at a much higher dose than V(V), any compound effective against both V(IV) and V(V) was probably functioning as both a chelating agent (for V(IV)) and a reducing agent (for V(V)). For example glutathione was slightly more effective for V(IV), perhaps indicating complex formation. The data on the dithiols sodium 2,3-dimercaptopropane-1-sulfonate and dimercaptosuccinic acid indicated that they are good reducing agents but weak chelators since they are only effective against V(V) (Jones and Basinger,1983).

In a similar study, Domingo et al (1985) compared the effectiveness of a smaller number of metal chelating agents and reducing agents, in terms of their antidotal effectiveness against the lethality of single intraperitoneal injections (0.33 mmol/kg) of V(V) (as $NaVO_3$) in adult male mice (Fig. 10.0). Chelating agents were administered intraperitoneally immediately after vanadium injection. The percent mortality in the control group was 40%. At a 5:1 ratio (chelating agent:vanadium), the percent mortality was - desferrioxamine (0) < Tiron (10) < EDTA (20) = DTPA (20) = l-cysteine (20) < d-penicillamine (30) < ascorbate (50) = dimercaptosuccinic acid (50) <

sodium salicylate (80) < diethyldithiocarbamate. The latter four agents actually increased mortality then. At a 10:1 At a 10:1 chelating agent:vanadium ratio, Tiron (0) < DES (10) = ascorbate (10) = cysteine (10) < EDTA (20) = DTPA (20) < dimercaptosuccinic acid (70) < sodium salicylate (80) < diethyldithiocarbamate (90) < d-penicillamine (100). Determination of the therapeutic indices for each compound (defined as the LD_{50} of each compound divided by its ED_{50}, where the ED_{50} is defined as the amount of compound in mmol/kg which protects 50% of the animals against the lethal effects of 0.61 mmol $NaVO_3$/kg) revealed that at this dosage level of vanadium, the percent mortality and therapeutic indices were: EDTA (95, < 5.3), DTPA (100, < 3.8), ascorbate (30, 95.2), l-cysteine (65, < 3.8), Tiron (5, 20.5), DES (30, 7.8). Ascorbate was suggested as being the best antidote for treatment of vanadium toxicity on the basis of its low ED_{50} and low toxicity.

One important feature of vanadium chelation by desferrioxamine and EDTA, among others, is the formation of complexes containing the *cis*-VO^{2+} are also formed (Amos and Sawyer,1972; Luterotti and Grdinic,1986). While both EDTA and desferrioxamine can be oxidized by vanadium under appropriate conditions, the extent to which this may occur *in vivo* is not known (Jones and Basinger,1983). Some of the conflicting reports concerning the effectiveness of chelating agents against vanadium-mediated phenomena may be due to the retention of catalytic activity by these vanadium chelates. Thus, V(V)-mediated inhibition of Na^+/K^+-ATPase is not reversed by EDTA (Josephson and Cantley,1977; Post et al,1979). Although EDTA eliminated the V(IV)-mediated inhibition of Na^+/K^+-ATPase under the experimental conditions employed by Post et al (1979), Svoboda et al (1984) found that EDTA did not eliminate V(IV)-mediated inhibition, likely as a result of the presence of an excess of Mg(II) (Post et al,1979; Svoboda et al,1984). In addition, V(IV) complexes with DES but not DTPA differ little from V(IV) alone in their ability to promote hemolysis of human erythrocytes (Hansen et al,1986). Complexes of V(IV) or V(V) with desferrioxamine are in many respects more reactive than the hydrated ions (Wu,1987).

D. SUMMARY

Clearly, reactions of vanadium with molecular O_2, reduced O_2 species or biological reducing agents differ from those of other physiologically and or toxicologically significant transition metal ions. The most unique aspects of the redox chemistry of vanadium are related to the presence of bound O_2 and its ability to form relatively stable complexed radical species in reactions with reduced O_2 species, in particular H_2O_2. For example, V(IV) alone effectively reduces O_2 at physiological pH and may catalyze OH· formation from H_2O_2 via Fenton mechanisms. However, in the presence of the high concentrations of ligands and reducing agents, reactions of V(IV) with both O_2 and H_2O_2 are inhibited, especially at the low pH and O_2 partial pressures found intracellularly. Further, decomposition of H_2O_2 to OH· may not be quantitatively important in reactions of V(IV) with H_2O_2 based on standard assays for OH· radicals (deoxyribose-TBA and DMSO assays). On the other hand, V(V) can react with O_2^-, and more importantly H_2O_2, to form peroxo complexes during the air autoxidation of V(IV). In the presence of biological reducing agents, these peroxo complexes may be important since they are stable enough to explore the cellular environment, but reactive enoug to yield a variety of radical products upon decomposition. Important topics to be addressed by future research include: (1) a detailed analysis of the mechanisms underlying reactions of V(IV) with H_2O_2, (2) determination of the effects of physiological ligands on the reactivity of both V(IV) and V(V) towards H_2O_2 and reducing agents, (3) analysis of the ability of free and bound V(IV) and V(V) to modify essential cellular components (enzymes, structural proteins, membranes) in the presence and absence of H_2O_2, (4) determination of the amplification mechanisms by which vanadium may act to exert its pharmacologic and toxic effects, and 5) the effect of redox state and redox reactions of vanadium on its ability to form biologically active esters of cellular regulatory molecules.

Figure 1.0

Reactions of vanadium species with oxygen and or reduced oxygen species

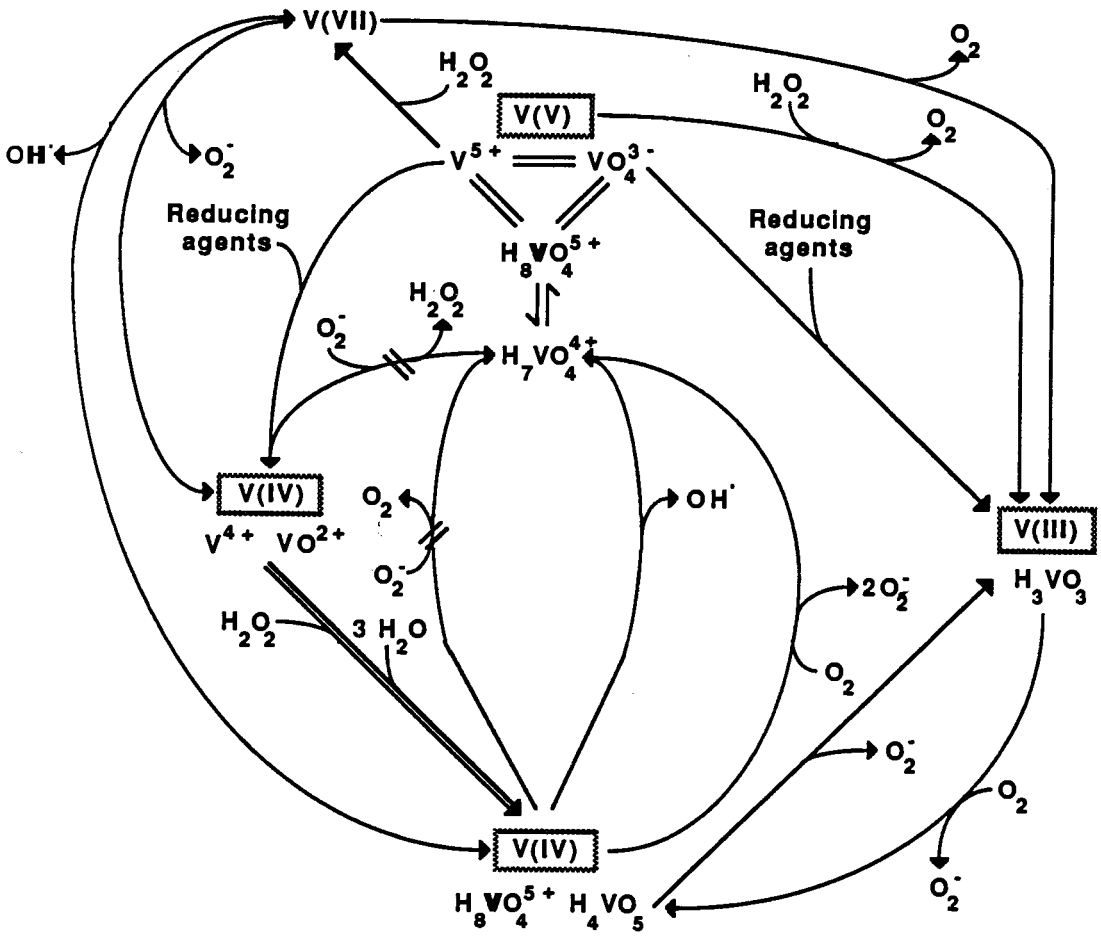


Figure 2.0

Oxygen consumption by Fe(II), V(III) or V(IV): effects of buffer type

Changes in $[O_2]$ for reactions involving 250 μM $FeSO_4$ (Fischer Scientific Co.), $VOCl_3$ or $VOSO_4$ (Aldrich Chemical Co.) were measured polarographically by means of a Clark-type electrode equipped with a magnetic stirrer and a water jacket and connected to a chart recorder. All stock solutions and buffer were made using dionized, distilled water (specific resistivity < 10 Mohm cm^{-3}). Individual reactions were conducted in both potassium phosphate (BDH Chemicals) and HEPES (Sigma Chemical Co.) buffers (air saturated, 50 mM, pH 7.0, 20° C, final volume 5.0 ml). All reactions were initiated by addition of the metal ion.

Figure 2.0

Oxygen consumption by Fe(II), V(III) or V(IV): effects of buffer type

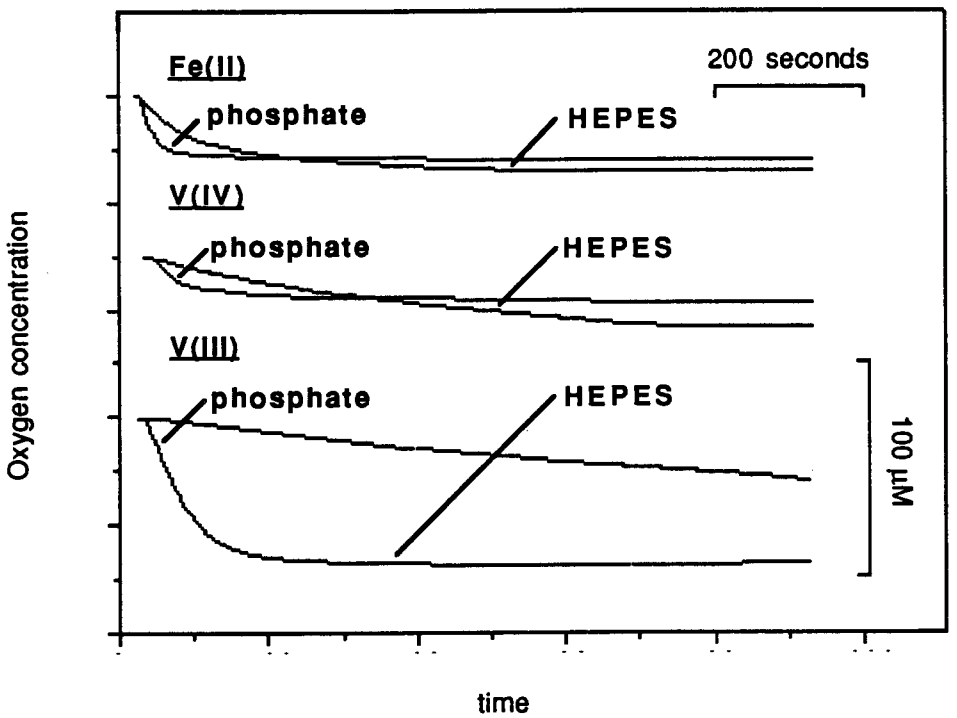


Figure 3.0

Oxygen consumption by V(III): effects of pH and ligands

Data replotted from Wu (1987). Changes in [O_2] for reactions involving 1.0 mM $VOCl_3$ (Aldrich Chemical Co.) alone or in the presence of 1.0 mM ATP (adenosine 5'-triphosphate; Sigma Chemical Co.), histidine (l-histidine hydrochloride monohydrate, HIS; Fisher Scientific Co.) , EDTA (disodium ethylenediaminetetraacetic acid; Fisher Scientific Co.), or DES (desferal mesylate, desferrioxamine; Ciba-Geigy Corp.), or 600 μg/ml bovine serum albumin (BSA; Sigma Chemical Co., fraction V, final concentration 600 μg/ml) were measured polarographically by means of a Clark-type electrode equipped with a magnetic stirrer and a water jacket, and connected to a chart recorder. Reactions were conducted in 50 mM MES (2[N-morpholino]-ethanesulfonic acid; Sigma Chemical Co.) (pH 6.0) or HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid; Sigma Chemical Co.) buffer ((pH 7.0, 7.4, air saturated, 25° C). The total volume was 3.0 ml. All reactions were initiated by addition of metal ion.

Figure 3.0

Oxygen consumption by V(III): effects of pH and ligands

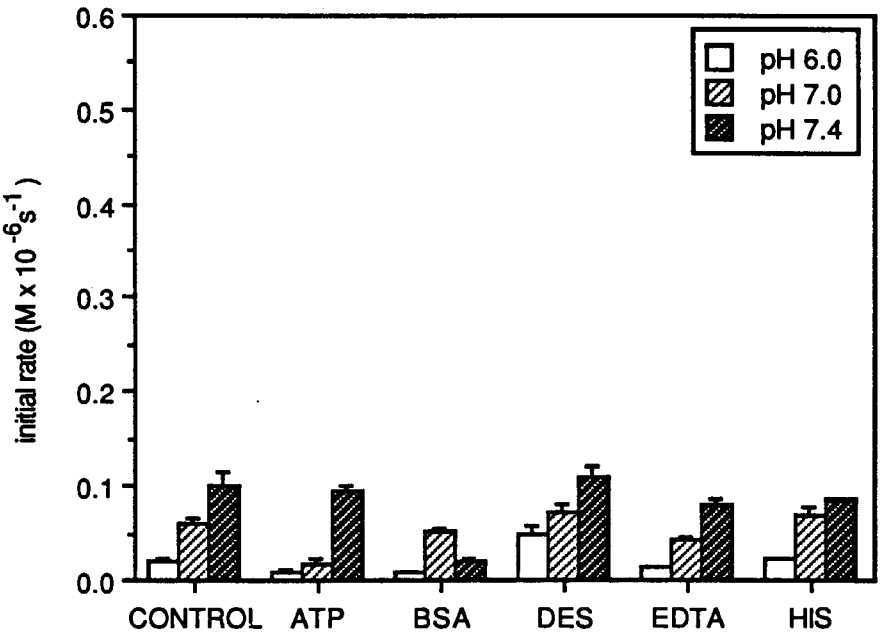


Figure 4.0

Oxygen consumption by V(IV): effects of pH and ligands

Data replotted from Wu (1987). Changes in [O_2] for reactions involving 1.0 mM $VOSO_4$ (Aldrich Chemical Co.) alone or in the presence of 1.0 mM ATP (adenosine 5'-triphosphate; Sigma Chemical Co.), histidine (l-histidine hydrochloride monohydrate, HIS; Fisher Scientific Co.) , EDTA (disodium ethylenediamine-tetraacetic acid; Fisher Scientific Co.), or desferrioxamine (desferal mesylate, DES; Ciba-Geigy Corp.), or 600 μg/ml bovine serum albumin (BSA; Sigma Chemical Co., fraction V) were measured polarographically by means of a Clark-type electrode equipped with a magnetic stirrer and a water jacket, and connected to a chart recorder. Reactions were conducted in 50 mM MES (2[N-morpholino]-ethanesulfonic acid; Sigma Chemical Co.) (pH 6.0) or HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Sigma Chemical Co.) (pH 7.0, 7.4) buffer (air saturated, 25° C). The total volume was 3.0 ml. All reactions were initiated by addition of metal ion.

Figure 4.0

Oxygen consumption by V(IV): effects of pH and ligands

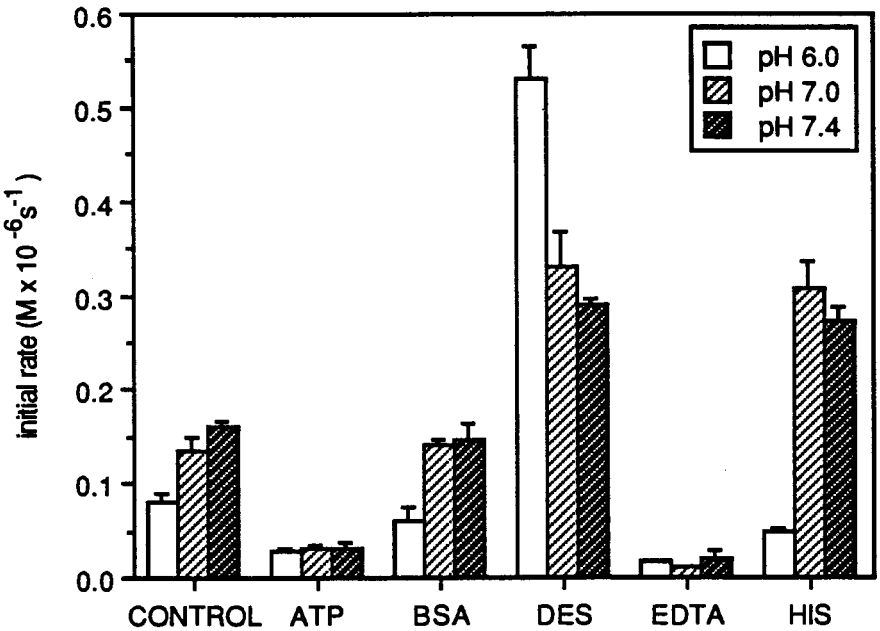


Figure 5.0

Oxygen consumption/hydrogen peroxide decomposition by V(IV): effects of hydrogen peroxide concentration and buffer type

Changes in $[O_2]$ for reactions involving 250 μM $VOSO_4$ (Aldrich Chemical Co.) alone or in the presence of equimolar or 10-fold excess H_2O_2 (BDH Chemicals) were measured polarographically by means of a Clark-type electrode (equipped with a magnetic stirrer and a water jacket) connected to a chart recorder. Individual reactions were conducted in both potassium phosphate (BDH Chemicals) and HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid; Sigma Chemical Co.) buffers (50 mM, pH 7.0, air saturated, 50 mM, 20° C). All reactions were initiated by addition of the metal ion.

Figure 5.0

Oxygen consumption/hydrogen peroxide decomposition by V(IV): effects of buffer type and hydrogen peroxide concentration

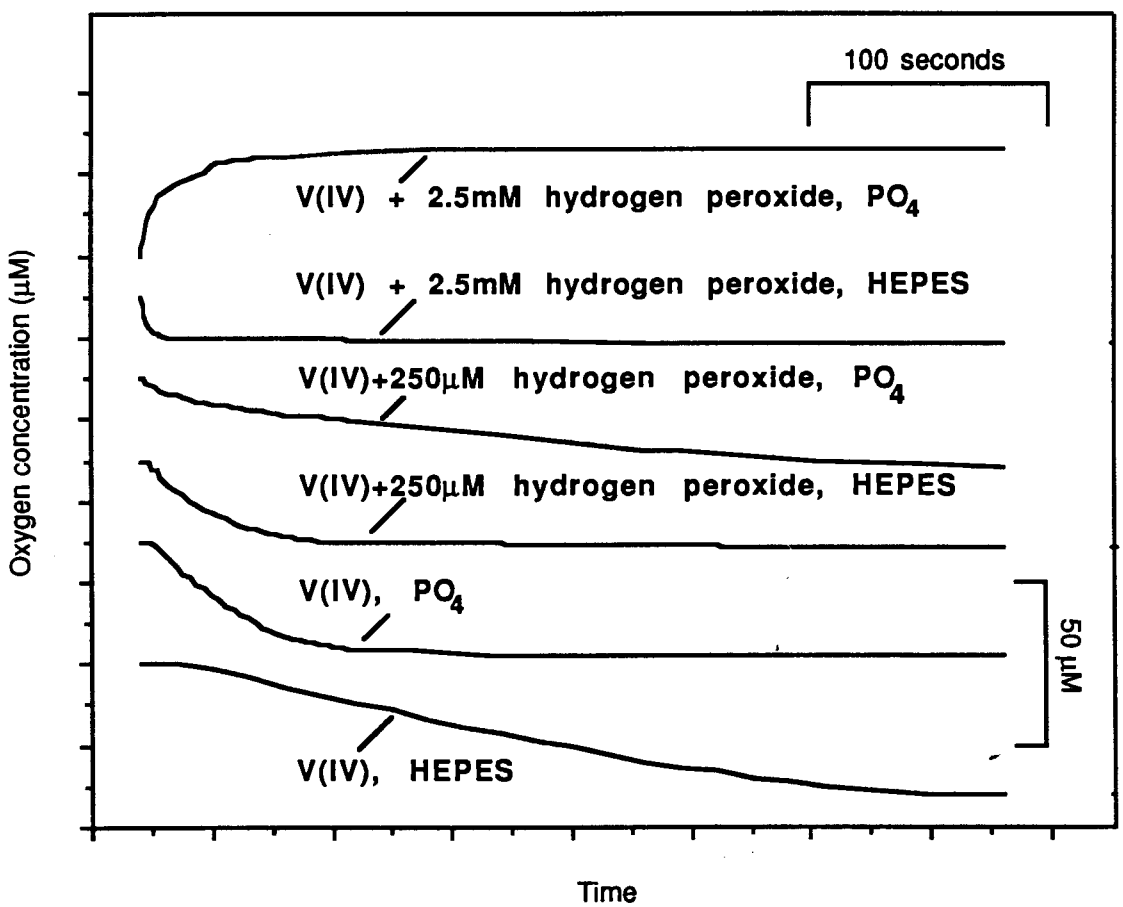


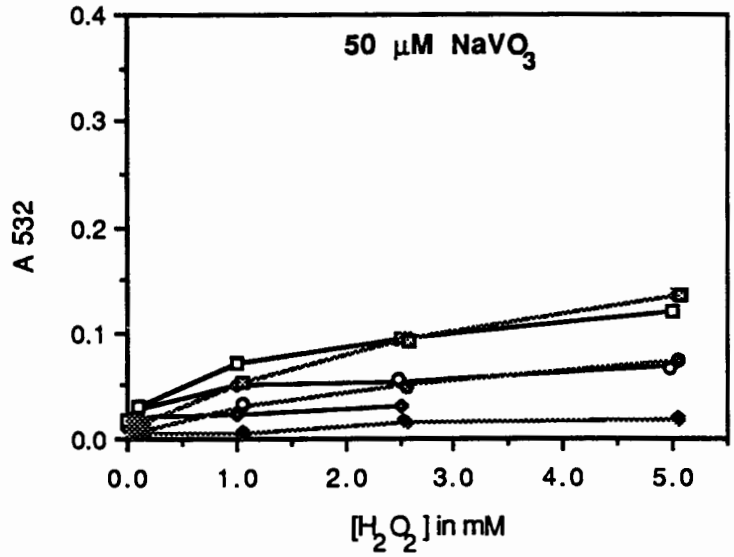
Figure 6.0

Production of TBA-reactive material from deoxyribose by Cu(II), Fe(II), Fe(III), V(IV) or V(V) alone and in the presence of exogenous H_2O_2

The formation of TBA-reactive substances from deoxyribose (2-deoxy-d-ribose; Sigma Chemical Co.) was measured for reactions involving 50 μM or 100 μM metal ions ($CuSO_4$, $FeSO_4$, $FeCl_3$, (Fisher Scientific Co.); $NaVO_3$ (Sigma Chemical Co.) or $VOSO_4$ (Aldrich Chemical Co.)) alone or with added H_2O_2 (BDH Chemicals; final concentration 0.25, 0.5, 1.0, 2.5, 5.0 mM). Data plotted represents the following conditions: ◘ 1.76 mM deoxyribose + metal, o 0.88 mM deoxyribose + metal, ◇ no deoxyribose + metal, ◙ 1.76 mM deoxyribose alone, ● 0.88 mM deoxyribose alone, ◆ no deoxyribose, no metal. Reactions were conducted in 50 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; Sigma Chemical Co.) buffer (pH 7.4, 25° C). Following a 15 minute incubation in a circulating water bath at 37° C, trichloroacetic acid (TCA; Fisher Scientific, final concentration 2.8%) and TBA reagent (4,6-dihydroxy-2-thiopyrimidine, 2-thiobarbituric acid; Sigma Chemical Co., final concentration 1%) were added. Tubes were then incubated for a further 15 minutes a second water bath at 100° C. Subsequently, tubes were removed and cooled in ice water, and the absorbance at 532 nm recorded. Individual reactions were conducted at least in triplicate.

Figure 6.0

Production of TBA-reactive substances from deoxyribose by $CuSO_4$, $FeSO_4$, $FeCl_3$, $NaVO_3$ or $VOSO_4$ alone and in the presence of exogenous H_2O_2



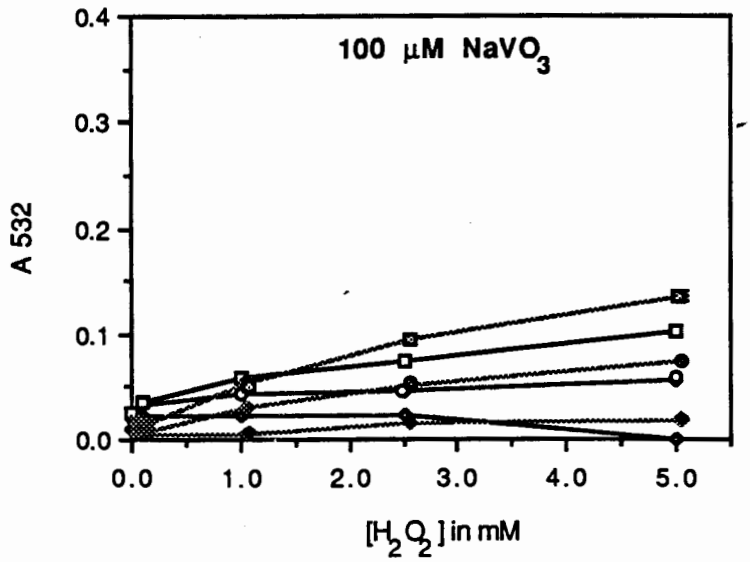


Figure 6.0

Production of TBA-reactive substances from deoxyribose by $CuSO_4$, $FeSO_4$, $FeCl_3$, $NaVO_3$ or $VOSO_4$ alone and in the presence of exogenous H_2O_2

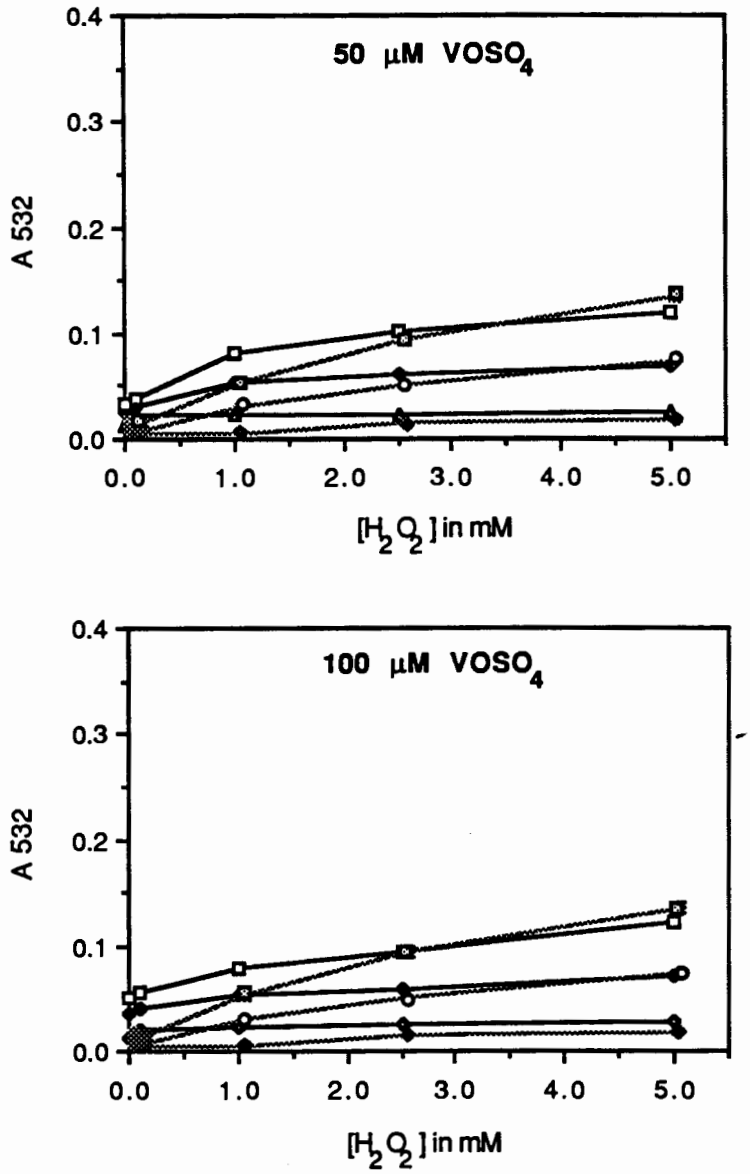
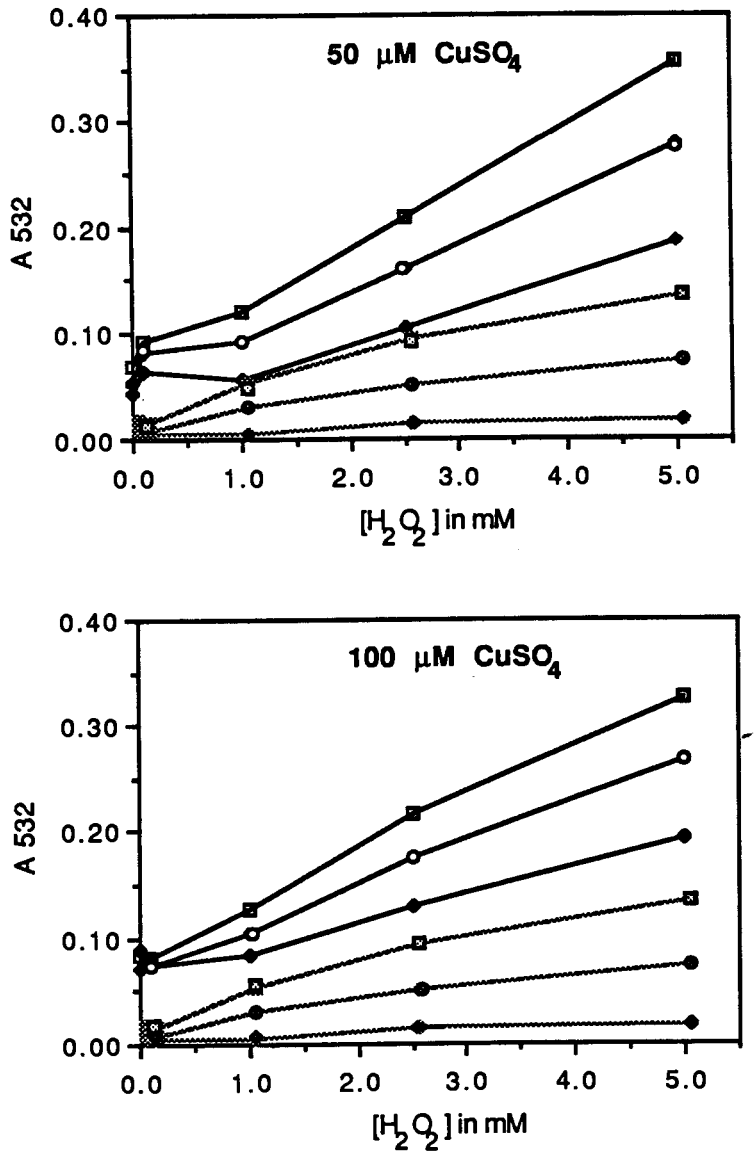


Figure 6.0

Production of TBA-reactive substances from deoxyribose by $CuSO_4$, $FeSO_4$, $FeCl_3$, $NaVO_3$ or $VOSO_4$ alone and in the presence of exogenous H_2O_2



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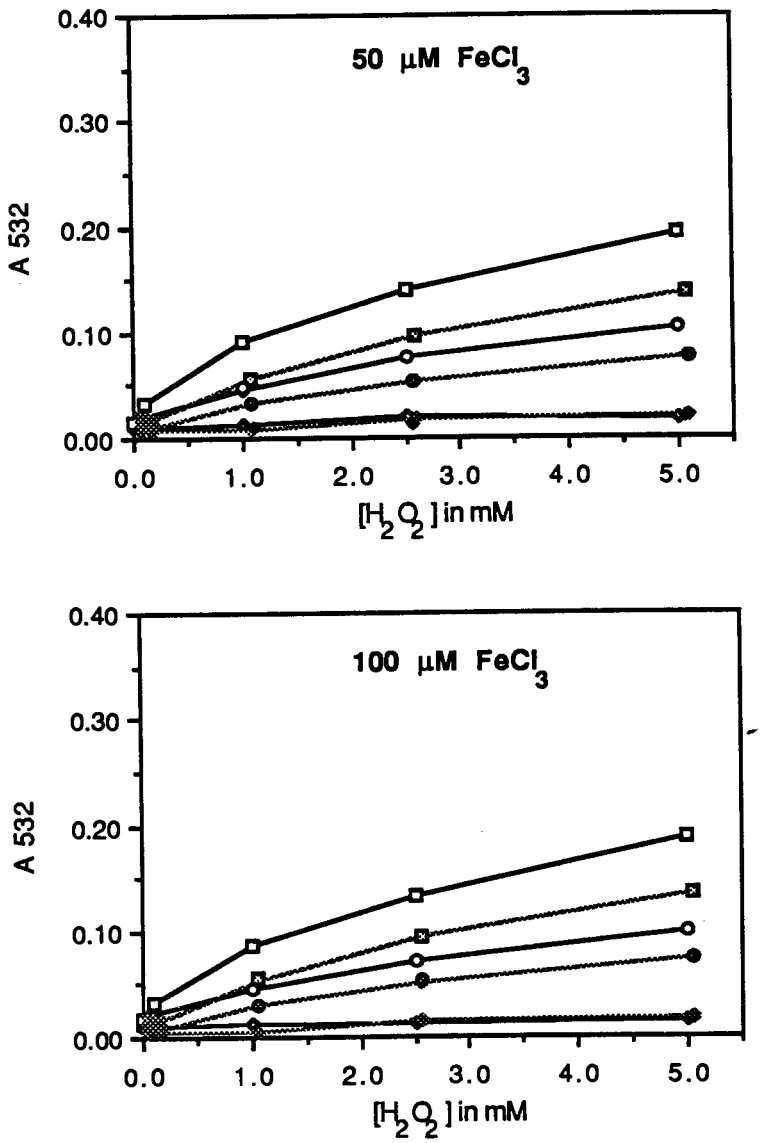
Production of TBA-reactive substances from deoxyribose by $CuSO_4$, $FeSO_4$, $FeCl_3$, $NaVO_3$ or $VOSO_4$ alone and in the presence of exogenous H_2O_2



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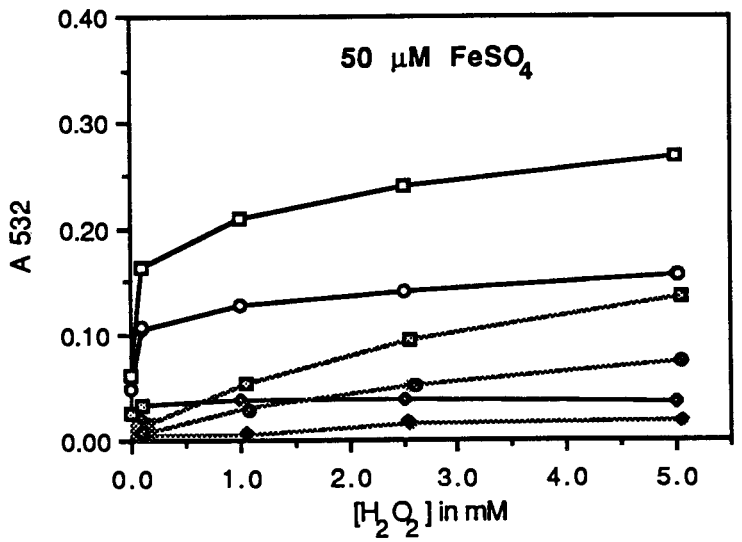
Production of TBA-reactive substances from deoxyribose by $CuSO_4$, $FeSO_4$, $FeCl_3$, $NaVO_3$ or $VOSO_4$ alone and in the presence of exogenous H_2O_2



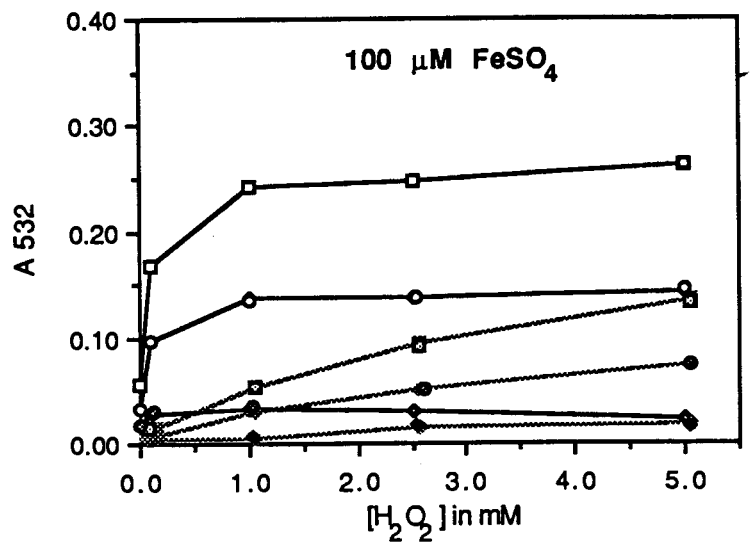


Figure 7.0

Oxygen consumption/hydrogen peroxide decomposition by V(V): effects of hydrogen peroxide concentration and buffer type

Changes in $[O_2]$ for reactions involving 250 μM $NaVO_3$ (Sigma Chemical Co.) alone or in the presence of equimolar or 10-fold excess H_2O_2 (BDH Chemicals) were measured polarographically by means of a Clark-type electrode (equipped with a magnetic stirrer and a water jacket) connected to a chart recorder. Individual reactions were conducted in both potassium phosphate (BDH Chemicals) and HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; Sigma Chemical Co.) buffers (air saturated, 50 mM, pH 7.0, 20° C). All reactions were initiated by addition of the metal ion.

Figure 7.0

Oxygen consumption/hydrogen peroxide decomposition by V(V): effects of buffer type and hydrogen peroxide concentration

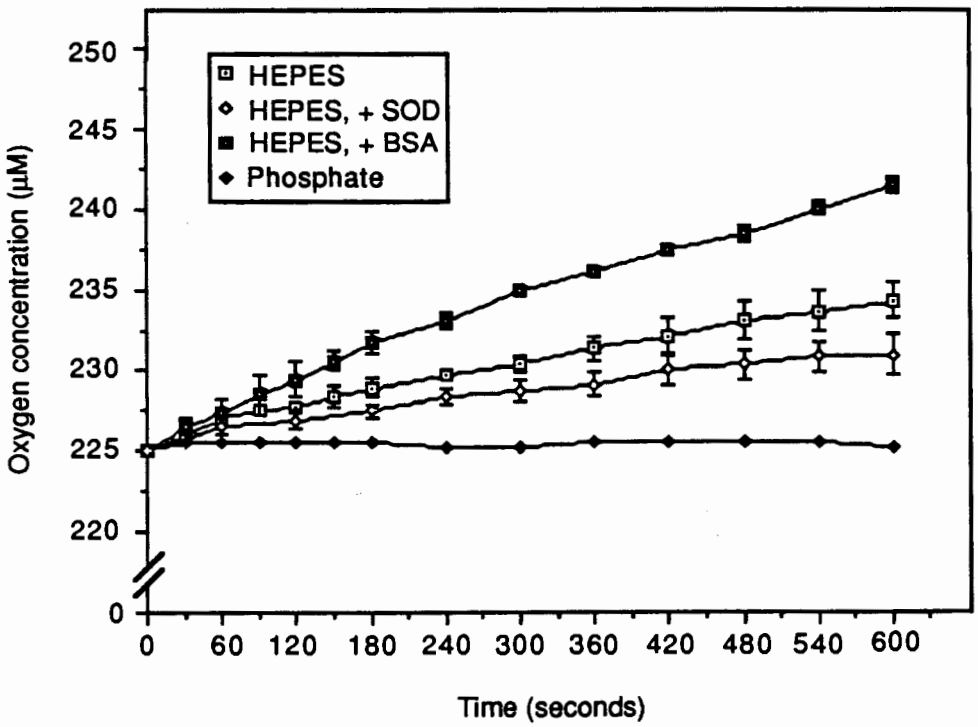


Figure 8.0

Oxygen consumption by V(IV) or V(V) alone and in the presence of l-ascorbic acid, NADH, l-cysteine, reduced glutathione, or l-norepinephrine

Changes in $[O_2]$ were monitored for reactions involving 250 μM $VOSO_4$ (Aldrich Chemical Co.) or $NaVO_3$ (Sigma Chemical Co.), l-ascorbic acid (BDH Chemicals), NADH, l-cysteine, reduced glutathione or l-norepinephrine (Sigma Chemical Co) alone or in combination. Oxygen consumption was measured polarographically by means of a Clark-type electrode (equipped with a magnetic stirrer and a water jacket) connected to a chart recorder. Individual reactions were conducted in both potassium phosphate (BDH Chemicals) and HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Sigma Chemical Co.) buffers (50 mM, pH 7.0, air saturated, 20° C). All reactions were initiated by addition of metal ion.

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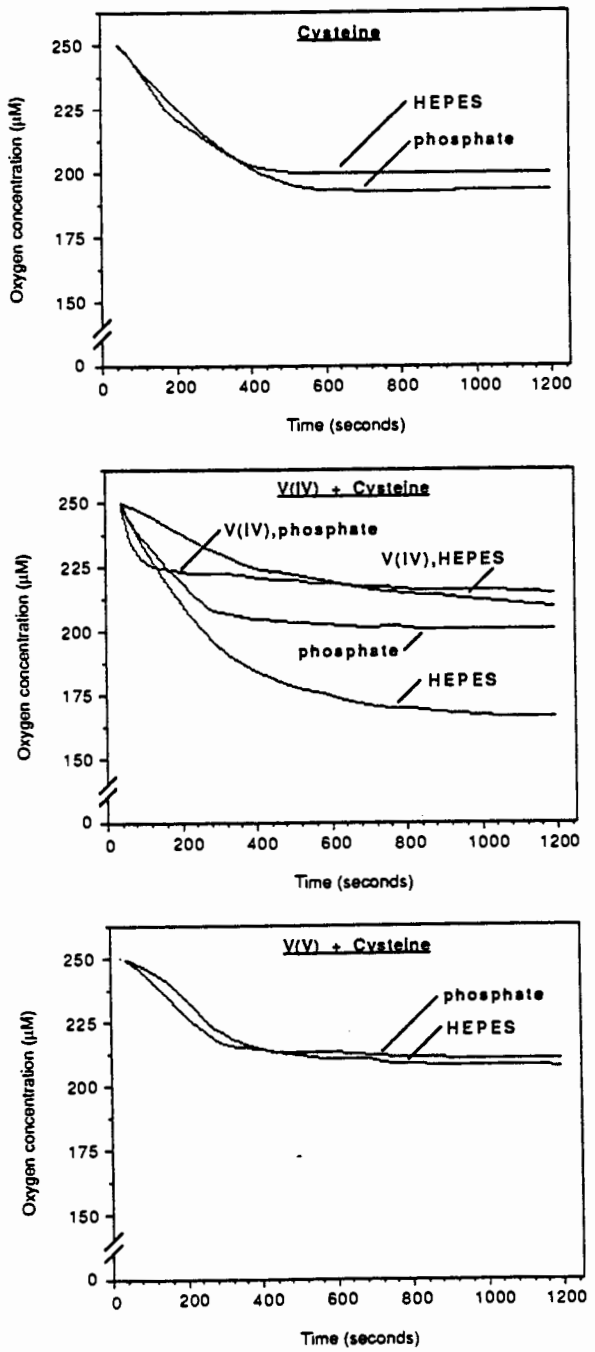

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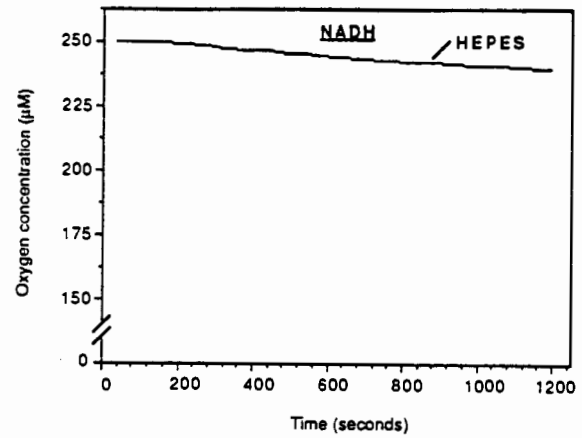
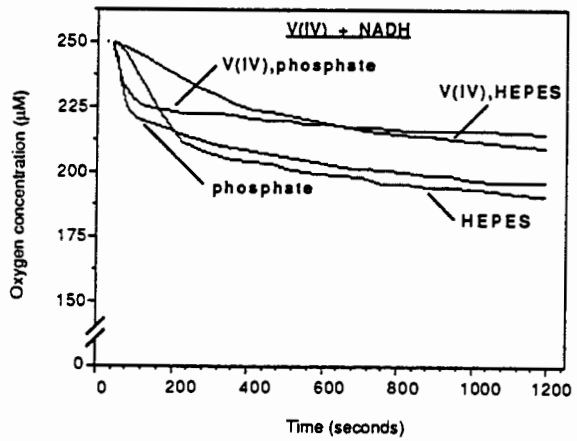
Oxygen consumption by V(IV) or V(V) alone and in the presence of l-ascorbic acid, NADH, l-cysteine, reduced glutathione, or l-norepinephrine





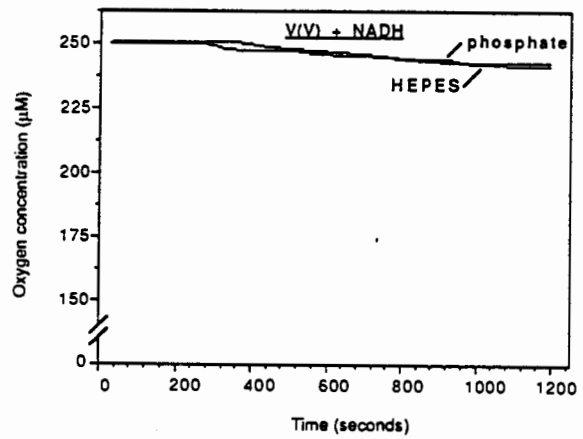
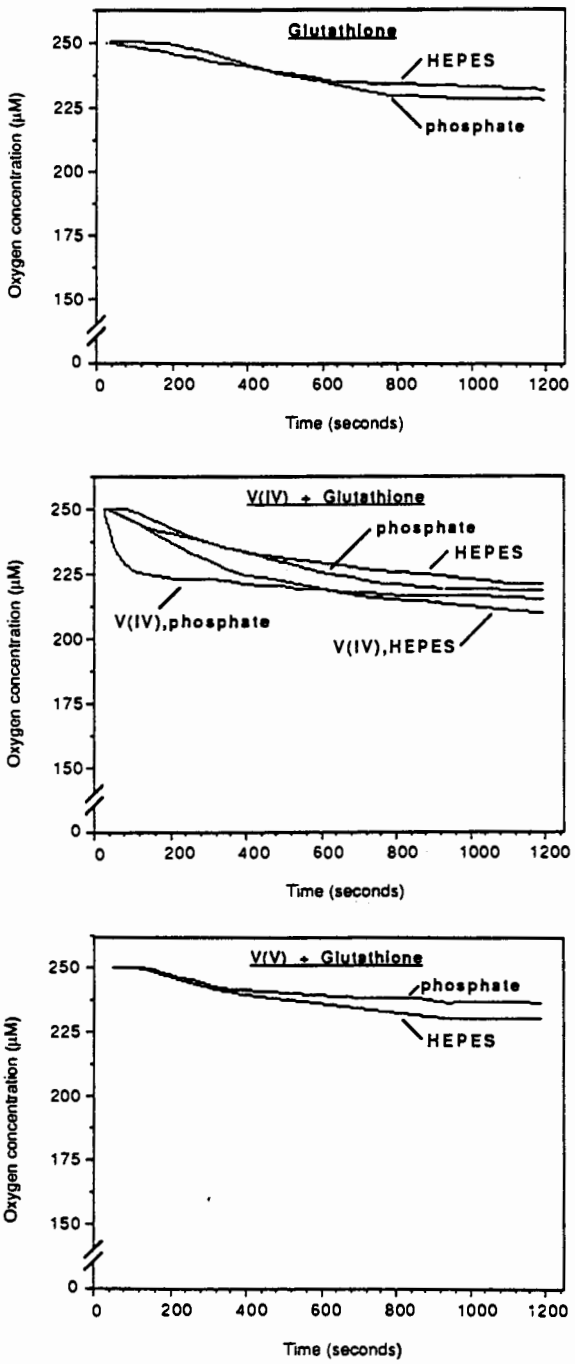


Figure 8.0

Oxygen consumption by V(IV) or V(V) alone and in the presence of l-ascorbic acid, NADH, l-cysteine, reduced glutathione, or l-norepinephrine



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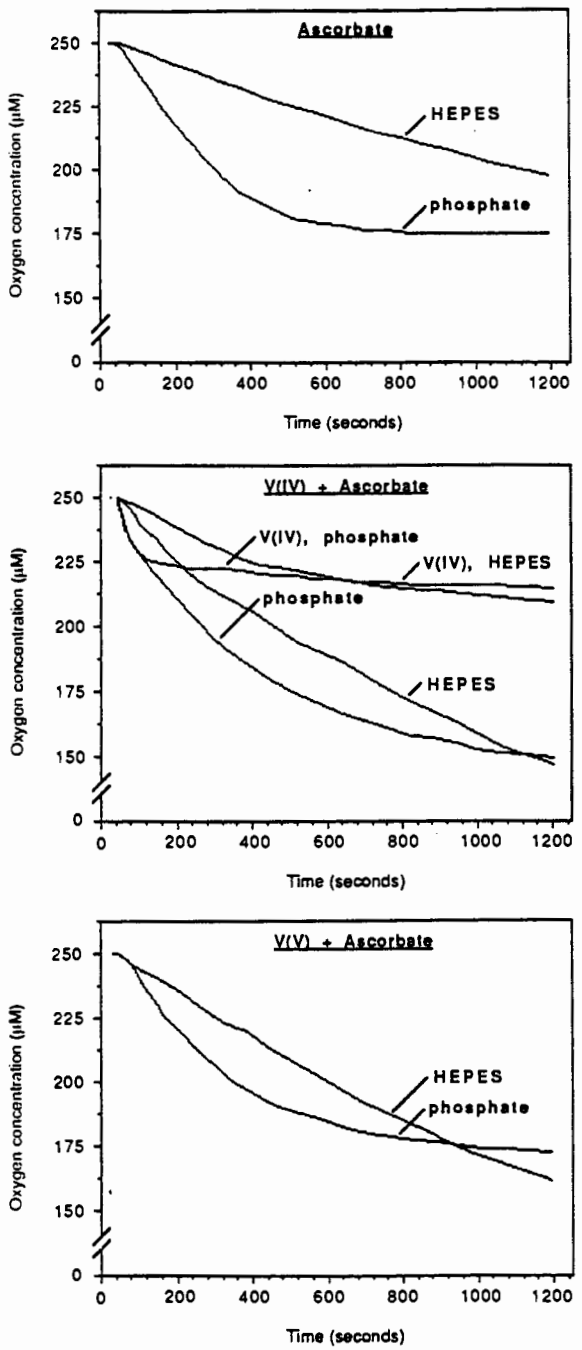
Oxygen consumption by V(IV) or V(V) alone and in the presence of l-ascorbic acid, NADH, l-cysteine, reduced glutathione, or l-norepinephrine



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Oxygen consumption by V(IV) or V(V) alone and in the presence of l-ascorbic acid, NADH, l-cysteine, reduced glutathione, or l-norepinephrine

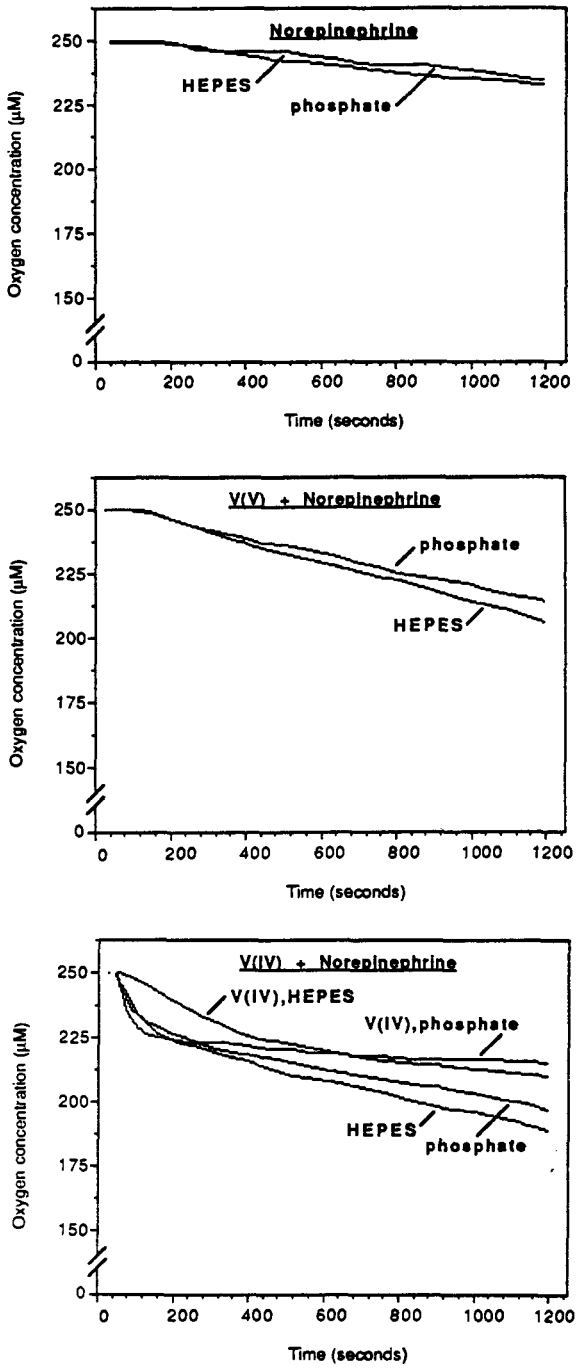


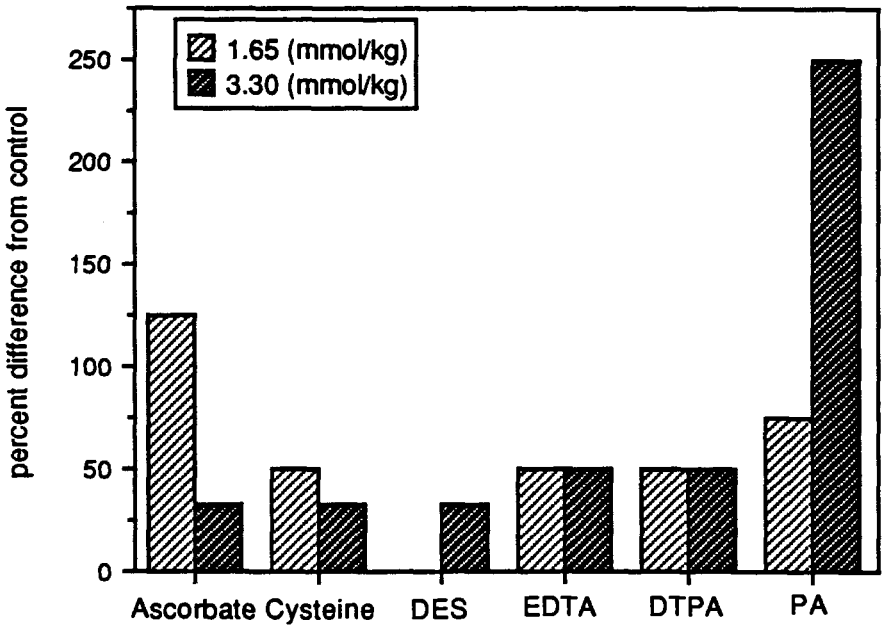
Figure 9.0

Effects of reducing and chelating agents on the acute toxicity of sodium metavanadate in mice

Data replotted from Domingo et al (1985). $NaVO_3$ (0.33 mmol/kg) was administered via a single intraperitoneal injection to male Swiss mice (25-28 g, 20 animals per group). Reducing (l-ascorbic acid, l-cysteine) or chelating agents (desferioxamine mesylate, $Na_2CaEDTA$, $Na_2CaDTPA$, d,l-penicillamine) were also administered i.p. immediately after injection of vanadium, all solutions being made in 0.9% saline. Solutions were adjusted to pH 7.0 with sodium bicarbonate where necessary. Animals were kept 14 days after $NaVO_3$ injection. Toxicity was measured as the percentage survival in each group of animals.

Figure 9.0

Effects of reducing and chelating agents on the acute toxicity of sodium metavanadate in mice



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CHAPTER 3

REDOX REACTIONS OF VANADIUM: COMPARATIVE EFFECTS OF VANADIUM, IRON AND COPPER ON 6-HYDROXYDOPAMINE OXIDATION

ABSTRACT

Vanadium-catalyzed oxidations of 6-hydroxydopamine (6-OHDA) were compared with oxidations by iron and copper. Anaerobically, both V(V) and V(IV) were strong oxidants of 6-OHDA. Vanadium (V) oxidized twice as much 6-OHDA as V(IV) and Cu(II), consistent with conversion of both forms to V(III). However, neither V(V) nor V(IV) were as effective as Fe(III), Fe(II) or Cu(II) in accelerating *para*-quinone product formation aerobically. Superoxide dismutase inhibited oxidations catalyzed by V(V), V(IV) or Fe(III), but did not inhibit in the presence of Fe(II) or Cu(II). This effect of superoxide dismutase implies that V(IV) (like Fe(III)) acts via an outer sphere mechanims in which superoxide is released to propagate the reaction, whereas propagation of Fe(II)- and Cu(II)-catalyzed oxidations is superoxide-independent. All added metals increased the maximal rate of O_2 consumption compared to 6-OHDA alone. Vanadium (V), Fe(III) and Cu(II) also increased the total O_2 consumed. Hydrogen peroxide (the major product of O_2 reduction in the control oxidation of 6-OHDA alone) was almost entirely consumed during the reaction in the presence of V(V), V(IV) or Fe(II). This results from catalatic actions of V(V) and V(IV), and a peroxidatic action of iron. In the presence of Cu(II), H_2O_2 accumulation was intermediate between that of other metals and the "uncatalyzed" reaction. The catalytic effects of vanadium on 6-OHDA oxidation differ significantly from those of iron and copper, being more dependent on oxidative recycling by reduced oxygen species.

A. INTRODUCTION

The pharmacological effects of vanadium are influenced by its unique redox chemistry. For example, catecholamines decrease the inhibitory effects of vanadium on Na^+/K^+=ATPases by reducing V(V) to V(IV) (Cantley et al,1978; Adam-Vizi et al,1981). Vanadium in turn promotes noradrenaline release from tissues (Torok et al,1982) and decreases brain catecholamine levels (Sharma et al,1986). Vanadium-catalyzed oxidations of NADH involve direct oxidation of NADH by V(V), as well as the formation of vanadium peroxo species and hydroxyl radicals (Vyskocil et

al,1980; Darr and Fridovich,1984; Liochev and Fridovich,1987). Similar mechanisms may mediate vanadium-catalyzed oxidations of catecholamines. Vanadium(V) is sometimes more effective than other metal ions including Cu(II) and Fe(II) in catalyzing oxidations of endogenous catecholamines (Martin et al,1960).

In a model system (oxidation of 6-hydroxydopamine, 6-OHDA) we compared the effects of V(V) and V(IV) with those of other transition metals (iron, copper) known to accelerate the reaction (Heikkila and Cabbat,1978; Sullivan and Stern,1981). Aerobically, 6-OHDA oxidation is initiated by metal/6-OHDA complexes (11, 12). Under certain conditions superoxide (O_2^-) propagates the reaction (low effective mertal concentrations, in the presence of metal chelators) (Sullivan and Stern,1981; Gee and Davison,1984; Bandy and Davison,1987). Hydrogen peroxide, while it can contribute (Liang et al,1976), does not accelerate the "uncatalyzed" (trace-metal-catalyzed) aerobic oxidation of 6-OHDA (Gee and Davison,1984, Bandy and Davison,1987, Liang et al,1976), but does accelerate in the presence of some added metal complexes (eg. Fe(III)/EDTA) (Sullivan and Stern,1981). We report the relative involvement of O_2^- and H_2O_2 in the actions of vanadium, ion and copper, and explain their relative effectiveness in terms of differing abflities to facilitate electron transfers within metal complexes, and to react with O_2^- and or H_2O_2.

B. RESULTS AND DISCUSSION

Anaerobically, metal should oxidize 6-OHDA in proportion to their capacity to accept electrons from it, and indeed 50 μM V(V) oxidized almost twice as mich 6-OHDA as V(IV) or Cu(II), oxidizing 26 μM as compared to 16 μM and 14 μM respectively (Fig. 1.0.0). Vanadium (V), V(IV) and Cu(II) are all good one electron oxidants ($E_0(VO_2^+/VO^{2+})$ = 1.0 V, E_0(Cu(II)/Cu(I)) = 0.15 V)) (Weast et al,1983), however only V(V), with a stable V(III) oxidation state is a good two electron oxidant ($E_0(VO_2^+/V(III))$ = 1.36 V). Only approximately one half the stoichiometrically predicted quantities of *p*-quinone were produced by V(V), V(IV) and Cu(II). This must be explicable in terms

of the equilibria of the relevant reduced metal/oxidized metal/reductant species. *p*-Quinone product formation could not be determined anaerobically in the presence of Fe(III) or Fe(II) because of interfering absorbance similar to that reported for Fe(III)- and Fe(II)-catceholamine complexes in the oxidation of adrenaline (Green et al,1956).

Aerobically, V(V) and V(IV), like Fe(III), were much less effective than Cu(II) and Fe(II) (Fig.2.0.0). Vanadium (V) caused an initial rapid increase in *p*-quinone formation reflecting stoichiometric oxidation of 6-OHDA, then a slower secondary increase dependent on reoxidation ofthe reduced metal. The anaerobic rate of oxidation of 6-OHDA by Fe(III) was insufficient to account for the rapidity of the aerobic (FeIII)-catalyzed reaction. This suggests that aerobically an iron-O_2 complex, and not Fe(III), acts as an oxidant of 6-OHDA, Cu(II) perhaps acting by a similar mechanism.

Superoxide dismutase inhibits the later phase of vanadium(V)-catalyzed oxidations which involves redox cycling of the metal, but predictably does not affect the direct oxidation of 6-OHDA by V(V). In contrast, for V(IV), inhibition by the enzyme was apparent from the beginning of the reaction. This implies that V(IV) reacts with molecular O_2 by an outer sphere mechanism to produce $O_2^{\cdot-}$. Thus, both V(IV)- and V(V)-, like Fe(III)-catalyzed oxidations of 6-OHDA are propagated by $O_2^{\cdot-}$. In copntrast, in the presence of Fe(II) or Cu(II), no inhibition by superoxide dismutase was observed. The effects of superoxide dismutase were primarily due to its catalytic activity rather than its ability to chelate metals in a non-specific manner since albumin had much smaller effects.

Maximal rates of O_2 consumption paralleled *p*-quinone formation, except that the V(V) rate was slower than Fe(II) (Fig.3.0.0). The smaller amount of O_2 consumed in the case of Fe(II) combined with a more rapid reaction can be accounted for on the basis of a peroxidative action of fe(II). The slower rate of O_2 consumption and lack of H_2O_2 accumulpation in the presence of V(V) is explicable

on the basis of a catalatic effect of the metal, evident in the O_2 evolved when V(V) is added to solution containing H_2O_2 (not shown). These catalatic and peroxidative actions, as well as the ability of Cu(II) to act as a Fenton donor explain the differences in accumulation of H_2O_2 as revealed by addition of catalase when reaction were near completion.

C. CONCLUSIONS

We conclude that the catalytic effects of vanadium on 6-OHDA oxidation differ significantly from those of iron and copper, being more dependent on oxidative recycling by reduced oxygen species. While V(V) is a strong direct oxidant of 6-OHDA, O_2^- mediates the catalytic activity of V(IV). In addition, vanadium accelerates the reduction of not only O_2, but also H_2O_2.

Figure 1.0

Effects of metals on anaerobic oxidation of 6-OHDA

Stock solutions and buffers were prepared using deionized, distilled water. 6-hydroxydopamine (6-OHDA) hydrobromide and metal ion stock solutions were made anaerobically under 100% nitrogen according to the procedure of Gee and Davison (1984). Reactions were initiated by addition of 6-OHDA or simultaneous addition of 6-OHDA and metal ion to HEPES/KOH buffer (50 mM, pH 7.4, 25° C, final volume 2.5 ml) contained in a sealed cuvette. Buffer aliquots were bubbled with for at least 30 minutes prior to initiation of individual reactions. Final concentrations were 125 μM 6-OHDA and 50 μM metal ions. *p*-Quinone product formation was followed at 490 nm. A molar extinction coefficient of 1892 M^{-1} s^{-1} was used to calculate *p*-quinone concentrations.

Figure 1.0

Effects of metals on anaerobic oxidation of 6-OHDA

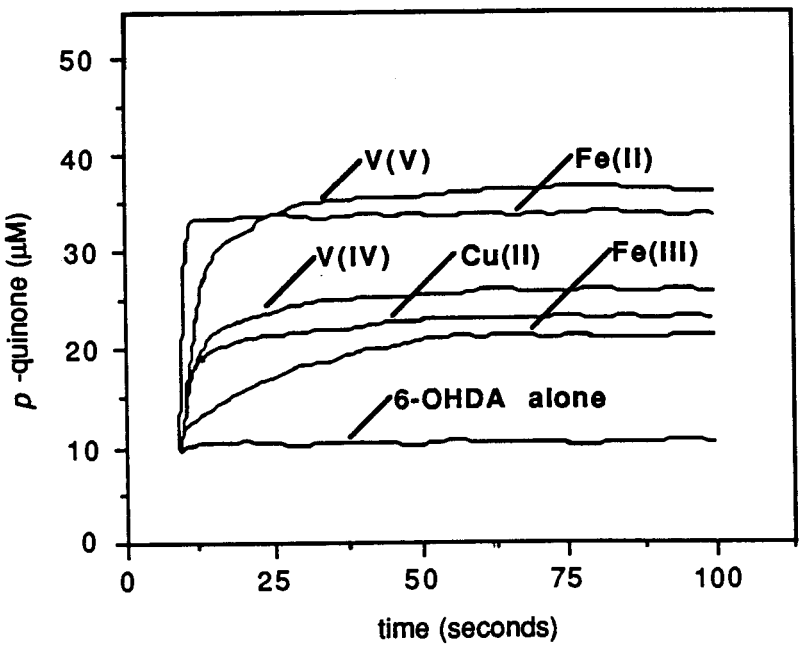


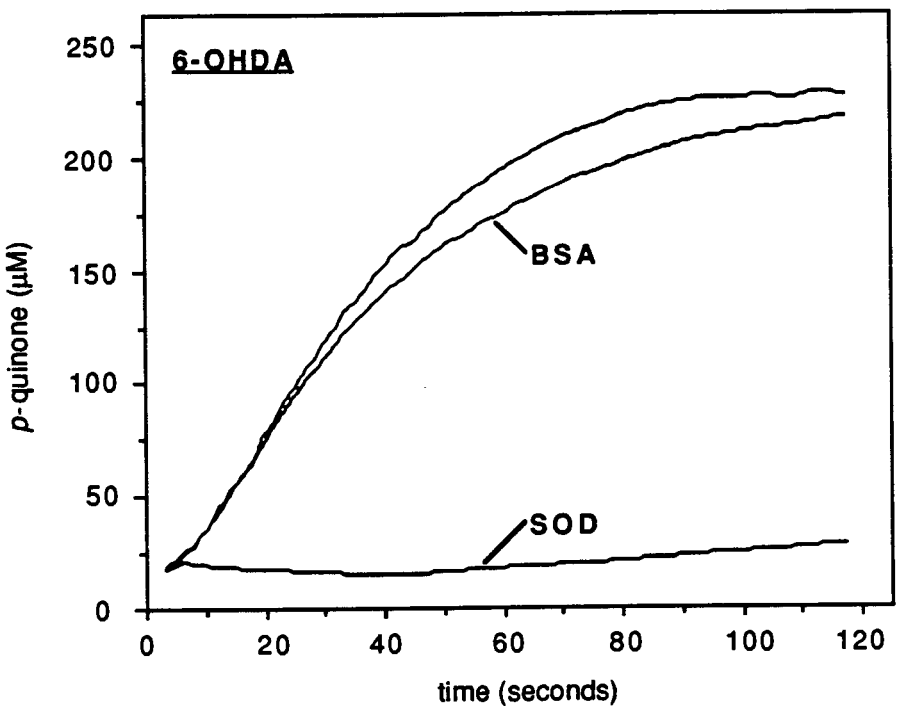
Figure 2.0

Effects of metals on aerobic oxidation of 6-OHDA

Conditions and procedures were as described in Fig. 1.0, with the exceptions that metal ion stock solutions were made aerobically and buffer was air saturated (initial O_2 concentration 246 ± 6 μM). Superoxide dismutase (final concentration 25 U/ml) or an equivalent weight of albumin were added as indicated.

Figure 2.0

Effects of metals on aerobic oxidation of 6-OHDA



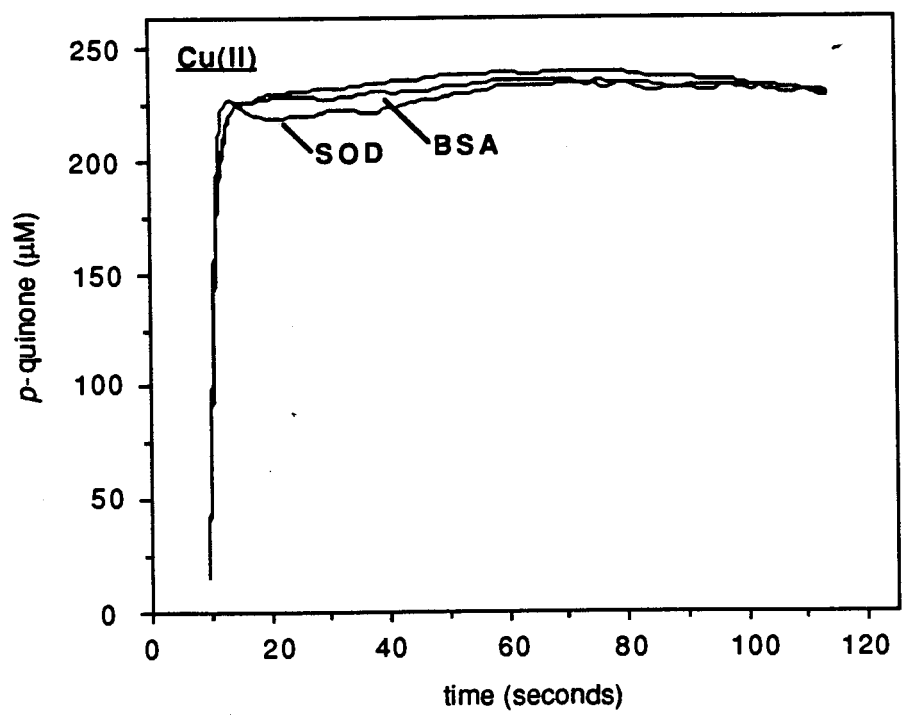
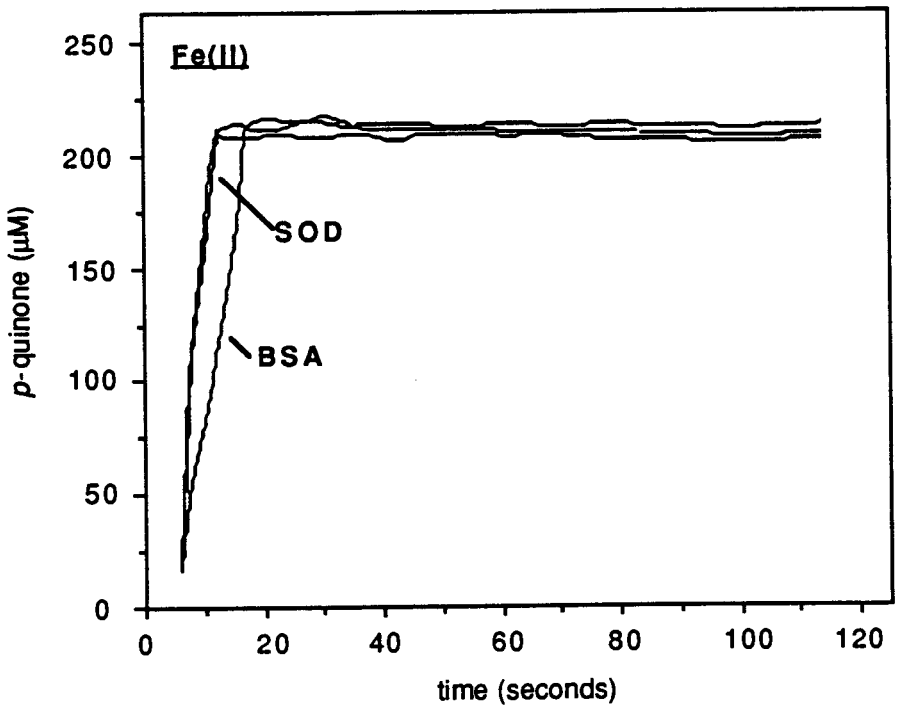


Figure 2.0

Effects of metals on aerobic oxidation of 6-OHDA



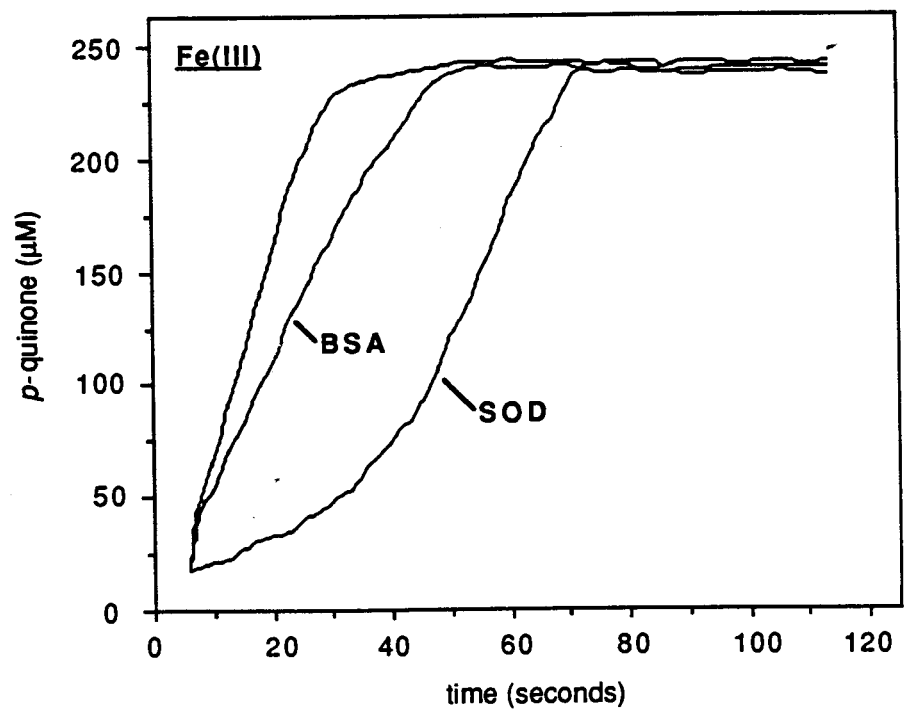
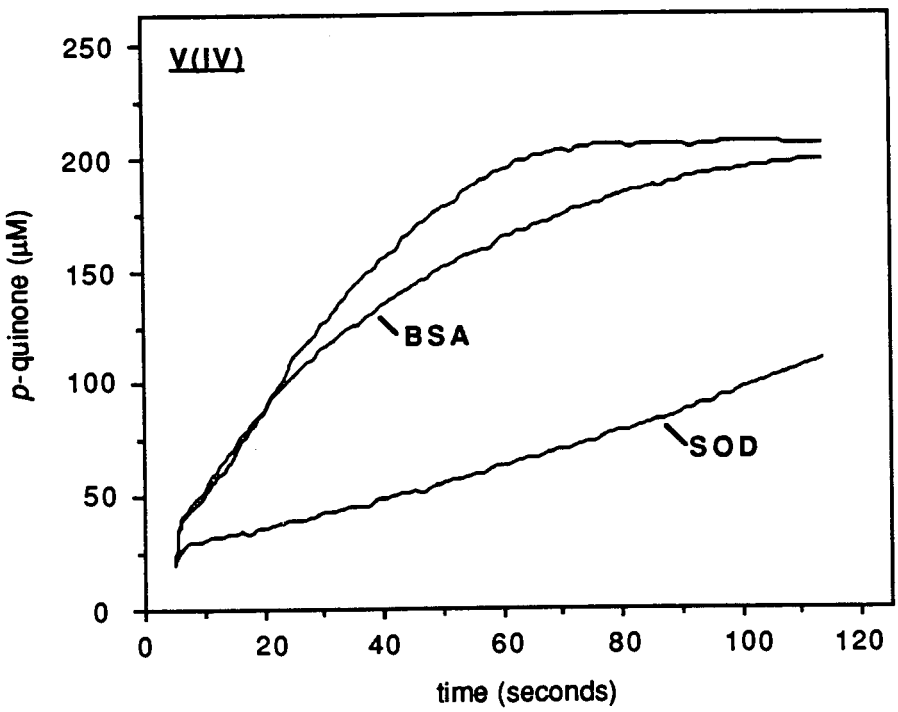


Figure 2.0

Effects of metals on aerobic oxidation of 6-OHDA



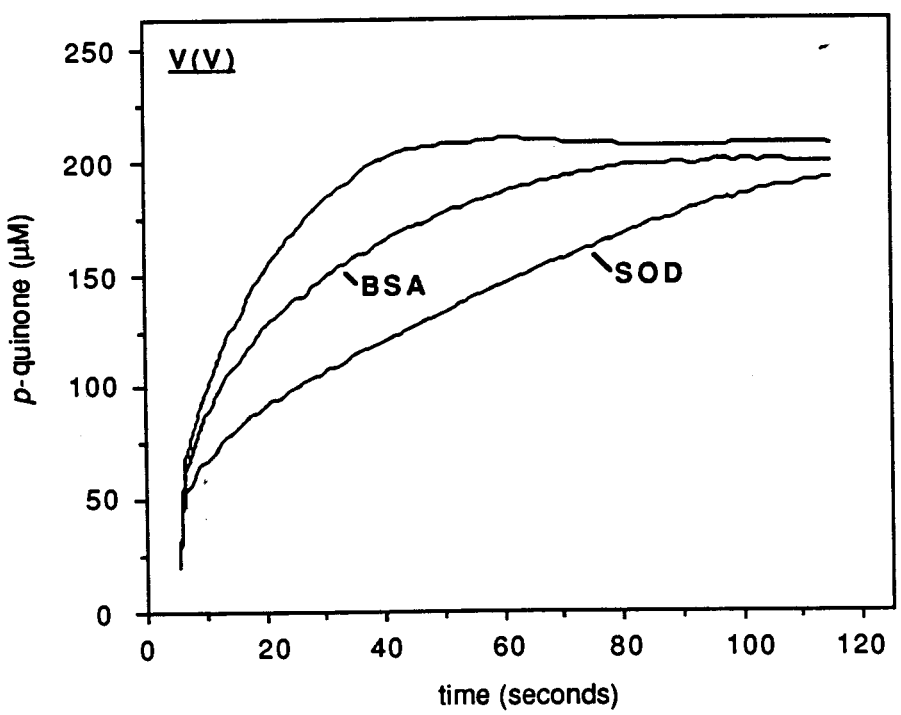


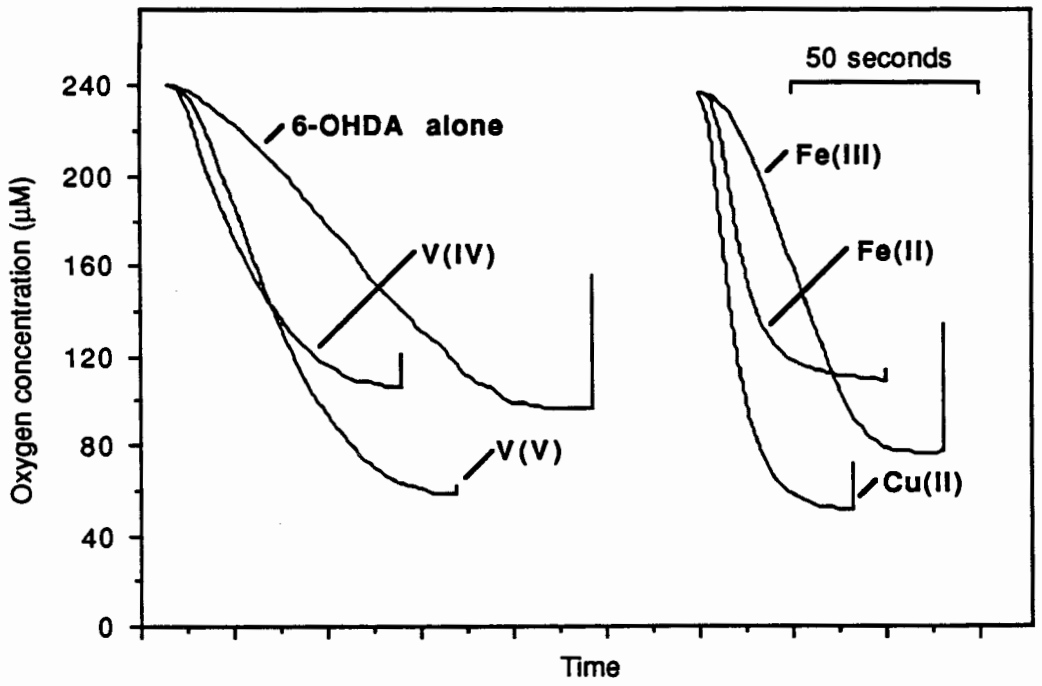
Figure 3.0

Effects of metals on oxygen consumption and hydrogen peroxide production by 6-OHDA

Procedures and conditions were as described for Fig. 1.0 with the exception that a final volume of 5.0 ml was employed. Oxygen consumption was measured by means of a Clark-type oxygen electrode connected to a circulating water bath and a chart recorder. Catalase was added when reactions were near completion to a final concentration of 20 U/ml.

Figure 3.0

Effects of metals on oxygen consumption and hydrogen peroxide production by 6-OHDA



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CHAPTER 4

ASCORBATE OXIDATION IN THE PRESENCE OF TRANSITION AND NON-TRANSITION METAL IONS: ROLES OF OXYGEN AND HYDROGEN PEROXIDE

ABSTRACT

Conflicts regarding the roles of metals in the oxidation of ascorbate have resulted from failure to consider the roles of buffer salts, pH, and extraneous ligands. These factors affect the reduction of metal by ascorbate, and also the relative contributions of oxygen and product hydrogen peroxide (product) as competing oxidants. At neutral pH, very few metals are reduced by ascorbate, and even fewer are reoxidized by molecular oxygen. We tested Al (III), Cd(II), Cu(II), Fe(II), Fe(III), Hg(II), Mn(II), Pb(II), V(IV), and V(V) for their ability to be reduced by ascorbate and to be reoxidized by O_2 or H_2O_2. Among these metal ions only Cu(II) redox cycles in the presence of ascorbate and oxygen. In the presence of added H_2O_2, only copper and vanadium redox cycle. Factors retarding redox cycling of other metals in the autoxidation and peroxidatic oxidation of ascorbate are: (1) failure of ascorbate to reduce metal ions other than Cu or Hg at neutral pH, (2) the presence of ligands, including phosphate ions and even ascorbate itself which slow reactions of reduced metal with oxygen or H_2O_2. In the absence of added metals, oxidations of ascorbate (100 μM) were faster in HEPES buffer than in phosphate, and faster at higher pH. Among the metal ions tested, only Cu(II) and Hg(II) are reduced by ascorbate or anaerobically or aerobically, under any reaction conditions. Anaerobically, 50 μM Cu(II) oxidized 13 μM ascorbate at pH 7.4 and 12 μM at pH 8.0 whereas Hg(II) oxidized 24 μM and 23 μM. The different stoichiometries observed for the two metals reflect the nature of the reactive metal species and reactions of the metal ions with semidehydro-ascorbate. Aerobically, Cu(II) (at 50 μM) redox cycled under all reaction conditions, oxidizing virtually all of the ascorbate initially present within 250 seconds. In contrast, the effects of Hg(II) were not notably changed by the admission of O_2. Direct oxidation of ascorbate by either Cu(II) and Hg(II) was faster in HEPES than in phosphate buffer. However, steady state rates of Cu(II) catalyzed oxidations were at least 30% faster in phosphate than in HEPES buffer (likely as a result of increased reactivity of Cu(I) with O_2). Of the metal ions other than Cu, only V(V) accelerated ascorbate oxidation, causing transient accelerations aerobically in phosphate (but not HEPES buffer) at pH 7.4 (+165%) and 8.0 (+86%). Air oxidation of the V(IV) produced by the initial oxidation of ascorbate by V(V) proceeds more rapidly in phosphate buffer. Superoxide

dismutase (5.0 U/ml) or catalase (20.0 U/ml) did not inhibit ascorbate oxidation whether or not metals were added. Only Cu(II) consistently accelerated maximum rates of O_2 consumption over that of ascorbate alone (by at least 378%). Both V(IV) (+16% to +76%) and Fe(II) (+19% to +249%) caused transient increases in the maximum rates of O_2 consumption attributable to direct oxidation of ascorbate by the metal. Despite the reactivity of either metal with both ascorbate and oxygen, neither Fe nor V underwent redox cycling. This was because ascorbate acting as a chelating agent almost completely prevented reactions of either V(IV) or Fe(II) with O_2. In vanadium-catalyzed oxidations of ascorbate all endogenous H_2O_2 was decomposed. Other added metals had no notable effects on H_2O_2 production or decomposition. However, exogenous H_2O_2 (to 2.5 mM) increased the rates of control oxidations of ascorbate. In phosphate buffer the acceleration by H_2O_2 was a modest 2.5- fold, but in HEPES it was 113- fold. In the presence of added metals addition of H_2O_2 also stimulated ascorbate oxidation to a much greater extent in HEPES buffer. The lower rates of ascorbate oxidation in phosphate buffer may reflect inhibition by phosphate of reactions of metal ions with H_2O_2. Among the metal ions listed above, V(V) , V(IV) , Cu(II), Fe(II) and Fe(III) accelerated the direct oxidation of ascorbate by H_2O_2. However, increases steady state rates of ascorbate oxidation were observed only for Cu(II) (11.4-, 9.6-fold) > V(V) (1.95-, 8.7-fold) > V(IV) (2.4-, 9.2-fold) in HEPES or phosphate buffers. Thus, copper and vanadium, but not iron redox cycle in the presence of ascorbate and excess H_2O_2 under the reaction conditions employed. The common assumption that ascorbate is an effective reductant, able to promote redox cycling of metal ions in the presence of O_2 requires reevaluation.

A. INTRODUCTION

1.0 REACTIONS WITH METAL IONS UNDERLY THE PROOXIDANT EFFECTS OF ASCORBATE

In living cells, l-ascorbic acid (ascorbate) at cellular pH serves important antioxidant functions (Bendich et al,1986). However, under certain conditions, ascorbate may also act as a prooxidant, and so promote tissue damage. Metal ions play critical roles in these deleterious phenomena. It has been suggested that the prooxidant activity of ascorbate is likely of little importance *in vivo* (Levine,1986; Bendich,1986). Nevertheless, further research into the free radical mechanisms underlying reactions of ascorbate with metal ions is warranted on the basis of evidence *in vitro* cytotoxicity (Omura et al,1978; Galloway and Painter,1979; Stich et al,1979; Norkus et al,1983; Higson et al,1988), damage to lipid membranes (Haase and Dunkley,1969a,b; Romer et al,1981; Niki et al,1982; Cheeseman et al,1983; Norkus et al,1983; Girotti et al,1985; Braughler et al,1987), enzymes (Samuni et al,1981; Samuni et al,1983; Shinar et al,1983; Davison et al,1986; Higson et al,1988), RNA (Murata and Uike,1976) and DNA (Morgan et al 1976; Omura et al,1978; Stich et al,1978; Stich et al,1979; Rosin et al,1980; Chiou et al,1984; Norkus et al,1983; Chiou,1984; Shamberger,1984; Chevion,1988; Aronovitch et al,1987). Moreover, ascorbate is implicated *in vivo* in the toxicity of a number of transition and non-transition metals including: copper and iron (Aust et al,1985), mercury (Blackstone et al,1974; Murray and Hughes,1974; Hill,1979,1980; Whanger,1981), lead (Holmes,1939; Clegg and Rylands,1966; Pal et al,1975; Suzuki and Yoshida,1979), cadmium (Spivey-Fox and Fry,1970; Loh,1973; Richardson et al,1974; Fox,1975) and vanadium (Mitchell and Floyd,1954; Roschin,1967; Chakraborty et al,1977; Hill,1979,1981,1983; Jones and Basinger,1983; Donaldson et al,1985). The semidehydroascorbate radical (Morgan et al,1976; Davison et al,1986), and the hydroxyl radical (OH·) (Shinar et al,1983; Samuni et al,1983; Chevion,1988; ; Higson et al,1988) have all been implicated in ascorbate-mediated tissue damage. Thus, the involvement of metal ions towards ascorbate under conditions where either O_2 or H_2O_2 serve as the ultimate electron acceptor are of interest.

1.1.0 Comparisons of the effects of different metal ions at physiological pH are needed

No generally accepted mechanism for metal-catalyzed oxidations of ascorbate exists (Khan and Martell,1967; Ogata et al,1968; Jameson and Blackburn,1975; Sanehi et al,1975; Schwertnerova et al,1976; Shtamm et al,1979; Gamp and Zuberbuhler, 1981). As noted by Bielski (1985), many of the most detailed studies been conducted at acid pH (Dekker and Dickinson,1940; Silverblatt et al,1943; Weissberger and LuValle,1944; Nord,1955; Khan and Martell,1967a,b; Ogata et al,1968; Shtamm et al,1974,1979; Sanehi et al,1975; Pelizetti et al,1978). Metals which are effective oxidants of ascorbate at acid pH include: Cu(II), Fe(III) and V(IV) (Khan and Martell,1967), V(V) (Kustin and Toppen,1973), Mn(III) and Co (III) (Pelizetti et al,1978), $HgCl_2$ (Sanehi et al,1975) and Pb (McNair Scott,1964). Yet at neutral pH, only Cu(II) and Hg(II) are effective (Sanehi et al,1975; Halliwell and Foyer,1976; Donaldson and LaBella,1983; Buettner,1986). Thus, added Fe(II) (Halliwell and Foyer,1976), Fe(III) (Halliwell and Foyer,1976; Buettner,1986,1988), V(IV) or V(V) (Adam-Vizi et al,1981; Donaldson and LaBella) do not accelerate ascorbate oxidation under physiological conditions. At pH $\geq$ 10.2 Fe(II) and Mn(II), but not Fe(III) are effective (Halliwell and Foyer,1976).

Changes in pH may alter the progress of the overall reaction through effects on the relative proportions of ascorbate species, and the state of protonation of metal (aquo) complexes (Khan and Martell,1967a; Buettner,1986, 1988). Other factors affecting ascorbate oxidation which are also affected by pH are side reactions involving adventitious metal ions (Dekker and Dickinson,1940; Shtamm et al,1979), the ability of reduced metal ions to reduce O_2 (Nord,1955; Zuberbuhler,1976; Koppenol and Butler,1985; Wu, 1987), and the half lives of radical reaction intermediates such as O_2^- and semidehydroascorbate (Bielski et al,1971; Bielski and Allen,1977; Bielski and Richter,1977; Nadezhdin and Dunford,1979; Ingraham and Meyer,1981; Halliwell and Foyer,1976; Cabelli and Bielski,1983). Direct comparisons of the effects of different metals of physiological and/or toxicological importance on ascorbate oxidation are lacking. Equally important, with the exceptions of the 'uncatalyzed' oxidation of ascorbate (Halliwell and Foyer,1976; Scarpa et

al,1983; Buettner,1986; 1988) Cu(II)-catalyzed oxidations at pH 7.4 (Lovstad,1987) and iron-catalyzed oxidations at pH 8.8 and 10.2 (Halliwell and Foyer,1976), little detailed information concerning inhibition of the effects of metal catalysts by free radical scavengers is available.

2.0.0 CATALYSIS BY METAL IONS IS DEPENDENT ON REDOX CYCLING

2.1.0 Metals may act as simple oxidants of ascorbate with variable stoichiometry

Metal catalysis of ascorbate oxidation usually involves redox cycling of metal ions. Thus, the most fundamental questions to be answered concern the relative abilities of different metal ions to accept electrons from ascorbate in the absence and presence of O_2, and to donate electrons to O_2 (or H_2O_2) in the presence of ascorbate. Simple oxidations of ascorbate at acid pH by Cu (II) (Dekker and Dickinson,1940; Mapson,1945; Erkama,1949; Ogata et al,1968; Shinar et al,1983; Shtamm et al,1974), Fe(III) (Taqui Khan and Martell,1967) and V(IV) (Khan and Martell,1967b) have been reported. At acid pH, Fe(III) oxidizes HA^- more effectively than Cu(II) or V(IV) (Khan and Martell,1967a,b; Martell,1982). All three metals reportedly act by inner sphere mechanisms in which complex formation between ascorbate and the oxidized metal ions is followed by reduction of the metal ion and concomitant formation of semidehydroascorbate. Thereafter, dissociation of the complex allows reaction of a second oxidized metal ion with semidehydro-ascorbate (Nord,1955; Ogata et al,1968; Martell,1982). Similarly, the aerobic oxidation of ascorbate by $HgCl_2$ in acidic media has been described in terms of sequential reactions with $HgCl_2$ molecules (Sanehi et al,1975). The mechanism suggested for Cu(II) by Shtamm et al (1974; 1979) on the basis of kinetic data differs in that two ascorbate-bound Cu(II) ions synchronously oxidize HA^- to dehydroascorbate in a single step. Either mechanism yields an overall stoichiometry of one ascorbate oxidized per two Cu(II) ions. Alternately, binuclear Cu(II) complexes may be simple oxidants (Jameson and Blackburn,1975), the stoichiometry expected in the absence of O_2 being uncertain. Other metal ions may also act as simple oxidants. In this case, as in other direct

oxidations of organic compounds by metals, the stoichiometry in the absence of O_2 depends on the redox potential of the complex.

2.2.0 Direct oxidation of ascorbate by metal ions may modulate the initial involvement of O_2

Because of the variability in stoichiometry, it is important to measure both O_2 consumption and ascorbate oxidation. Of the kinetic studies located in the literature, only that of Shtamm et al (1979) involved such measurements.Even in this case O_2 consumption was measured indirectly and changes in ascorbate concentration were monitored intermittently rather than continuously. Direct measurement of these variables provides additional information concerning the mechanism of catalysis. For example, variations in the ability of metal ions to oxidize ascorbate directly may also modulate the involvement of oxygen through reactions with semidehydroascorbate and/or differences in rates of redox cycling. As noted by Ogata et al (1968), a number of workers have reported that the dependency of the reaction rate of Cu(II)-catalyzed oxidations of ascorbate in acidic solution deviates from proportionality (Barron et al,1936; Silverblatt et al,1943; Nord,1955c; Butt and Hallaway,1961). Even in the presence of O_2, direct oxidation of ascorbate by monomeric Cu(II) may occur. The initiation step proposed for aerobic Cu(II)-catalyzed reactions by Shtamm et al (1979) is identical to that proposed by the same authors to explain the reaction in the absence of O_2 . Here two Cu(II) ions act coopertatively to oxidize ascorbate to dehydro-ascorbate. The data have implication in terms of O_2 consumption for initation steps involving other metal species such as Cu(II) binuclear complexes (Jameson and Blackburn,1975), $HgCl_2$ (Sanehi et al,1975) or perhaps V(V).

2.3.0 Dependence of the effects of metal catalysts on O_2 is modulated by reaction conditions

The observed dependence of Cu(II)-catalyzed reactions on the O_2 partial pressure has been a key element in arguments for various mechanisms to explain the overall oxidation reaction. The reaction order with respect to O_2 has been established to be 0.5 (Jameson and Blackburn,1975, 1976a,b; Shtamm et al,1974,1977,1979; Shtamm and Skurlatov,1974), although the reaction order is dependent on reaction conditions (Jameson and Blackburn,1975; Shtamm et al,1974,1979). In addition, the reported reaction orders with respect to both Cu(II) and ascorbate vary from 0.5 to 1.0, varying with reaction conditions (Barron et al,1936; Weissberger et al,1944; Taqui Khan and Martell,1967a; Jameson and Blackburn,1975; Shtamm et al,1974; 1979). These include: experimental procedures such as the rate of stirring (Ogata et al,1968), the presence of ligands (Shtamm et al,1979; Jameson and Blackburn,1976a; Schwetnerova et al,1976) and the nonadditive influence of adventitious metals such as Fe(III) (Dekker and Dickinson,1940; Shtamm et al,1979). At pH < 3.85 Fe(III) oxidizes ascorbate more effectively than Cu(II) both in the absence and presence of O_2 (Taqui Khan and Martell,1967a; Martell,1982), at neutral pH ca. 7.0, little or no catalysis is observed (Euler et al,1933; Barron et al,1936; Buettner,1986; Thomas et al,1988). In fact, addition of exogenous Fe(III) (10 μM) to chelex-treated phosphate buffer at pH 7.0 actually slows the rate of oxidation as compared to ascorbate alone in untreated buffer while Cu(II) causes rapid oxidation of ascorbate (Buettner,1986, 1988). The relative concentrations of ascorbate and metal catalyst may also be important (Silverblatt et al,1943; Ogata et al,1968). For example, at lower relative ascorbate concentrations (pH ca 3.0) the rate of Cu(II)-catalyzed oxidation of ascorbate is independent of the oxygen partial pressure (Ogata et al,1968). In addition, at very low Cu(II) concentrations, the rate of ascorbate oxidation increases faster than the concentration of the metal. In contrast, at higher concentrations of the metal, the rate increase becomes proportional to the increase in copper concentration (Weissberger and LuValle,1944). Under a given set of reaction conditions then, the dependence of the effects of a metal catalyst on the O_2 partial pressure is

primarily a reflection of the ability of the metal to propagate the overall reaction through the reduction of O_2.

2.4.0 Reduction of O_2 by different metal ions in the presence of ascorbate requires more complete characterization

One of the objections to the "metal-catalyzed Haber-Weiss reaction" as a source of OH· has been the fact that O_2^- is a less effective reductant than many other biological molecules. The relative ability of reduced metal ions to donate an electron to oxygen in the presence of ascorbate may determnine steady state levels of O_2^-.With the exception of Cu(I) (Dekker and Dickinson,1940; Shtamm et al,1979), the relative abilities of different metal ions to reduce oxygen in the presence of ascorbate are not known. Iron(II) and Mn(II) reportedly catalyze electron transfer from ascorbate to O_2 only at high pH (Euler et al,1933; Barron et al,1936; Taqui Khan and Martell,1967a; Halliwell and Foyer,1976; Thomas et al,1988). For example at pH 7.3 in phosphate buffer 10 μM Fe(II) caused little or no loss of ascorbate (100 μM) (Matsumura and Pigman,1965). Reduction of V(IV) to V(II) was suggested by Taqui Khan and Martell (1967b) to explain V(IV)-catalyzed oxidation of the neutral form of ascorbate in the pH range 1.75-2.85. However, the V(IV)-stimulated oxidation of NADH is dependent on air oxidation of the metal ion to V(V) (Liochev and Fridovich,1987).

2.5.0 O_2^- does not propagate metal-catalyzed oxidations of ascorbate

Ascorbate and semidehydroascorbate are both relatively unreactive towards O_2 (Yamazaki and Piette,1961, 1963; Bielski and Richter,1973; Bielski et al,1975; Schuler, 1977). For this reason, the air autoxidation of reduced metal ions is the most likely source of any O_2^- formed during ascorbate oxidation. Ascorbate is oxidized by O_2^- (Allen and Hall,1973; Nishikimi,1975; Nanni et al,1980; Cabelli and Bielski,1983; Thomas et al,1988) and Shtamm et al (1979) report that O_2^- is involved in Cu(II)-catalyzed oxidations of asorbate. Nevertheless, a role for O_2^- as an intermediate in the air oxidation of ascorbate either in the absence or presence of added metals is unlikely (Halliwell

and Foyer,1976; Scarpa et al,1983). This is because superoxide dismutase inhibits no more than equivalents of albumin (Halliwell and Foyer,1976; Gutteridge and Wilkins,1982; Lovstad,1987). The lack of involvement of O_2^- in Cu(II)-catalyzed reactions may be due to two electron transfer to O_2 from a Cu(II) redutant complex (Jameson and Blackburn,1975). Iron(II)-catalyzed oxidations of ascorbate at pH 10.2, (but not lower pH) are inhibited by superoxide dismutase since at this pH Fe(II) rapidly reduces O_2. The resultant O_2^-, not the Fe(III)) oxidizes ascorbate (Halliwell and Foyer,1976). Further, participation of O_2^- in aerobic autoxidations of Fe(II) and V(IV) may be buffer dependent (Tadolini,1987; Wu, 1987). Reactions of manganese with O_2^- involve MnO_2^+ transients and Mn(III) complexes which oxidize both ascorbate and dehydroascorbate (Bielski,1985; Thomas et al,1988). Vanadium peroxo complexes reportedly formed by V(V) or V(IV) and O_2^- can oxidize NADH (Darr and Fridovich,1984,1985). However, no other data concerning the possible involvement of such radicals or O_2^- in oxidations of ascorbate by other metal catalysts have been presented to date.

2.6.0 Endogenously generated H_2O_2 has little effect on the rate of ascorbate oxidation

The extent to which H_2O_2 to can substitute for, or compete with O_2 in ascorbate oxidation catalyzed by different metals is of interest. Thus, hydrogen peroxide may amplify the effects of metal ions by accelerating redox cycling, and also through metal-catalyzed decomposition to OH· which effectively oxidizes ascorbate (Anbar and Neta,1967). Catalase fails to slow oxidations of ascorbate, and therefore, accumulation of H_2O_2 has little effect either in the absence or presence of added Fe(III) or Cu(II) at acid pH (Decker and Dickinson, 1940; Silverblatt et al,1943; Breslow and Lukens,1960; Khan and Martell,1967a; Ogata et al,1978). In the 'uncatalyzed' reaction, H_2O_2 formation varies with pH, and the amount of H_2O_2 formed does not increase linearly with ascorbate concentration (Silverblatt et al,1943). In Cu(II)-catalyzed reactions, H_2O_2 production is faster at higher ascorbate and/or metal concentrations and at higher pH. Nevertheless, steady state levels of H_2O_2 decrease as a result of slow H_2O_2 decomposition by Cu(II) (Silverblatt et

al,1943; Weissberger et al,1944; Taqui Khan and Martell,1967a). When catalase inhibits Cu(II)-catalyzed oxidations at pH 7.4, it is as a result of binding of copper ions (Lovstad,1987). Information concerning the effects of other metal ions on endogenous H_2O_2 production from ascorbate is needed. Vanadium was unique in that in V(V)- or V(IV)-catalyzed oxidations of 6-OHDA, no H_2O_2 is produced (Steele et al,1988). This reflects a catalase-like activity of vanadium. In contrast, iron demonstrates considerable peroxidatic activity, and Cu(II) a more limited catalatic activity (Zuberbuhler,1967; Steele et al,1988).

2.7.0 Exogenous H_2O_2 may increase the effectiveness of a number of different metals in oxidizing ascorbate

Higher concentrations of H_2O_2 may amplify the effects of metal catalysts through acceleration of redox cycling and/or the formation of powerful oxidant species such as OH·. Addition of exogenous H_2O_2 increases the rate of Cu(II)-catalyzed ascorbate oxidation in unbuffered solutions (Dekker and Dickinson,1940). A thiobarbituric acid-reactive product is reportedly formed from ascorbate as a result of attack by OH· formed from Fenton decomposition of H_2O_2 by Cu(II) (Gutteridge and Wilkins,1982). Iron (II) and V(IV) also decompose H_2O_2 to OH· (Fenton,1894; Keller et al,1987; Liochev and Fridovich,1987) and so may oxidize ascorbate via similar mechanisms. Reactions of this type constitute "non-classical" Fenton reactions in which ascorbate rather than O_2^- serves as the primary reductant of metal ions. In addition, V(V) peroxo complexes oxidize a variety of organic substrates (Mimoun et al,1983; Djordjevic and Wampler,1984) and may also oxidize ascorbate. The effects of these and other metal ions on ascorbate oxidation in the presence of added H_2O_2 deserve clarification.

The current study addresses the need for a comprehensive survey of the effects of different metals of physiological and/or toxicological significance on ascorbate oxidation at pH close to neutrality. Both transition (Cu(II), Fe(II), Fe(III), Mn(II), V(IV), V(V)) and non-transition (Al(III), Cd(II),

Hg(II), Pb(II)) metal ions were tested. The specific questions we sought to answer were - (1) To what extent can different metals oxidize ascorbate directly in the absence of O_2?, (2) Are reactions of metals with semidehydroascorbate quantitatively significant?, (3) To what extent does redox cycling of metal ions contribute to their observed catalytic effects in the presence of O_2?, (4) To what extent do reaction conditions (buffer type, pH) modify the mechanisms of action of individual metal catalysts, ? (5) Do endogenous O_2^- and/or H_2O_2 contribute significantly to the rate of oxidation either in the 'uncatalyzed' reaction or in the presence of metal catalysts?, (6) In the presence of exogenous H_2O_2, are there observable differences in the ability of metal different metal catalysts to redox cycle and thus participate in classical or non-classical Fenton reactions?, (7) On the basis of experiments with added metal ions, can the catalytic effects of adventitious metals present in water supplies and/or buffer reagents be attributed to one or more specific metal ions?, (8) Can general principles concerning the ability of metal ions to participate in free radical reactions in the presence of ascorbate and perhaps other biological reducing agents be formulated? The results of the current experiments confirm significant differences among the metal ions tested with regard to effectiveness in oxidizing ascorbate.

B. MATERIALS AND METHODS

4.0.0 MATERIALS

Distilled, deionized water (specific resistivity >10 megaohm cm^{-3}) was used to prepare all stock solutions. All stock solutions were prepared fresh daily, immediately prior to the commencement of each experiment. All other reagents were of reagent grade. l-Ascorbic acid, potasssium phosphate and ferrous sulphate were from BDH Chemicals. HEPES (N-2-Hydroxyethyl-piperazine-N'-2-ethane sulfonic acid) buffer, cadmium chloride, mercuric chloride, sodium orthovanadate were from Sigma Chemical Company, cupric acetate and ferric chloride from Fisher Scientific, vanadyl sulfate trihydrate from Aldrich Chemical Company, lead chloride from Matheson, Coleman and Bell, aluminum chloride from Anachemia Ltd. and manganous sulphate monohydrate from Malinckrodt. Crystalline, bovine blood superoxide dismutase (3300 units/mg protein) and bovine serum albumin (Type V) were from Sigma Chemical Company. Catalase (from bovine liver; 65,000 units/mg) was obtained from Boehringer-Mannheim.

5.0.0 METHODS

Ascorbate stock solutions (and in the case of anaerobic reactions all stock solutions) were prepared under anaerobic conditions. An atmosphere of nitrogen (Linde high purity grade, < 5 ppm oxygen) was introduced into rubber-capped vials by means of a VirTis gas manifold by at least five cycles of evacuation to boiling and reintroduction of nitrogen to a slight positive pressure so as to minimize subsequent contamination by oxygen. Aliquots were then withdrawn using a gas-tight syringe. The potency of ascorbate stock solutions was confirmed by recording of the change in absorbance of control reactions involving ascorbate alone at fixed time intervals.

Aerobically, buffers were bubbled with water-saturated air (previously bubbled through deionized distilled water) for at least 30 min. prior to each experiment in order to achieve air saturation. For anaerobic reactions, aliquots of the appropriate buffer were placed in a quartz

Capcell™ spectrophotometer cell (TCS Medical Products), then bubbled for the same period of time with nitrogen (previously bubbled through an aqueous solution of sodium sulfite so as to further remove contaminant oxygen) and introduced into the cuvette by means of a fine gauge hypodermic needle inserted through the top of the sealed spectrophotometer cell. Aliquots from anaerobic stock solutions were then added by means of hypodermic syringes, the cuvette and all stock solutions being immediately resealed. All (aerobic and anaerobic) reactions were initiated by addition of metal ions alone or concomitantly with H_2O_2. Oxygen consumption was monitored by means of a YSI Clark polarographic O_2 probe in a 10.0 ml cell equipped with a stirrer and thermostatted water jacket connected to a Beckman strip chart recorder. Fresh O_2 probe membranes were installed prior to commencement of each experiment so as to avoid contamination by metal ions and/or buffers. Where proteins were added to reaction mixtures, the measurement chamber was thoroughly washed between individual reactions since it was determined that progressive adsorption of enzymes and/or serum albumin biased measurements. Ascorbate oxidation was followed at 265 nm (McDermott et al,1968; Puget and Michelson,1974). A molar extinction coefficient of $E_{265}=1.5 \times 10^4$ $M^{-1}s^{-1}$ (Scarpa et al,1983) was used for calculations of the stoichiometries observed. Final volumes of 2.5 ml and 5.0 ml were employed for spectrophotometric and polarographic assays respectively. Wavelength scans conducted for reactions involving ascorbate alone and in the presence of added metal ions in both potassium phosphate and HEPES buffers (50 mM, pH 7.4, 20 °C) indicated no intereference from metal ions at 265 nm.

Statistical analysis of the data was accomplished by means of t-tests and one-way ANOVA, with the level required for significance determined in advance to be $p < .05$.

C. RESULTS

6.0.0. OXIDATION OF ASCORBATE DEPENDS ON REACTION CONDITIONS AND THE PRESENCE OF METALS

6.1.0 Autoxidation of ascorbate is faster at pH 8.0 than pH 7.4, and in phosphate than HEPES buffer

The extent to which even trace amounts of contaminant metal ions in water supplies and buffer reagents may accelerate ascorbate oxidation is well documented (McNair Scott,1961; Shtamm et al,1979; Buettner,1986, 1988). The steady state rates of control oxidations (ascorbate alone) varied with buffer type (phosphate > HEPES) and pH (7.4 > 8.0). This variability is due to adventitious transition metal ions. Using typical analytical reagent grade buffer salts, oxidations of ascorbate occur at rates of 2.7×10^{-7} μM s^{-1} and 9.5×10^{-8} μM s^{-1} in phosphate and HEPES buffers respectively at pH 7.4 , and 3.7×10^{-7} μM s^{-1} and 2.6×10^{-7} μM s^{-1} phosphate and HEPES buffers at pH 8.0. Thus, the rates observed in the same buffer at pH 8.0 and phosphate buffer at pH 7.4 and 8.0 were 2.6- , 4- and 3.4-fold greater than the rate in HEPES buffer at pH 7.4. Superoxide dismutase (5.0 U/ml) inhibited reactions with and without added metal ions. However, this inhibition could not be attributed to its catalytic activity since an equivalent weight of albumin inhibited to the same or a greater extent. Thus, under the experimental conditions employed, the contribution (if any) of O_2^- to the effects of even the most effective of the metal ions tested was insignificant. Similarly, H_2O_2 was not involved since catalase (20 U/ml) had no observable effect under any conditions.

6.2.0 Anaerobically only Cu(II) and Hg(II) accelerated ascorbate oxidation

Substitution of phosphate buffer for HEPES slowed oxidations in the presence of Cu(II) or Hg(II). HEPES buffer was used to minimize possible interference resulting from complexation of metals by buffer ions , anaerobic reactions were conducted in HEPES buffer (50 mM, pH 7.4 and 8.0, 25°C). Of the metal ions tested, only Cu(II) and Hg(II) produced measurable ascorbate oxidation anaerobically (Fig. 1.0). The other metals tested were Al(III), Cd(II), Fe(II), Fe(III), Pb(II), Mn(II), V(IV) and V(V). For all of these, accelerations were less than 0.5% and attributable to

contamination of the reaction mixture by small amounts of oxygen (data not shown). Of the initial 100 μM ascorbate present, 50 μM Cu(II) oxidized 13 μM and 12 μM at pH 7.4 and 8.0 respectively, and 50 μM Hg(II) oxidized 24 μM and 23 μM (Figure 2.0) (E_{265}=1.5 x 10^4 M^{-1} s^{-1}; Scarpa et al,1983). The different stoichiometries observed for the two metals must be explicable in terms of the nature of the reactive metal species and reactions of the metal ions with semidehydroascorbate.

6.3.0 Aerobically only Cu(II), Hg(II) and V(V) accelerated oxidation of ascorbate

Aerobically (as anaerobically) only Cu(II) and Hg(II) accelerated ascorbate oxidation under all conditions of buffer type and pH. Each metal ion produced a distinctive pattern of ascorbate oxidation (Fig. 2.0). In the presence of Cu(II), virtually all of the ascorbate present was oxidized within approximately 250 seconds. However, Hg(II) exhibited effects similar to those observed anaerobically, causing a rapid decrease in A_{265} initially, whereupon the rate of oxidation fell to a value similar to those for control oxidations of ascorbate alone. Phosphate decreased the maximal (initial) rates of oxidation by both Cu(II) (-80% at pH 7.4 and -73% at pH 8.0) and Hg(II) (-23%, -24%) as compared to the maximal rates observed in HEPES buffer (Fig. 3.0). Thus, in phosphate buffer the initial rates of ascorbate oxidation for Hg(II)-catalyzed reactions were at least 2.5-fold greater than those for Cu(II)-catalyzed reactions, while in HEPES buffer no significant difference was observed. Steady state rates of aerobic ascorbate oxidation in reactions involving Cu(II) were at least 5-fold greater than those involving Hg(II) under all conditions of pH and buffer type (Fig. 4.0). For reactions involving Cu(II), steady state rates of ascorbate oxidation were 6.7-fold greater than control rates in phosphate buffer at both pH 7.4 and 8.0, and 10.8- and 4.0-fold greater in HEPES buffer at pH 7.4 and 8.0 respectively, while the steady state rates in phosphate buffer exceeded those in HEPES by +29% at pH 7.4 and + 63% at pH 8.0. Steady state rates of oxidation in the presence of Hg(II) did not differ from the rates observed for control oxidations of ascorbate. Thus Cu(II) but not Hg(II) redox cycles in the presence of ascorbate and O_2.

Further, neither Cu(II)- nor Hg(II)-catalyzed reactions were inhibited by superoxide dismutase (5 U/ml) or catalase (20 U/ml).

Vanadium(V), although less effective than Cu(II) or Hg(II), caused a transient acceleration in the rate of oxidation in phosphate buffer at both pH 7.4 (+165 %) and 8.0 (+86 %) but not in HEPES buffer (Fig. 5.0). The maximum rates of ascorbate oxidation observed in the presence of all other added metal ions in all combinations of buffer type and pH were virtually identical to those for ascorbate alone. Not surprisingly, superoxide dismutase (5 U/ml) or catalase (20 U/ml) were also ineffective in the presence of these other metal ions (data not shown).

7.0.0 REDUCTION OF O_2 BY ASCORBATE IS AFFECTED BY REACTION CONDITIONS, Cu(II)

7.1.0 Effects of H_2O_2 decomposition on measures of O_2 consumption

As noted by previous workers, O_2 evolution as a result of decomposition of endogenous H_2O_2 may bias measurements of O_2 consumption by ascorbate, alone and especially in the presence of added metals. The extent of hydrogen peroxide decomposition was significant in all reactions including ascorbate controls since although approximately one half of the total O_2 consumed should be regenerated on addition of catalase (20 U/ml) if H_2O_2 was the only product, in every case, only approximately one quarter was regenerated. This effect is attributable to the catalytic activity of adventitious metal ions towards hydrogen peroxide. However, since (with the exception of reactions involving vanadium) H_2O_2 decomposition in the presence of added metals did not differ significantly from that for controls reactions (ascorbate alone) (see section 7.4.0). We have therefore reported the observed (uncorrected) rates of O_2 consumption and total amounts of O_2 consumed.

7.2.0 Maximum rates of O_2 consumption by ascorbate alone were highest in phosphate buffer at pH 8.0

Maximum rates of O_2 consumption by ascorbate (alone) were lowest in HEPES buffer at pH 7.4 (8.6×10^{-8} μM s^{-1}), with the observed rates in phosphate buffer at pH 8.0 and HEPES buffer at pH 7.4 and 8.0 being 2.8-, 2.9- and 3.9-fold greater (Fig. 6.0). Reactions in HEPES were more susceptible to modulation by pH. Maximum rates of O_2 consumption and ascorbate oxidation were similar under all conditions (Fig x.x). The average amount of H_2O_2 remaining when control reactions of ascorbate alone were near completion was smaller in phosphate buffer (16 and 17 μM at pH 7.4 and 8.0) as compared to HEPES buffer (32 and 28 μM). These differences cannot be accounted for solely on the basis of the small differences in total oxygen consumption (93, 100 μM in phosphate and 104 and 122 μM HEPES buffer at pH 7.4 and 8.0). The average total oxygen consumption for ascorbate alone and with added metal ions increased in the order phosphate pH 7.4 (86 μM) < phosphate pH 8.0 (95 μM) < HEPES pH 7.4 (106 μM) < HEPES pH 8.0 (108.3 μM) (Fig.6.0).

7.3.0 Only Cu(II) accelerated O_2 consumption by ascorbate

Only Cu(II) of all the metals tested consistently accelerated the maximum rates of O_2 consumption over ascorbate controls under all conditions (Fig. 6.0, 7.0). Oxygen consumption for reactions involving ascorbate alone and in the presence of Cu(II) or Hg(II) in 50 mM HEPES buffer, pH 7.4, 20° C is shown in Fig. 6.0. As a percentage of the rates for control reactions of ascorbate alone, increases of 378%, 548%, 1012% and 461% were observed in phosphate and HEPES buffers at pH 7.4 and 8.0 respectively. In contrast to spectrophotometric studies where the effects of buffer type on catalysis by Cu(II) were greater than those of pH, the reverse was true with regard to oxygen consumption. Thus in phosphate buffer the rate at pH 8.0 was 8-fold greater than that at pH 8.0, while in HEPES buffer a 2-fold difference was observed. In phosphate buffer at pH 7.4 slightly less (-12.5%) oxygen was consumed as compared to ascorbate controls, and in HEPES buffer at pH 7.4 33.4% more, however approximately equal amounts were consumed at pH 8.0 in both

buffers (Fig.8.0a,b). Maximum rates of O_2 consumption in the presence of Hg(II) differed from ascorbate controls by -2.3%, -32.9%, -1.5% and +6.8 % in phosphate and HEPES buffers at pH 7.4 and 8.0 respectively. Total O_2 consumption for Hg(II)-catalyzed reactions ranged from 11% to 36% less than for reactions involving ascorbate alone. Maximum rates of ascorbate oxidation exceeded maximum rates of O_2 consumption for both Cu(II) and Hg(II) under all conditions (Fig. 9.0). Cu(II) or Hg(II) did not consistently increase or decrease H_2O_2 production under any reaction conditions (Fig. 8.0).

7.4.0 Iron or vanadium had little effect on O_2 consumption, but decreased the yield of H_2O_2. at pH 7.4

As shown in Fig. 6.0, with the exception of reactions involving Fe(II) in phosphate buffer at pH 8.0, neither the reduced nor the oxidized forms of iron or vanadium greatly accelerated the maximum rates of O_2 consumption over ascorbate controls. The lack of effect of Fe(II) and V(IV) in particular on the maximum rates of O_2 consumption, is surprising since both metal ions effectively reduce oxygen in both buffers at pH 7.4 and 8.0. Both V(IV) and Fe(II) increases the initial (maximum) rates of O_2 consumption in phosphate buffer at pH 7.4 (+50%; +77%) and pH 8.0 (+76%; +249%), and in HEPES buffer at pH 8.0 (+16%; +19%). Only Fe(II) caused a similar effect in HEPES buffer at pH 7.3 (+78%). However, neither vanadium nor iron affected the progress of the overall reaction, these effects on ascorbate oxidation and oxygen consumption being transient. These effects are explicable in terms of ascorbate-mediated inhibition of O_2 consumption by Fe(II) or V(IV) alone. Under all reaction conditions, ascorbate inhibited the maximum rates of oxygen consumption by V(IV) and Fe(II) alone by at least 80% and 83% respectively, and total oxygen consumption by at least 80% and 75%. In contrast, only minimal inhibition was observed in the presence of 5 U/ml superoxide dismutase (20%, 22%) or 20 U/ml catalase (10%,12%) (data not shown).

Total O_2 consumption for reactions involving Fe(II) or Fe(III) did not differ significantly from ascorbate controls in either phosphate or HEPES buffer at pH 7.4 or 8.0. However, under all reaction conditions except in HEPES buffer at pH 8.0, both metal ions decomposed slightly more H_2O_2 than ascorbate controls. The apparent total O_2 consumptions for V(IV)- and V(V)-containing reaction mixtures were slightly lower than for ascorbate controls in phosphate buffer at both pH 7.4 (-6%, -26%) and pH 8.0 (-41%, -26% at pH 8.0). However, these values greatly underestimate the true values since virtually all the H_2O_2 formed during the course of the reaction was either decomposed via catalatic actions of V(V) (Brooks and Sicilio,1967) or tied up in the form of V(V)-peroxo complexes. In contrast to reactions involving Cu(II) or Hg(II), under all conditions, maximal rates of ascorbate oxidation were equal to or slightly less than maximal rates of O_2 consumption (Fig. 10.0).

7.5.0 Al, Cd, Mn or Pb had no effect on either O_2 consumption or H_2O_2 decomposition

As in the case of ascorbate oxidation *per se,* reactions involving Al(III), Cd(II), Mn(II), Pb(II)) demonstrated maximum rates of O_2 consumption and total O_2 consumption virtually identical to control rates (Fig.6.0, 8.0). However, Al, Cd, Mn and Pb slowed H_2O_2 decomposition under all conditions. Maximal rates of ascorbate oxidation and O_2 consumption were very similar under all conditions (Fig. 10.0).

8.0.0 COPPER, IRON AND VANADIUM REDUCE H_2O_2 IN THE PRESENCE OF ASCORBATE

8.1.0 Exogenous H_2O_2 dramatically accelerates oxidation of ascorbate in HEPES buffer

The effects of exogenous H_2O_2 on oxidations of ascorbate alone were buffer dependent (Fig.11.0). In phosphate and HEPES buffers (50 mM, pH 7.4, 20° C), addition of exogenous H_2O_2 (final concentration 2.5 mM) increased the maximum rates of ascorbate oxidation in control reactions by 2.5-fold and 113-fold respectively. Thus, in the presence of O_2 alone the maximum rates of

oxidation for ascorbate alone at pH 7.4 were almost 3-fold higher in phosphate buffer than in HEPES buffer. However, in the presence of added H_2O_2 the rates in HEPES buffer were significantly greater than those in phosphate. Further, although exogenous H_2O_2 caused a linear decrease in A_{265}, an curvilinear decrease was apparent in HEPES buffer.

8.2.0 Exogenous H_2O_2 Cu(II), Fe(II) and V(IV) or V(V) accelerated ascorbate oxidation

In combination with exogenous H_2O_2, added metal ions increased the maximum and/or steady state rates of oxidation (Fig. 11.0). As for control reactions (ascorbate alone), both the absolute rates and relative increase over reactions without added H_2O_2 were consistently lower in phosphate than HEPES buffer. Equally as important, in both buffers, only V(V) > V(IV) > Fe(II) > Cu(II) increased the absolute maximum rates of ascorbate oxidation over that for ascorbate plus H_2O_2, while increases in the steady state rates of ascorbate oxidation were observed only for Cu(II) (11.4-, 9.6-fold) > V(V) (1.95-, 8.7-fold) > V(IV) (2.4-, 9.2-fold). The relative increases in the rates of ascorbate oxidation were again lowest in phosphate buffer. These values indicate the extent to which all metals added in phosphate buffer, and Fe(III), Mn(II), Al(III), Cd(II), Pb(II) in HEPES buffer, moderated the stimulatory effects of added H_2O_2 as compared to reactions of ascorbate, O_2 and H_2O_2 alone. Although the initial rapid oxidations of ascorbate by Hg(II) were relatively unaffected by the addition of H_2O_2 (Fig.12.0), the later phases of the reactions were accelerated in comparison with reactions involving Hg(II) and ascorbate alone, but were actually slower than the steady state rates for ascorbate and H_2O_2.

D. DISCUSSION

The present results will be discussed in terms of: 1) the extent to which ascorbate reduces specific metal ions, 2) the extent to which individual metal ions reduce O_2 in the presence of ascorbate, 3) the extent to which metal ions as opposed to O_2^- serve to propagate the overall oxidation reaction, and 4) the reaction mechanisms underlying generation of propagating radical species.

9.0.0 ROLES OF METAL IONS

9.1.0 Kinetic and steric factors overshadow reduction potentials in determining the reactivity of different metal ions towards ascorbate

Kinetic and steric factors overshadow reduction potentials in determining the reactivity of different metal ions towards ascorbate. Reduction potentials for species related to ascorbate are presented in Fig. x.x. On the basis of reduction potentials alone, the order of effectiveness of different metals in direct oxidations of ascorbate to dehydroascorbate should be: copper (E^o (Cu(II)/Cu(I)) = 0.15 V) < iron (E^o (Fe(III)/Fe(II)) = 0.77 V) < $HgCl_2$ < (E^o (2Hg(II)/Hg_2^{2+}) = 0.92 V) < V(V) (E^o (VO_2^+/VO^{2+})) =1.00 V and (E^o (VO^{2+}/V(III)) = 1.36 V) < Mn(II) (E^o (Mn(III)/Mn(II)) =1.53 V). Yet only Hg(II) > Cu(II) were effective in the absence of O_2. Among these, in increasing order of effectiveness, Cu(I) > Fe(II) > V(IV) > Mn(II) should reduce O_2 ($E^{o'}(O_2/HO_2\cdot)$ = 0.3 V at pH 7.0). In the presence of oxygen, the most effectively redox cycled metals should be those with a reduction potential somewhere between that of the $HA^-/A\cdot$ couple and the one electron reduction potential for O_2. However none of the metals in the current study had a reactivity fully explicable on this basis. Thus, full explanations of the anaerobic and aerobic effects of each metal must necessarily encompass steric and kinetic factors.

9.2.0 Stoichiometries of anaerobic oxidations of ascorbate are explained by the nature of the active metal species, and reactions of the metal with semidehydroascorbate

Of the metals tested, only Cu and Hg can accept electrons from ascorbate. Explanations of the anaerobic effects of these two metals involve questions relating to their reduction potentials, the nature of the active metal species, and reactions of the metal ions with semidehydroascorbate.

Ascorbate is a carboxylic acid which in the furanose or pyranose forms has a bifunctional ene-diol group built into a heterocyclic lactone ring (Smith and Martell,1977). At both pH 7.4 and 8.0, HA^- is the major ascorbate species, with low concentrations of H_2A and A^{2-}. The most likely metal binding sites are the hydroxyl groups associated with carbon atoms 2 and 3 (Khan and Martell,1967a,b; Ogata et al,1968; Jameson and Blackburn,1975; Martell,1982). Perhaps the best studied ascorbate:metal complexes involve Cu(II). Although only relatively weak 1:1 metal chelates of ascorbate have been reported (Martell,1982), some workers have suggested mechanisms involving 1:1 (Cu:ascorbate) complexes (Ogata et al,1968). Other mechanisms involve cooperative oxidation of ascorbate by two Cu(II) ions. For example, Shtamm et al (1974, 1979) explained the anaerobic oxidation of ascorbate by Cu(II) at pH 2.7 - 4.0 in terms of the sequential binding of two Cu(II) ions:

$$Cu^{2+} + H_2A \leftrightarrow CuHA^+ + H^+ \leftrightarrow CuHA \leftrightarrow Cu_2A + H^+ \qquad (1)$$

Similarly, oxidation by Cu(II) binuclear complexes (Jameson and Blackburn,1975) also implies oxidation of one ascorbate molecule per two Cu(II) ions. Yet in the present study, anaerobically, Cu(II) oxidized only approximately one quarter of the amount of ascorbate expected by Ogata et al (1968) (12 μM at pH 7.4 in and 13 μM at pH 8.0).

9.3.0 Ligands and reaction conditions modulate the reactivity of Cu towards ascorbate

The redox properties of copper are strongly influenced by ligands (Jameson,1981). Phosphate decreased the maximum rate of ascorbate oxidation at either pH 7.4 or 8.0 in compasriosn with

reactions in HEPES buffer, in the presence or absence of O_2. This is due at least in part to impaired of cooperative interactions between Cu(II) ions and ascorbate and to modulation of the redox potential of the metal ion. Similarly, the differences between the present results and those of Shtamm et al (1974,1979) may be due to differences in reaction conditions. The concentrations of metal ions and ascorbate were much lower in the present study. Thus, whereas the current results were obtained in HEPES buffer at pH 7.4, 20° C using metal concentrations of 50 μM and and initial ascorbate concentration of 100 μM, Shtamm et al (1974,1979) conducted their studies in unbuffered solutions in the pH range 2.7 - 4.0, using Cu(II) concentrations of 5×10^{-5} - 10^{-3} M and ascorbate concentrations of 5×10^{-4} - 10^{-2} M. The occurrence of side reactions as a result of the higher absolute concentrations of reagents may also account in part for the discrepant stoichiometries.

9.4.0 Oxidation of ascorbate by Hg(II) results in insoluble Hg(I)

On the other hand, Hg(II) oxidized 24 and 23 μM ascorbate at pH 7.4 and 8.0 respectively. This is consistent with the mechanism of Sanehi et al (1975) in which one molecule of $HgCl_2$ reacts with ascorbate and a second with the semidehydroascorbate formed. The insoluble mercurous chloride thus formed precipitates out of solution.

$$HgCl_2 + AH^- \rightarrow AH\cdot + HgCl + Cl^- \quad (1)$$

$$AH\cdot + HgCl_2 \rightarrow A + HgCl + Cl^- \quad (2)$$

$$HgCl + HgCl \rightarrow Hg_2Cl_2 \quad (3)$$

Phosphate had relatively less effect on the initial rapid phase of ascorbate oxidation by Hg(II), probably because of the stronger oxidizing potential of this metal ion, and because cooperation between Hg(II) ions is not required.

9.5.0 Ascorbate does not reduce Fe(III) or V(V)

If in phosphate or HEPES buffer, most of the added Fe(III) or V(V) binds ascorbate, the inability of either metal ion to accept an electron seems to require a thermodynamic explanation. Since the data are not in the direction predicted by the redox potentials, the true explanation must be kinetic. Thus, both Fe(III) and V(V) must oxidize ascorbate via outer-sphere mechanisms, these being much more susceptible ot modulation by a variety of kinetic factors including pH and the effects of ligands. Two lines of evidence support this contention. First, addition of EDTA greatly increases the rate of ascorbate oxidation by Fe(III) (Taqui Khan and Martell,1967a; Martell,1982; Scarpa et al,1983; Buettner,1986). The reduction potential of Fe(III) is decreased when it complexes with EDTA (Noguchi and Nakano,1974), and the resulting Fe(III)-EDTA complex can catalyze ascorbate oxidation (Halliwell and Foyer,1976) via an inner sphere mechanism. Presumably this is because EDTA increases the reactivity of Fe(II) with O_2, so that redox cycling of iron is facilitated.

The inability of the other metal ions tested to oxidize ascorbate in the absence of oxygen at neutral pH is of interest since direct oxidations of ascorbate by Fe(III) and V(IV) at acid pH have previously been reported (Taqui Khan and Martell,1967a,b). At pH < 3.85 Fe(III) oxidizes ascorbate more effectively than Cu(II) both in the absence and presence of O_2 (Khan and Martell,1967a; Martell,1982). However, the lack of effect of added Fe(III) at neutral pH in the present study is in agreement with the results of Euler et al (1933); Barron et al (1936), Buettner (1986) and Thomas et al (1988). On the other hand at neutral pH V(V) oxidizes NADH (Vyskocil et al,1981) and 6-OHDA (Steele et al,1988) directly, so it is somewhat surprising that it demonstrates so little reactivity towards ascorbate. In the case of Fe(III) precipitation of the metal ion out of solution (Taqui Khan and Martell,1967a; Buettner,1986) may account in part for the lack of effect of Fe(III), however V(V) which would not be expected to precipitate out of solution is also virtually ineffective.

The reducing power of ascorbate in comparison with catecholamines, or steric factors relating to the nature of metal binding to the ascorbate molecule (Khan and Martell,1967a; Martell,1982; Buettner,1986) must underly the lack of reduction of these other metals. For example, the effects of Cu(II) and Hg(II) anaerobically may be due to their redox potentials, as well as the tightness of their binding to ascorbate. The redox potential of the Fe(II)/Fe(III) couple, as well as the weaker chelates that Fe(II) and likely Fe(III) form with ascorbate preclude electron transfer. The lack of effect of V(IV) and V(V) may be explained in similar terms. The reduction potentials for V(V)/V(IV) (E^o (VO_2^+/VO^{2+})) = 1.0V) and V(V)/V(III) (E^o (VO_2^+/V(III)))=1.36V) both favor ascorbate oxidation. The oxo group of VO^{2+} reportedly affects the rate-determining electron transfer step in the V(IV)-catalyzed oxidation of ascorbate at acid pH (Taqui Khan and Martell,1967b). Mn(II)-ascorbate chelates are also weaker than those of Cu(II) (Smith and Martell,1977), and those of Al(III) and Cd(II), Pb(II) stronger. More obviously, Fe(II) and Mn(II) do not generally demonstrate lower oxidation states while Al(III), Cd(II) and Pb(II) are not generally redox active.

10.0.0 ROLES OF OXYGEN

10.1.0 The ability of metal ions to oxidize ascorbate directly modulates the involvement of O_2

As in the case of other "autoxidizable" organic compounds such as catechols and thiols, in the absence of catalytic metals ascorbate does not reduce O_2 at neutral pH (Buettner,1988). Consumption of O_2 in the presence of ascorbate is therefore contingent upon the ability of the metal to oxidize ascorbate directly. The ability of the metal to oxidize ascorbate catalytically is contingent upon autoxidation of the metal. For example, Cu(II) reacts preferentially with ascorbate and O_2, while Hg(II) reacts with only with ascorbate. Thus, O_2 is not required for ascorbate oxidation by either Cu(II) or Hg(II), and O_2 consumption is increased only by Cu(II) - as a result of redox cycling of the metal ion. In the case of Cu(II), it has been suggested that O_2 may promote electron transfer within

ternary metal-ascorbate-O_2 complexes (Khan and Martell,1967; Schwertnerova et al,1976; Shtamm et al,1979). In agreement with the mechanism of Shtamm et al (1979) then, initiation of ascorbate oxidation by Cu(II) is dependent upon the cooperative actions of two Cu(II) ions, and does not require O_2. The possibility that cuprous metal-O_2-ascorbate complexes may serve as the primary oxidations of ascorbate in the propagation phase of the reaction (Schwertnerova et al,1976; Shtamm et al,1979) cannot be ruled out. However given the rapidity of the overall oxidation reaction, the lack of a requirement for O_2, the lack of a role for O_2 as a propagating species, and evidence for the involvement of the OH· (Morgan et al,1976; Shinar et al,1983; Samuni et al,1983; Chevion,1988; ; Higson et al,1988) or semidehydroascorbate (Morgan et al,1976; Davison et al,1986), and the site-specific nature of the toxic effects of Cu(II) and ascorbate in combination, the involverment of the proposed Cu(I)-O_2 species as propagating agents may be relatively

10.2.0 The dependence of Cu-catalyzed oxidations on O_2 partial pressure is modulated by ligands and adventitious Fe

Much controversy has surrounded the dependence of Cu(II)-catalyzed oxidations of ascorbate on O_2 partial pressure (reviewed in Zuberbuhler,1981). Cu(I) reportedly autoxidizes at approximately the same rate in the absence of ascorbate ($3.5 \times 10^4\ M^{-1}\ s^{-1}$) (Shtamm et al,1979) as in its presence ($5 \times 10^4\ M^{-1}\ s^{-1}$) (Zuberbuhler,1970). Further, the rate at which ascorbate is oxidized aerobically by Cu(II) is determined by the rate at which Cu(I) is reoxidized by O_2 (Shtamm et al,1979). The dependence of the 'uncatalyzed' reaction at neutral pH on O_2 partial pressure (Weissberger et al,1943) may be rationalized on the basis that reoxidation of Cu(I) is at least partly rate determining (Nord,1955a). In the present study, phosphate and HEPES buffers at pH 7.4 and 8.0, the maximum rates of copper-promoted ascorbate oxidation were 1.8-, 1.3-, 11- and 5-fold greater than the maximum rates of O_2 consumption. The rate-determining process in the redox cycling of the metal is therefore reoxidation. On this basis, the effects of phosphate on the steady state rates of oxidation, inhibition at pH 7.4 and acceleration at pH 8.0 (compared to reactions in HEPES buffer

at the same pH), represent mainly effects on reactivity towards O_2. Although phosphate decreases the ability of Cu(II) to oxidize ascorbate directly, it promotes reactions of copper with O_2 since steady state rates of Cu(II)-catalyzed reactions were greater in phosphate buffer.

Variations in the rates of ascorbate oxidation *per se* in the 'uncatalyzed' reaction in phosphate and HEPES buffer are likely partly due to the greater Cu(II) content of the former, differences in the Fe content may also modulate the rate of O_2 reduction. Untreated phosphate buffer solutions reportedly contain approximately 0.13 μM Cu(II) and 0.7 μM Fe (Buettner,1988). HEPES buffer reagents contain smaller amounts of both metals by virtue of their lesser affinity for metals. Notably, although added Fe(II) or Fe(III) does not accelerate ascorbate oxidation, some reactions catalyzed by Cu(II) are further accelerated by iron salts (Price,1898, Brode 1901, Schilow and Buligen,1913). This view agrees with previous findings, indicating a synergistic effect of Fe(III) on Cu(II)-catalyzed oxidations of ascorbate (Dekker and Dickinson,1940; Shtamm et al,1979).

10.3.0 Ascorbate inhibits oxidations of Fe(II) and V(IV) by O_2

Although Cu(I) readily reduces O_2 in the presence of ascorbate, ascorbate inhibits oxidations of Fe(II) and V(IV) by O_2. Except at high pH (≤ 10.2) or in the presence of EDTA (Michelson,1973; Halliwell and Foyer,1976), Fe(II) does not react rapidly enough with oxygen to allow redox cycling or O_2^- formation. Scavenging of O_2^- by ascorbate further limits redox cycling. Increased O_2^- production by Fe(II) in the presence of EDTA may also contribute to the stimulatory effects of this chelating agent. Reduction of O_2 by V(IV) also occurs more rapidly at higher pH. Both the rate and extent of O_2 consumption are greater than for Fe(II) (Wu, 1987; Steele, unpublished results). The ability of ascorbate to inhibit reduction of O_2 by V(IV) is thus even more remarkable, particularly since any V(V) and O_2^- formed should react to form strongly oxidizing peroxo complexes of vanadium (Darr and Fridovich,1984,1985). The extent to which ascorbate inhibited the air oxidation of Fe(II) and V(IV) in the present study dramatically illustrates the degree to which

redox cycling and propagation of the reaction by O_2^- are dependent on ligand binding by these metal ions.

The mechanisms underlying air autoxidation of Fe(II) differ in phosphate and HEPES buffers (Tadolini,1987a). In both buffers, increased ascorbate oxidation at high pH is most likely due to the increased rate of reaction of Fe(II) with O_2. (Michelson,1973; Halliwell and Foyer,1976). This is confirmed by the observation that the increase in pH from 7.4 to 8.0 had little effect on either the reactivity of added Cu(II), or the state of protonation of ascorbate. EDTA, which inhibits catalysis by Cu(II) (Samuni et al,1983; Shinar et al,1983; Lovstad,1987; Buettner, 1988), promotes ascorbate oxidation by by Fe(III). Thus, Fe(III)-EDTA oxidizes ascorbate directly (Halliwell and Foyer,1976) and EDTA accelerates the air autoxidation of Fe(II) (Kaden et al,1961; Halliwell and Foyer,1976; Samuni et al,1983; Buettner,1988). EDTA only partially inhibits the uncatalyzed oxidation of ascorbate (Halliwell and Foyer,1976; Buettner, 1988). Thus, iron rather than copper is responsible for the reaction in the presence of EDTA.

10.4.0 Superoxide does not contribute significantly to ascorbate oxidations at neutral pH

Under all conditions of buffer type and pH, and even at the lowest concentrations employed (5 U/ml), superoxide dismutase acted in a non-specific manner to chelate metals since equal amounts of albumin exerted identical or greater degrees of inhibition (Lovstad,1987). Therefore, superoxide does not contribute significantly to ascorbate oxidations at netral pH. There are also strong kinetic arguments for a lack of involvement of O_2^- in Cu(II)-catalyzed reactions (Shtamm et al, 1979). Some O_2^- is formed in the course of the Cu(II)-catalyzed reaction. However the mechanisms are uncertain, particularly since reactions of Cu(I) with O_2 may not result in the formation of O_2^-, but rather copper-oxygen species (Gampp and Zuberbuhler,1981). Aerobically semidehydro-ascorbate does not propagate Cu(II)-catalyzed ascorbate oxidations (Shtamm et al,1979). Reduction of O_2 reduction by Hg in any oxidation state is not thermodynamically feasible, and although significant

amounts of semidehydroascorbate may be formed in Hg(II)-catalyzed oxidations, it is does not reduce O_2. This is in keeping with the results of Bielski et al (1977) showing that even at pH 8.6, semidehydroascorbate reacts more rapidly with itself ($2.8 \pm 0.2 \times 10^5$ $M^{-1}s^{-1}$), dopamine ($3.6 \pm 0.4 \times 10^2$ M^{-1} s^{-1}) and cytochrome c (than with O_2 ($< 5 \times 10^2$ M^{-1} s^{-1}). Thus, the semidehydro-ascorbate radical is relatively unreactive and decays by disproportionation (Bielski et al,1977).

As noted in the preceding section, at neutral pH, reduction of O_2 by Fe(II) is relatively slow, and O_2^- appears to play no role in propagating the overall oxidation of ascorbate. Halliwell (1975) proposed that Fe(II)-EDTA, but not Fe(II) could react rapidly with O_2^-, while Fe(III)-EDTA but not Fe(III) oxidizes ascorbate (Halliwell and Foyer,1976). Thus, for reactions involving Fe(II) or Fe(III), the enhanced reduction of O_2 at high pH may serve to promote ascorbate oxidation by increasing O_2^- formation, and so increasing the rate of Fe(III) reduction by this species. We conclude that, since O_2^- involvement is difficult to demonstrate in experimental systems such as that employed in the present study, O_2^- is even less likely to propagate ascorbate oxidation*in vivo*.. However, O_2^-may be important as a source of H_2O_2.

11.0.0 ROLES OF HYDROGEN PEROXIDE

11.1.0 Reactions of some metals with H_2O_2 accelerate reactions with ascorbate

Hydrogen peroxide appears to be relatively stable towards ascorbate. Thus, H_2O_2 does not affect the rate-limiting step in either the uncatalyzed or metal-catalyzed reactions (Khan and Martell,1967). The primary pathway for H_2O_2 reduction in the presence of ascorbate at pH>7.0 has been may be side reactions with HA^- and/or its oxidation products (Weissberger et al,1943). Nevertheless, in the presence of added metals, ascorbate-driven Fenton-type H_2O_2 reduction reactions leading to formation of substantial amounts of $OH^\cdot$ may dominate (Winterbourn,1979; Kang,1987). If so, then the apparent consumption of H_2O_2 by Fe(II) at pH 7.4 and by V(IV) and V(V) at both pH 7.4 and 8.0 may be important. On this basis, the air autoxidations of either Fe(II)

or V(IV) involve formation of reactive intermediates by reactions of the metal ions with H_2O_2 product (Tadolini, 1987a; Steele et al,1988). Ascorbate oxidation by these metals may thus be limited by the availability of H_2O_2. Decomposition of H_2O_2 by other metals may occur to some extent, but be masked at higher pH by the presence of other metals and the greater instability of H_2O_2.

In the human body, the ratio of iron to copper is approximately 80:1. Ascorbate may therefore function as an effective antioxidant, restricting the involvement of iron in free radical reactions either by binding to the metal or by scavenging radical species. For example, Munkres (1979) found that treatment of germinated condidia of *Neurospora crassa* with 0.2 mM Fe(II) was 14- and 50-fold more mutagenic than UV irradiation or x-rays respectively. Ascorbate (2 mM) inhibited both the lethality and mutagenicity of Fe(II), the residual lethality of Fe(II) being completely inhibited by superoxide dimsutase. At the same time, neither the oxidized nor the reduced forms of either iron or vanadium *inhibited* ascorbate oxidation. If the majority of the catalytic effects of adventitious metals can be attributed to Cu(II) (Buettner,1986,1988), then iron- and vanadium- complexes with ascorbate may be susceptible to attack by Cu(II). However, in the subsequent section we will contend that ascorbate binding to iron or vanadium is likely to constitute a hazard by virtue of their reduction of H_2O_2.

11.2.0 The effects of Fe and V on ascorbate oxidation reflect a balance between reactions of the metal ions with O_2 and H_2O_2

Present in excess, H_2O_2 may allow more metals to contribute to ascorbate oxidation by facilitating redox cycling of the reduced metal ion, or by forming more powerful oxidizing species such as a metal-active oxygen complex, free $OH^{\cdot}$ or $OH^{\cdot}$ -like species. The acceleration of oxidation observed on addition of exogenous H_2O_2 to ascorbate in air further supports the importance of metal-H_2O_2 interactions. The effects of Fe(II) and Fe(III) are strongly influenced by the relative concentrations

of reactants. This follows from the observation that although exogenous H_2O_2 increased the maximum rate of oxidation in the initial phase of the reaction, the subsequent rates of oxidation were slower than for ascorbate alone. In the presence of H_2O_2, both V(IV) and V(V) oxidized ascorbate almost as rapidly as Cu(II). However, ascorbate oxidation in the presence of Hg(II) was actually decreased by addition of H_2O_2. Ascorbate oxidation is slowed by Al(III), Cd(II), Mn(II) and Pb(II), indicating either chelation of ascorbate by the metal or scavenging of some intermediate species Thus roles of Fe and V in ascorbate oxidation reflect a balance between lack of ability to promote O_2 consumption and a profound ability to promote H_2O_2 decomposition.

E. CONCLUSION

The conclusions to be drawn from the present study include the following: 1) Ascorbate readily oxidizes aerobically. However, despite the high reduction potential of ascorbate, kinetic barriers prevent its direct oxidation by O_2 or metal ions other than Cu or Hg. 2) Oxygen is not required for the reaction if stoichiometric amounts of Cu(II) are present. 3) Of these two metals, only Cu(II) redox cycles in the presence of ascorbate and O_2 , acting in a true catalytic fashion. 4) The actual or potential contributions of individual metal catalysts are significantly modified by reaction conditions, including the presence of O_2, buffer type (ligands) and pH. 5) Endogenous O_2^- and/or H_2O_2 do not contribute as kinetically accessible intermediates in the oxidation of ascorbate either in the 'uncatalyzed' reaction or in the presence of added metals.; 6) The presence of H_2O_2 allows catalysis by copper and vanadium and (to a limited extent) iron. Thus, Cu, Fe or V potentially participate in classical or non-classical Fenton reactions, and 7) Most if not all of the catalytic effects of adventitious metal ions can be attributed to Cu(II). This follows from the effects of added metals shown in Figs. 3.0, 4.0 and 5.0, and are in agreement with the results of Buettner (1988).

Since the catalytic effects of adventitious metals present in water supplies or buffer reagents (Buettner,1986,1988), can be can be mainly attributed to Cu(II), ascorbate oxidation is not in general Fe-mediated. The possible implications of this point with regard to interactions of ascorbate with metals in body fluids and tissues merit further investigation. Similarly, the differing abilities of Cu(II) and Hg(II) to oxidize ascorbate directly and redox cycle may have some bearing on ascorbate-mediated mechanisms of toxicity, particularly in tissues rich in ascorbate such as the central nervous system. Thus, superoxide dismutase does not inhibit DNA scission by Cu(II) and ascorbate, although catalase and OH· scavengers are effective (Morgan et al,1976). Superoxide dismutase also does not inhibit cleavage of phage R17 RNA in the presence of Cu(II) and ascorbate, although catalase and hydroxyl radical scavengers are effective (Wong et al,1974). The nature of the mechanisms underlying the catalytic effects of Fe or V in the presence of H_2O_2, in particular the yields of specific radical species also require reevaluation, particularly insofar as reactions of these metals with ascorbate are features of their toxic effects biologically. Finally, although manganese, cadmium and lead for example demonstrated no catalytic activity in the present experiments, the considerable body of indirect evidence linking the toxic effects of the latter two metals in particular to ascorbate metabolism *in vitro* and *in vivo* suggests that more detailed explanations, perhaps involving interactions with other metal ions such as copper or iron are required.

Fig.1.0

Anaerobic oxidation of ascorbate by successive additions of $CuSO_4$ or $HgCl_2$

The anaerobic oxidation of ascorbate (100 μM) in air-saturated HEPES/KOH buffer (50 mM, pH 7.4, 20° C) was followed spectrophotometrically in A_{265}. Buffer andstock solutions were made anaerobic according to the procedures described in the Methods section. Aliquots (5 μl) from $CuSO_4$ or $HgCl_2$ stock solutions were added where indicated, the initial metal ion concentration being 50 μM. The final volume was 2.5 ml. The upper and lower curves represent ascorbate oxidation in the presence of 50 μM $CuSO_4$, and $HgCl_2$ respectively.

Fig.1.0

Anaerobic oxidation of ascorbate by successive additions of $CuSO_4$ or $HgCl_2$

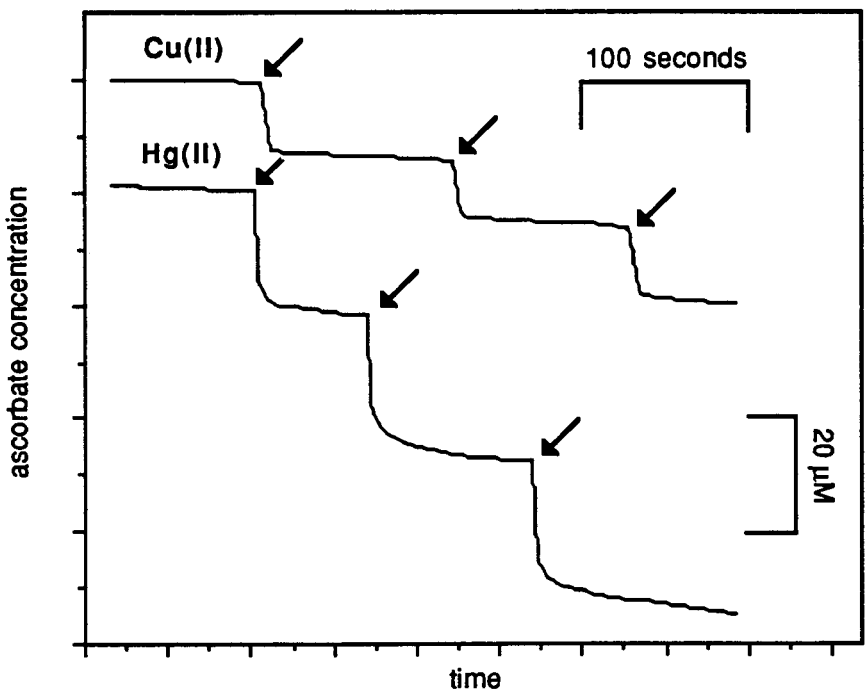


Fig. 2.0

Aerobic oxidation of ascorbate by $CuSO_4$ or $HgCl_2$: progress of reactions

The anaerobic oxidation of ascorbate (100 μM) in air-saturated HEPES/KOH buffer (50 mM, pH 7.4, 20° C) was followed spectrophotometrically in A_{265}. Buffer andstock solutions were made anaerobic according to the procedures described in the Methods section. Aliquots (5 μl) from $CuSO_4$ or $HgCl_2$ stock solutions were added where indicated to give a final metal ion concentration of 50 μM. The final volume was 2.5 ml. The top curve represents ascorbate oxidation in the absence of added metal, and the bottom left and right curves ascorbate oxidation for reactions involving 50 μM $CuSO_4$, and 50 $HgCl_2$ respectively.

Fig.2.0

Aerobic oxidation of ascorbate by $CuSO_4$ or $HgCl_2$: progress of reactions

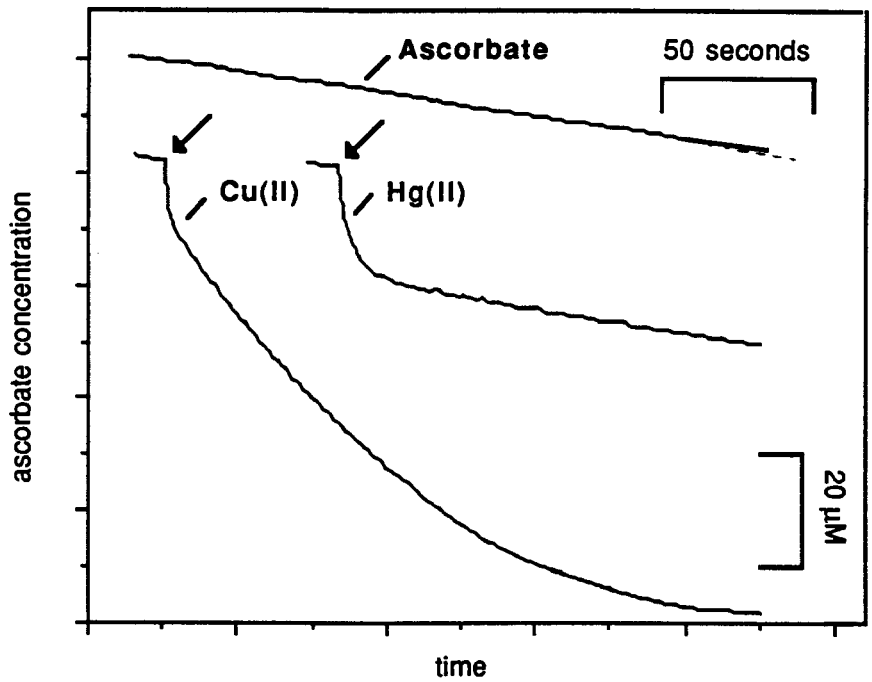


Fig. 3.0

Maximum rates of ascorbate oxidation by $CuSO_4$ or $HgCl_2$ under aerobic conditions

Maximum rates of ascorbate oxidation for reactions involving 100 μM ascorbate in the absence and presence of 50 μM $CuSO_4$ or $HgCl_2$ were followed as the decrease in A_{265}. Individual reactions were conducted in both potassium phosphate and HEPES/KOH buffers (50 mM, pH 7.4 or 8.0, 20° C, air-saturated), in a final volume of 2.5 ml. Reactions were initiated by addition of metal ion. Bars represent the means of at least 5 separate reactions, and error bars standard deviations.

Fig. 3.0

Maximum rates of ascorbate oxidation by $CuSO_4$ or $HgCl_2$ under aerobic conditions

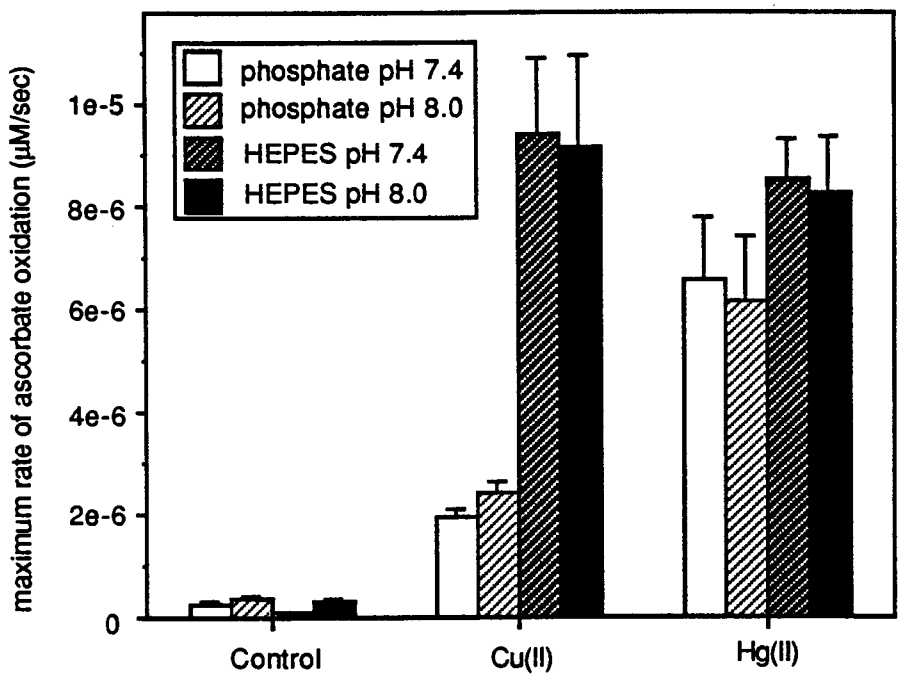


Fig. 4.0

Steady state rates of ascorbate oxidation by $CuSO_4$ or $HgCl_2$ under aerobic conditions

Steady state rates of ascorbate oxidation for reactions involving 100 μM ascorbate in the absence and presence of 50 μM $CuSO_4$ or $HgCl_2$ were followed as the decrease in A_{265}. Individual reactions were conducted in both potassium phosphate and HEPES/KOH buffers (50 mM, pH 7.4 or 8.0, 20° C, air-saturated), in a final volume of 2.5 ml. Reactions were initiated by addition of metal ion. Bars represent the means of at least 5 separate reactions, and error bars standard deviations.

Fig. 4.0

Steady state rates of ascorbate oxidation by $CuSO_4$ or $HgCl_2$ under aerobic conditions

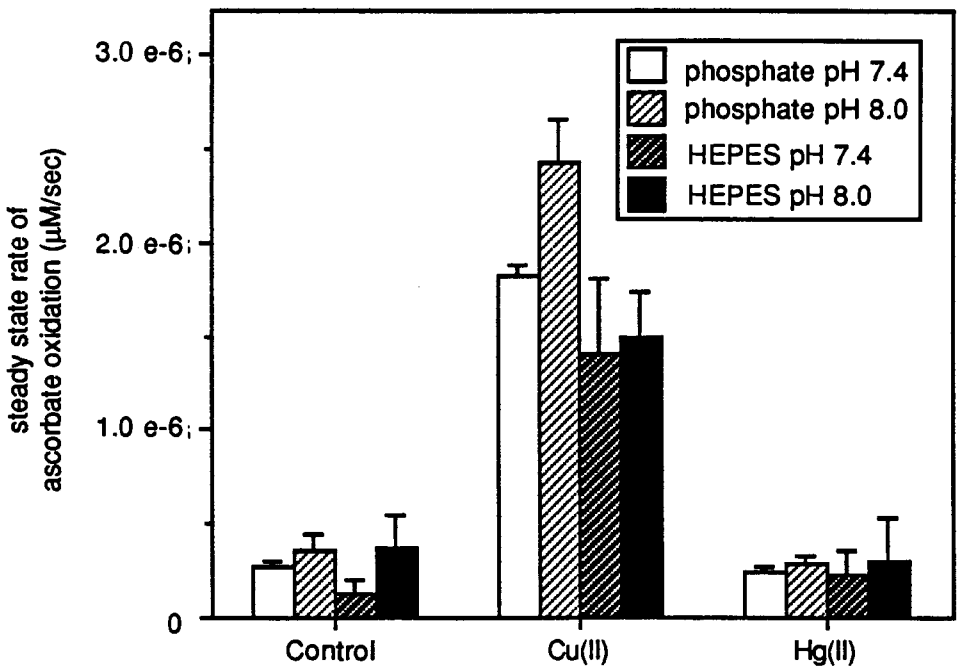


Fig. 5.0

Maximum rates of ascorbate oxidation by $AlCl_3$, $CdCl_2$, $FeSO_4$, $FeCl_3$, $MnSO_4$, $PbCl_2$, $VOSO_4$ or $NaVO_3$ under aerobic conditions

Maximum rates of ascorbate oxidation for reactions involving 100 μM ascorbate in the absence and presence of $AlCl_3$, $CdCl_2$, $FeSO_4$, $FeCl_3$, $MnSO_4$, $PbCl_2$, $VOSO_4$ or $NaVO_3$ were followed as the decrease in A_{265}. Individual reactions were conducted in both potassium phosphate and HEPES/KOH buffers (50 mM, pH 7.4 or 8.0, 20° C, air-saturated), in a final volume of 2.5 ml. Reactions were initiated by addition of metal ion. Bars represent the means of at least 5 separate reactions, and error bars standard deviations.

Fig.5.0

Maximum rates of ascorbate oxidation by $AlCl_3$, $CdCl_2$, $FeSO_4$, $FeCl_3$, $MnSO_4$, $PbCl_2$, $VOSO_4$ or $NaVO_3$ under aerobic conditions

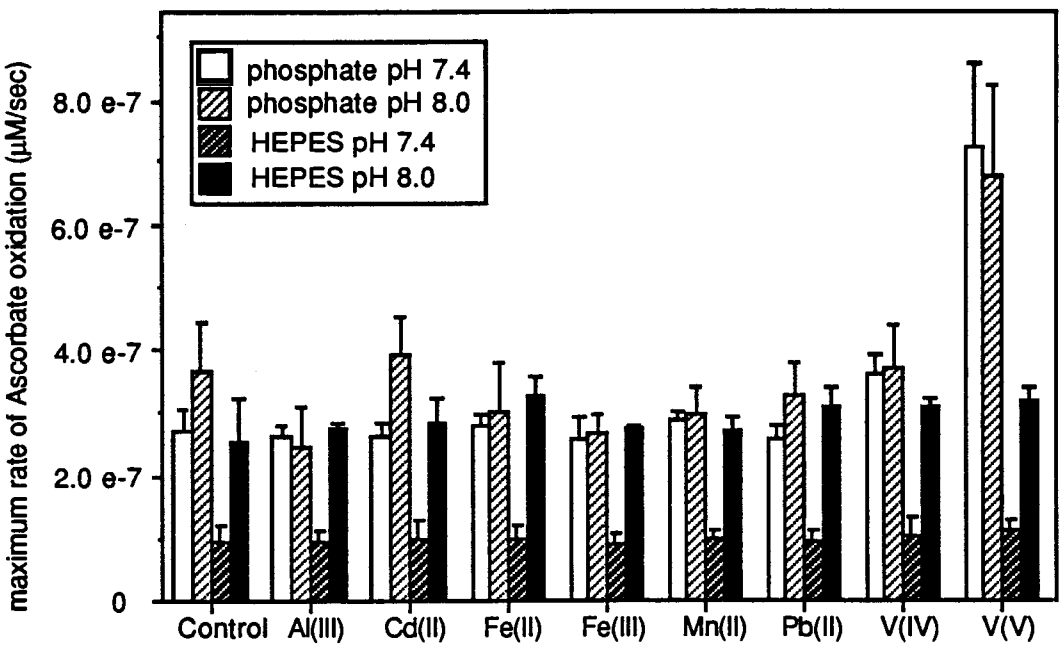


Fig. 6.0

Effects of added metal ions on maximum rates of oxygen consumption by ascorbate

Maximum rates of oxygen consumption were measured polarographically, as described in the Methods section for reactions involving 100 μM ascorbate in the absence and presence of 50 μM $AlCl_3$, $CdCl_2$, $CuSO_4$, $FeSO_4$, $FeCl_3$, HgI_2, $MnSO_4$,$PbCl_2$, $VOSO_4$ or $NaVO_3$. Individual reactions were conducted in both potassium phosphate and HEPES/KOH buffers (50 mM, pH 7.4 or 8.0, 20° C, air-saturated), in a final volume of 5.0 ml. The initial oxygen concentration was 256 ± 6 μM. Reactions were initiated by addition of metal ion. Bars represent the means of at least 5 replicate reactions, and error bars standard errors.

Fig. 6.0

Effects of added metal ions on maximum rates of oxygen consumption by ascorbate

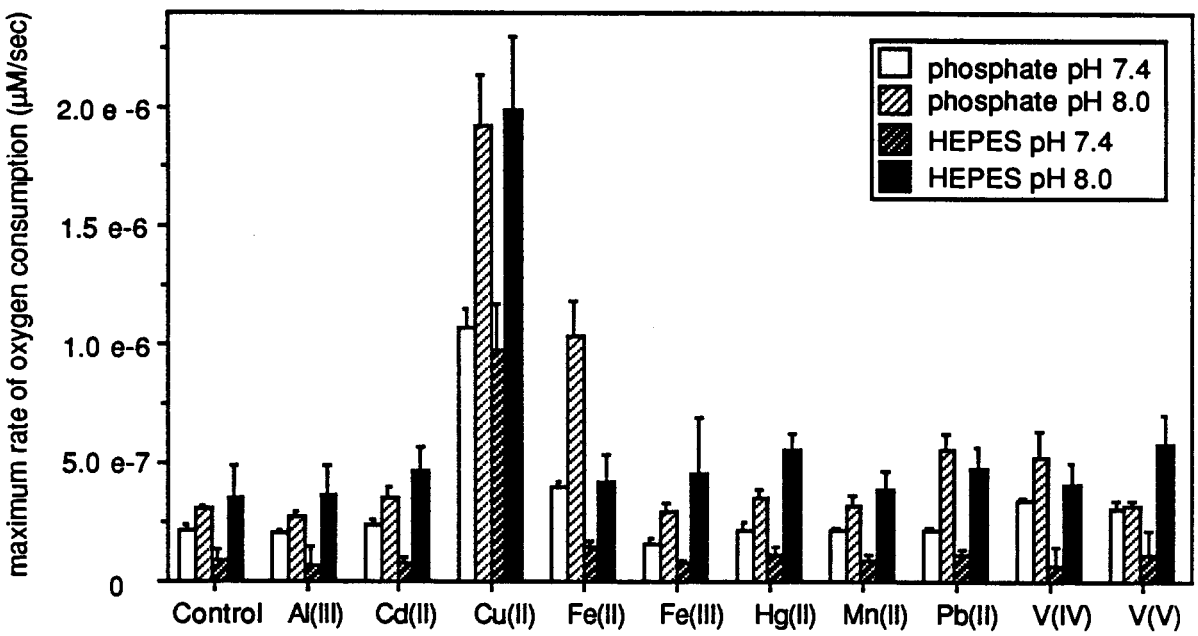


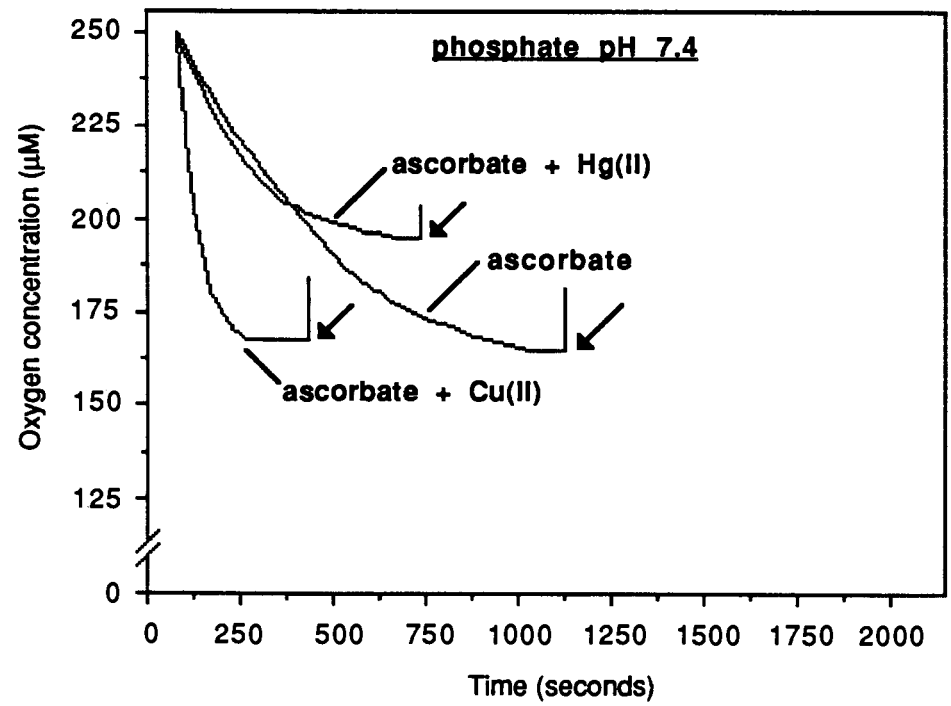
Fig. 7.0

Oxygen consumption by ascorbate in the absence and presence of added $CuSO_4$ or $HgCl_2$

Oxygen consumption by reaction mixtures involving ascorbate (100 μM) in the absence and presence of 50 μM $CuSO_4$ or Hgl_2 was followed polarographically according to the procedures described in the methods section. Reactions were conducted in both potassium phosphate and HEPES/KOH buffers at pH 7.4 and 8.0 (50 mM, 20° C, air-saturated) in a final volume of 5.0 ml. Reactions were initiated by addition of metal ions. Catalase (20 U/ml) was added where indicated in order to quantify the amount of hydrogen peroxide present.

Fig. 7.0

Oxygen consumption by ascorbate in the absence and presence of added $CuSO_4$ or $HgCl_2$ in phosphate or HEPES buffer at pH 7.4



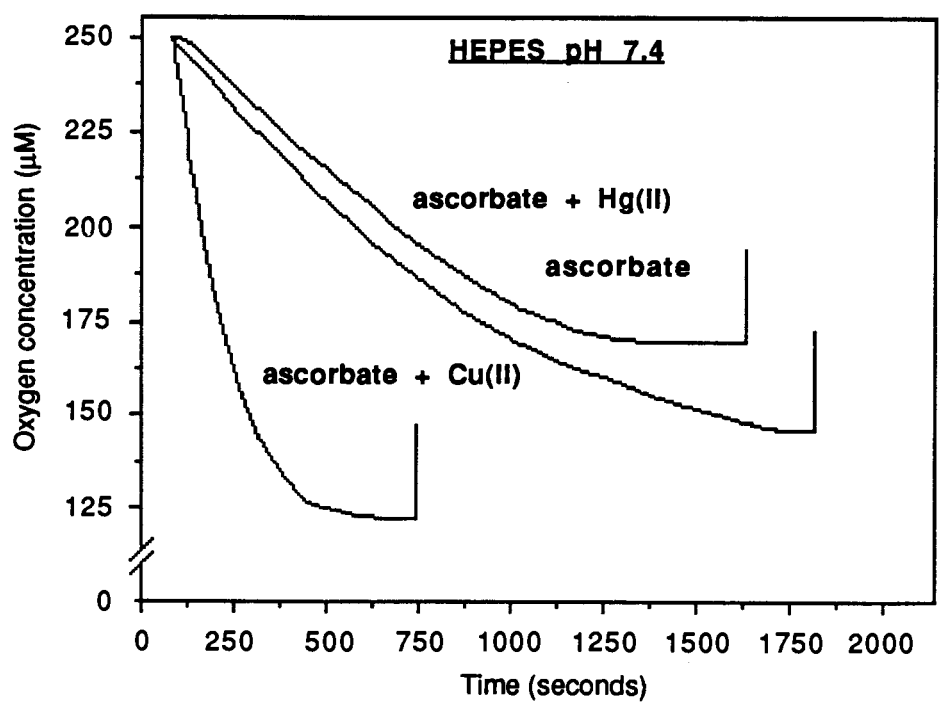
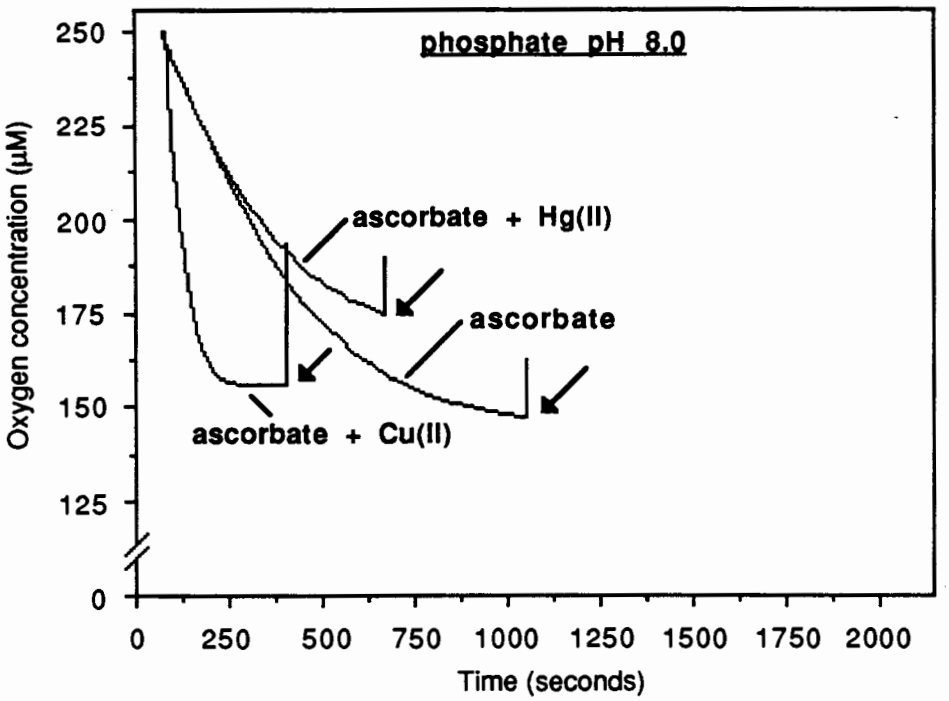


Fig. 7.0

Oxygen consumption by ascorbate in the absence and presence of added $CuSO_4$ or $HgCl_2$ in phosphate or HEPES buffer at pH 8.0



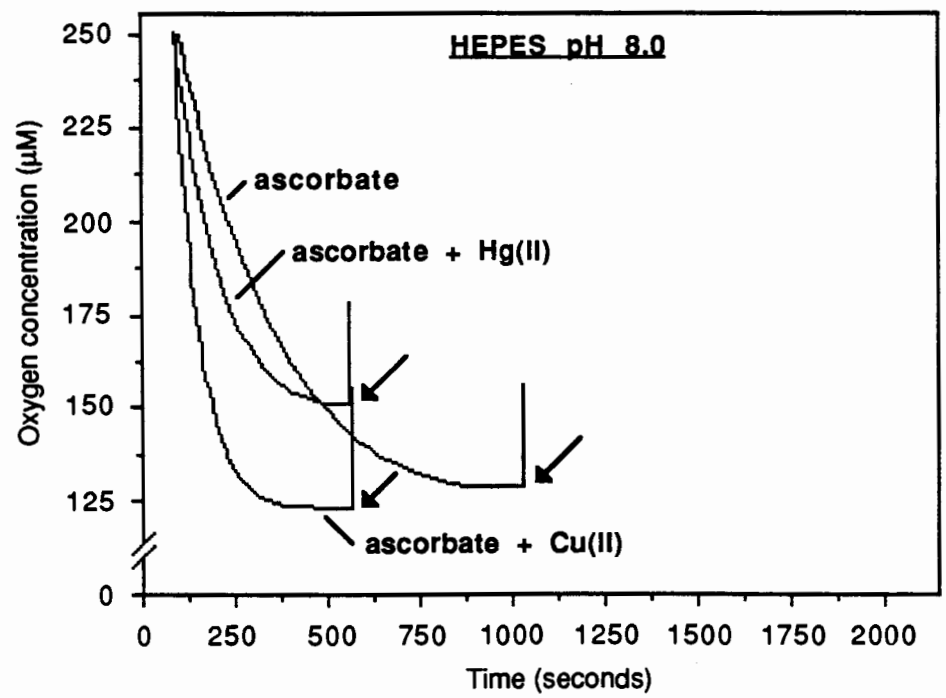


Fig. 8.0

Effects of added metal ions on total oxygen and hydrogen peroxide consumption by ascorbate

Total oxygen and hydrogen peroxide consumption were measured polarographically, as described in the Methods section for reactions involving 100 μM ascorbate in the absence and presence of 50 μM $AlCl_3$, $CdCl_2$, $CuSO_4$, $FeSO_4$, $FeCl_3$, HgI_2, $MnSO_4$,$PbCl_2$, $VOSO_4$ or $NaVO_3$. Individual reactions were conducted in both potassium phosphate and HEPES/KOH buffers (50 mM, pH 7.4 or 8.0, 20° C, air-saturated), in a final volume of 5.0 ml. The initial oxygen concentration was 256 ± 6 μM. Reactions were initiated by addition of metal ion. Bars represent the means of at least 3 replicate reactions, and error bars, standard errors. The amount of hydrogen peroxide remaining when reactions were near completion was measured by addition of catalase (20 U/ml) where indicated.

Fig. 8.0

Effects of added metal ions on total oxygen and hydrogen peroxide consumption by ascorbate

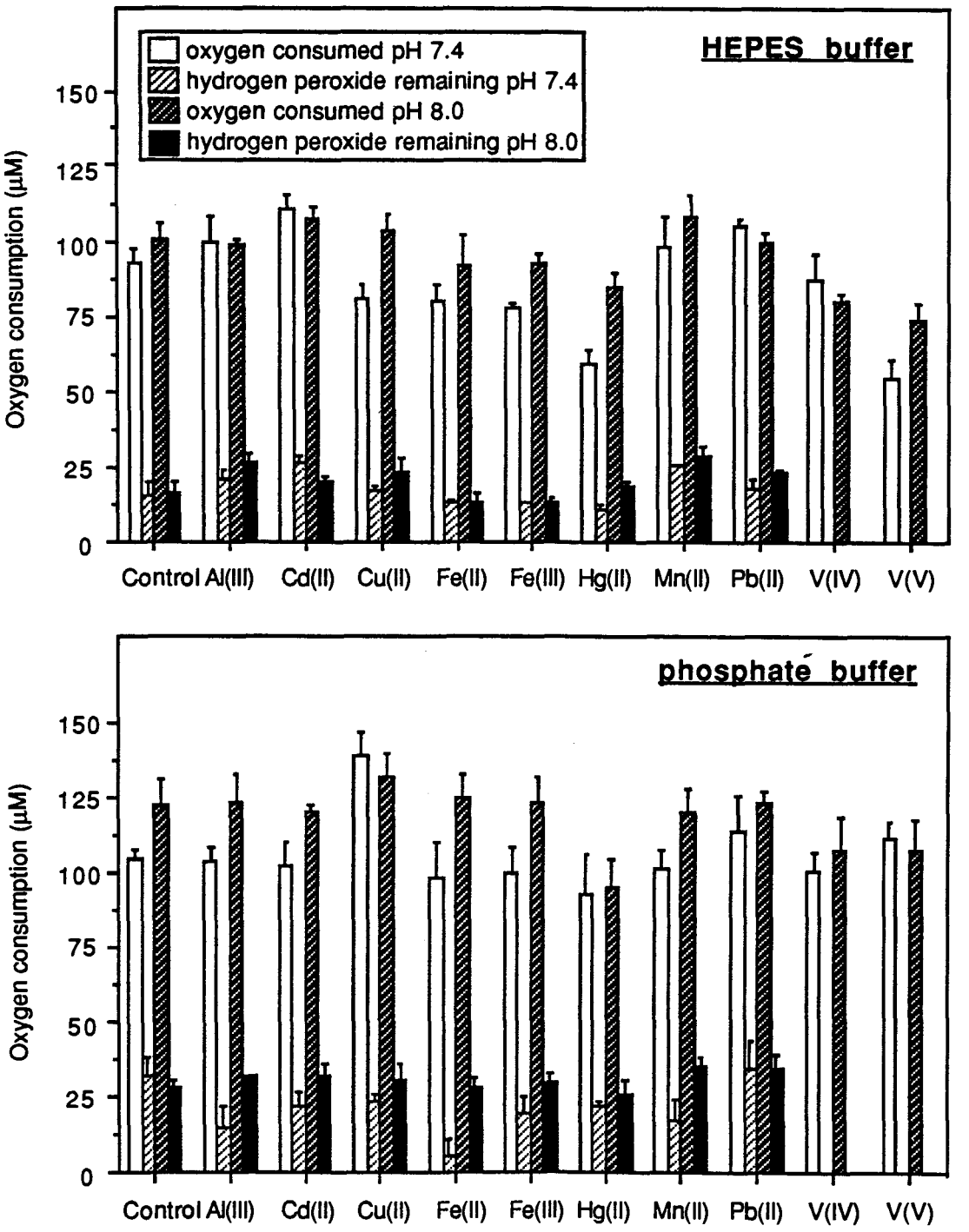


Fig. 9.0

Maximum rates of ascorbate oxidation versus maximum rates of oxygen consumption by ascorbate in the absence and presence of added $CuSO_4$ or $HgCl_2$

Maximum rates of ascorbate oxidation and oxygen consumption for ascorbate (100 μM) in the absence and presence of 50 μM $CuSO_4$ or $HgCl_2$ were measured in both potassium phosphate (Fig. 10.0a) and HEPES (Fig. 10.0b) buffers (50 mM, pH 7.4 and 8.0, 20° C, air-saturated) according to the procedures described in the Methods section and legends to Figs.3.0, 5.0 and 6.0. The data presented here is that from these previous figures, replotted so as to facilitate comparison of the maximum rates of ascorbate oxidation and oxygen consumption.

Fig. 9.0

Maximum rates of ascorbate oxidation versus maximum rates of oxygen consumption by ascorbate in the absence and presence of added $CuSO_4$ or $HgCl_2$

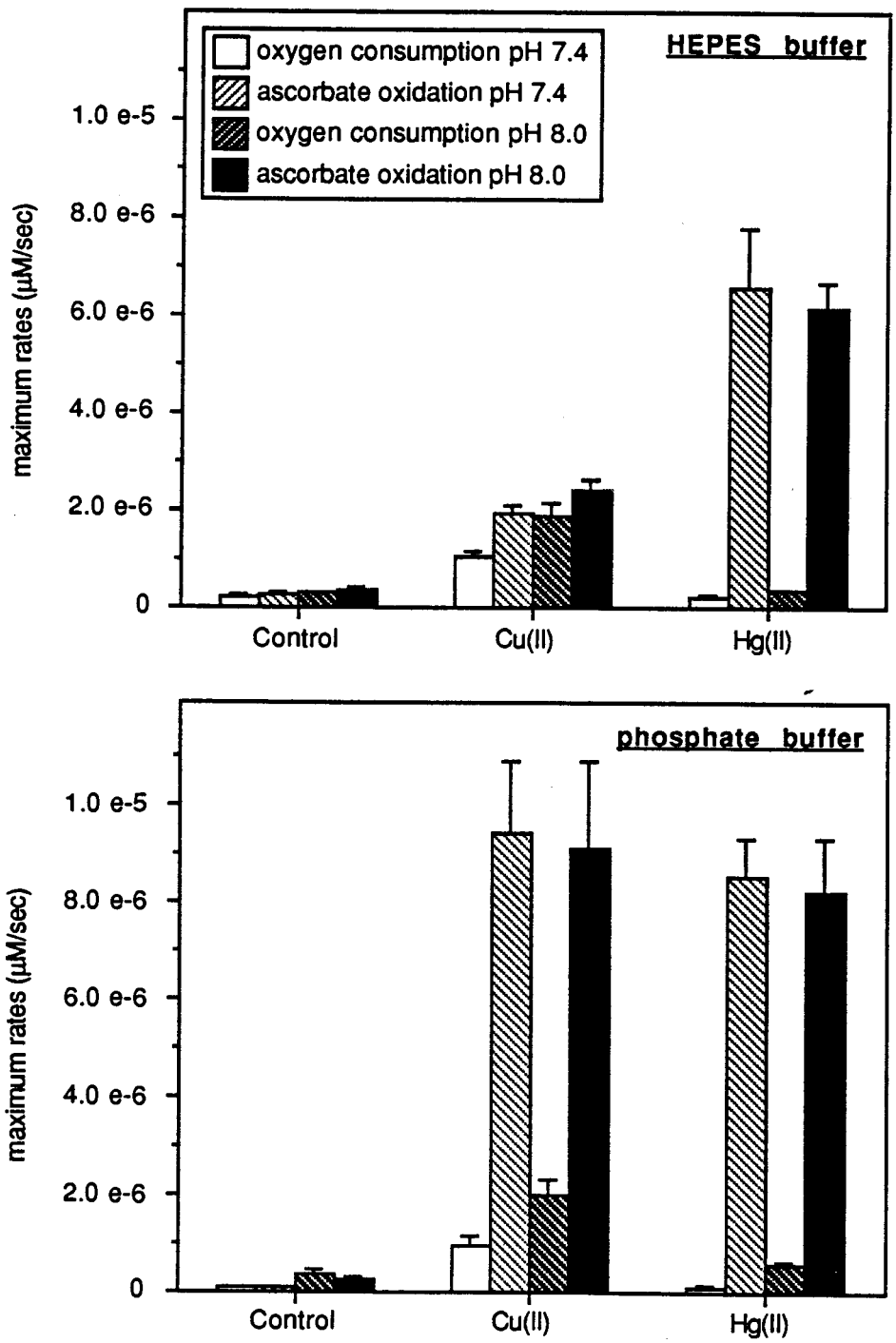


Fig. 10.0

Maximum rates of ascorbate oxidation versus maximum rates of oxygen consumption by ascorbatein the absence and presence of added $AlCl_3$, $CdCl_2$, $FeSO_4$, $FeCl_3$, $MnSO_4$, $PbCl_2$, $VOSO_4$ or $NaVO_3$

Maximum rates of ascorbate oxidation and oxygen consumption for ascorbate (100 μM) in the absence and presence of 50 μM $AlCl_3$, $CdCl_2$, $FeSO_4$, $FeCl_3$, $MnSO_4$,$PbCl_2$, $VOSO_4$ or $NaVO_3$ were measured in both potassium phosphate (Fig. 10.0a) and HEPES (Fig. 10.0b) buffers (50 mM, pH 7.4 and 8.0, 20° C, air-saturated) according to the procedures described in the Methods section and legends to Figs.3.0, 5.0 and 6.0. The data presented here is that from these previous figures, replotted so as to facilitate comparison of the maximum rates of ascorbate oxidation and oxygen consumption.

Fig. 10.0

Maximum rates of ascorbate oxidation versus maximum rates of oxygen consumption by ascorbatein the absence and presence of added $AlCl_3$, $CdCl_2$, $FeSO_4$, $FeCl_3$, $MnSO_4$, $PbCl_2$, $VOSO_4$ or $NaVO_3$

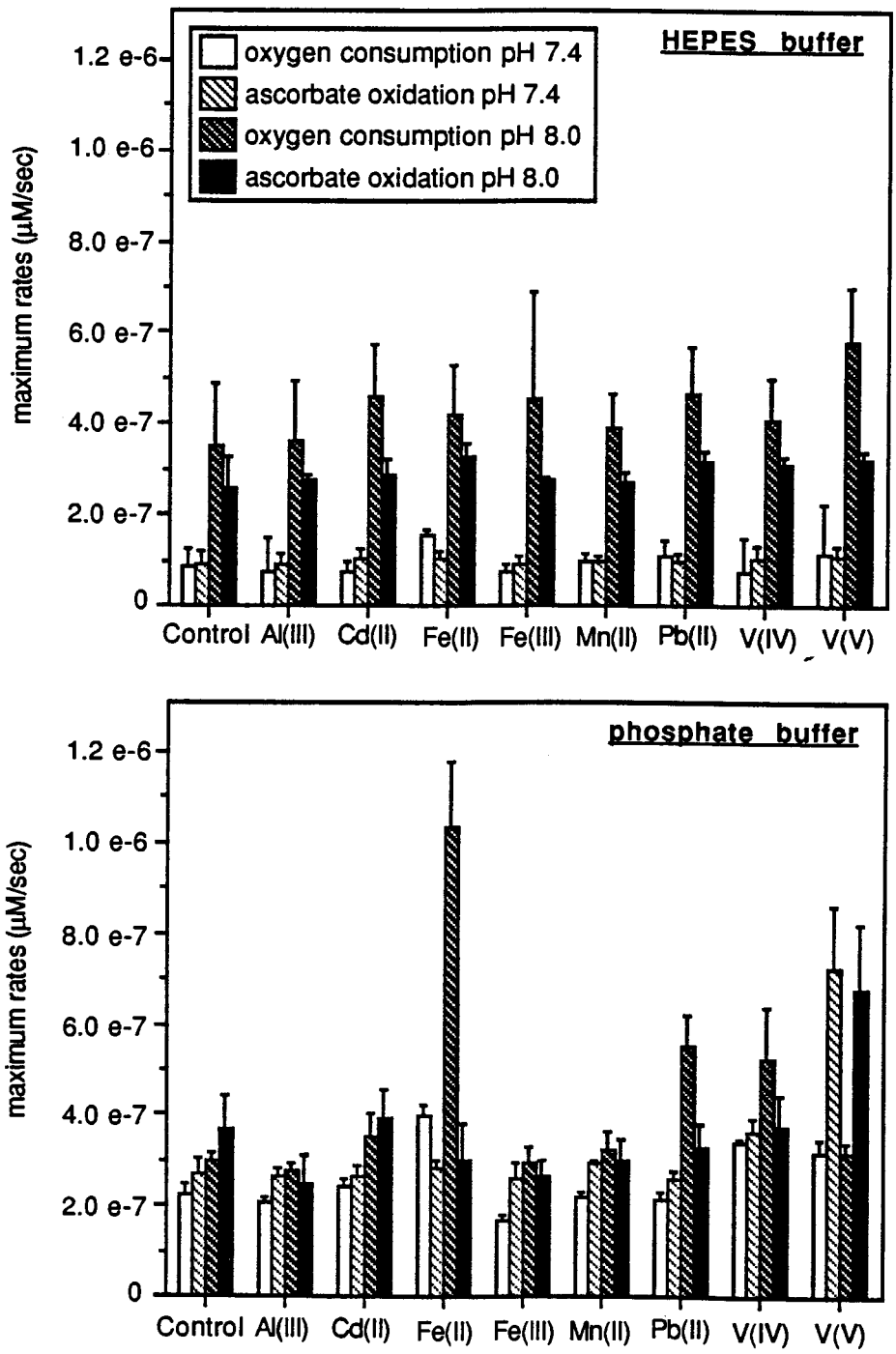


Fig. 11.0

Maximum rates of ascorbate oxidation by ascorbate in the absence and presence of added $AlCl_3$, $CdCl_2$, $CuSO_4$, $FeSO_4$, $FeCl_3$, $HgCl_2$, $MnSO_4$,$PbCl_2$, $VOSO_4$ or $NaVO_3$ and/or hydrogen peroxide

Maximum rates of ascorbate oxidation for reactions involving 100 μM ascorbate in the absence and presence of 50 μM $AlCl_3$, $CdCl_2$, $CuSO_4$, $FeSO_4$, $FeCl_3$, $HgCl_2$, $MnSO_4$,$PbCl_2$, $VOSO_4$ or $NaVO_3$, and/or 2.5 mM hydrogen peroxide were followed as the decrease in A_{265}. Individual reactions were conducted in both potassium phosphate and HEPES/KOH buffers (50 mM, pH 7.4 or 8.0, 20° C, air-saturated), in a final volume of 2.5 ml. Reactions were initiated by addition of metal ion. Bars represent the means of at least 5 separate reactions, and error bars standard deviations.

Fig. 11.0

Maximum rates of ascorbate oxidation by ascorbate in the absence and presence of added $AlCl_3$, $CdCl_2$, $CuSO_4$, $FeSO_4$, $FeCl_3$, $HgCl_2$, $MnSO_4$, $PbCl_2$, $VOSO_4$ or $NaVO_3$ and/or hydrogen peroxide

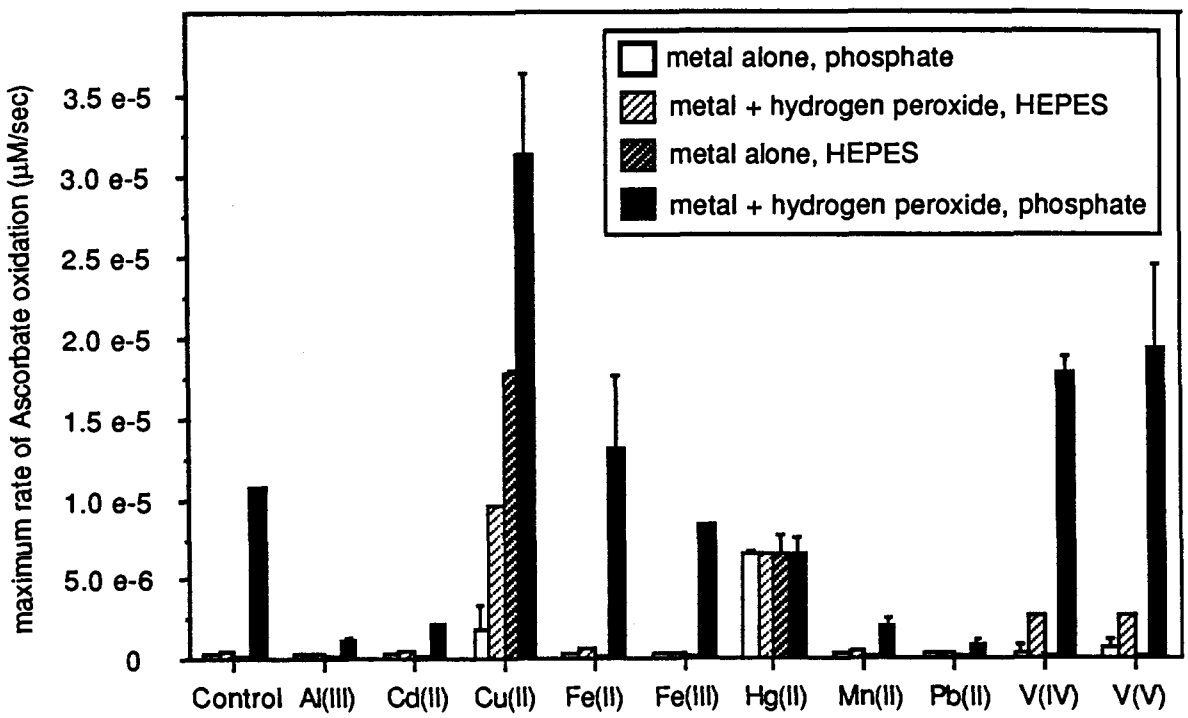


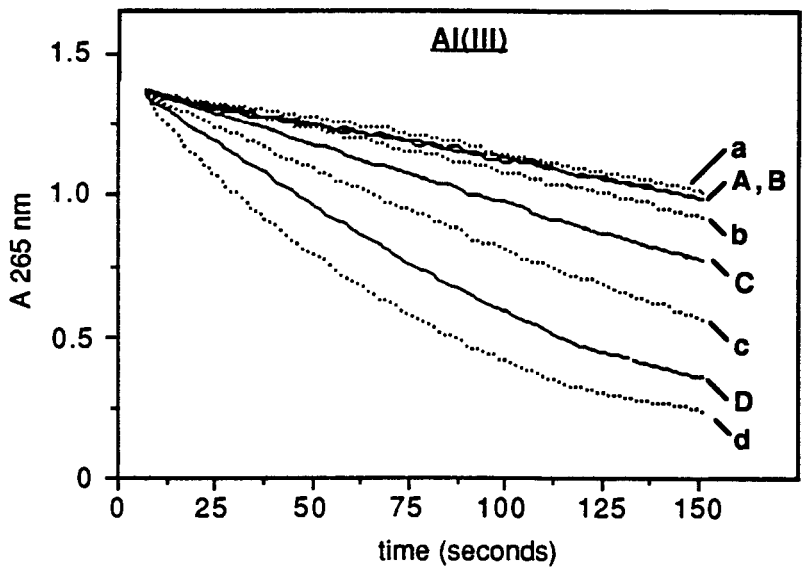
Fig. 12.0

Ascorbate oxidation in the absence and presence of added $AlCl_3$, $CdCl_2$, $CuSO_4$, $FeSO_4$, $FeCl_3$, $HgCl_2$, $MnSO_4$,$PbCl_2$, $VOSO_4$ or $NaVO_3$ and/or hydrogen peroxide: progress of reactions

The progress of ascorbate oxidation for reactions involving 100 μM ascorbate in the absence and presence of 50 μM $AlCl_3$, $CdCl_2$, $CuSO_4$, $FeSO_4$, $FeCl_3$, $HgCl_2$, $MnSO_4$,$PbCl_2$, $VOSO_4$ or $NaVO_3$, and/or 2.5 mM hydrogen peroxide were followed as the decrease in A_{265}. Individual reactions were conducted in both potassium phosphate and HEPES/KOH buffers (50 mM, pH 7.4 or 8.0, 20° C, air-saturated), in a final volume of 2.5 ml. Reactions were initiated by addition of metal ion. Bars represent the means of at least 5 separate reactions, and error bars standard deviations. Conditions are labelled as: **a** - ascorbate alone, HEPES buffer, **b** - ascorbate alone, phosphate buffer, **c** - ascorbate + H_2O_2, HEPES buffer, **c** - ascorbate + H_2O_2, phosphate buffer,**A** - ascorbate + metal, HEPES buffer, **B** - ascorbate + metal, phosphate buffer, **C** - ascorbate + metal + H_2O_2, HEPES buffer, **D** - ascorbate + meta; + H_2O_2, phosphate buffer.

Fig. 12.0

Ascorbate oxidation in the absence and presence of added $AlCl_3$, $CdCl_2$, $CuSO_4$, $FeSO_4$, $FeCl_3$, $HgCl_2$, $MnSO_4$,$PbCl_2$, $VOSO_4$ or $NaVO_3$ and/or hydrogen peroxide: progress of reactions



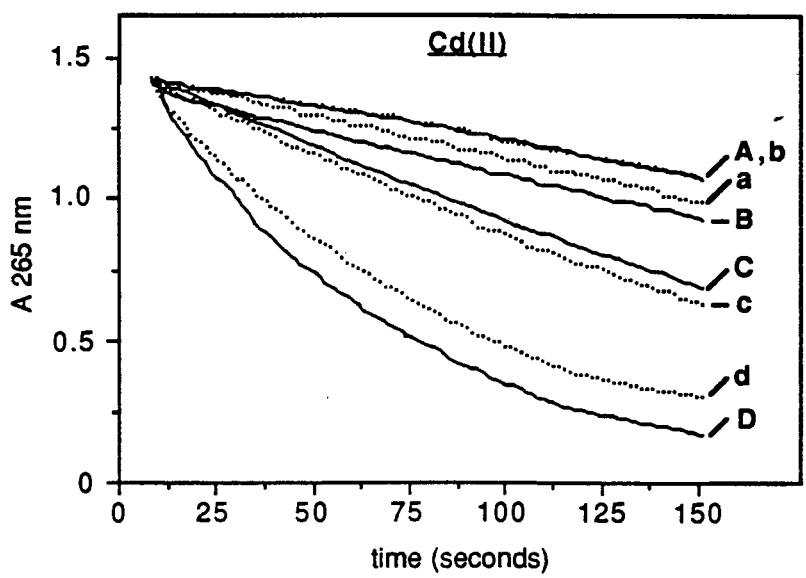


Fig. 12.0

Ascorbate oxidation in the absence and presence of added $AlCl_3$, $CdCl_2$, $CuSO_4$, $FeSO_4$, $FeCl_3$, $HgCl_2$, $MnSO_4$,$PbCl_2$, $VOSO_4$ or $NaVO_3$ and/or hydrogen peroxide: progress of reactions

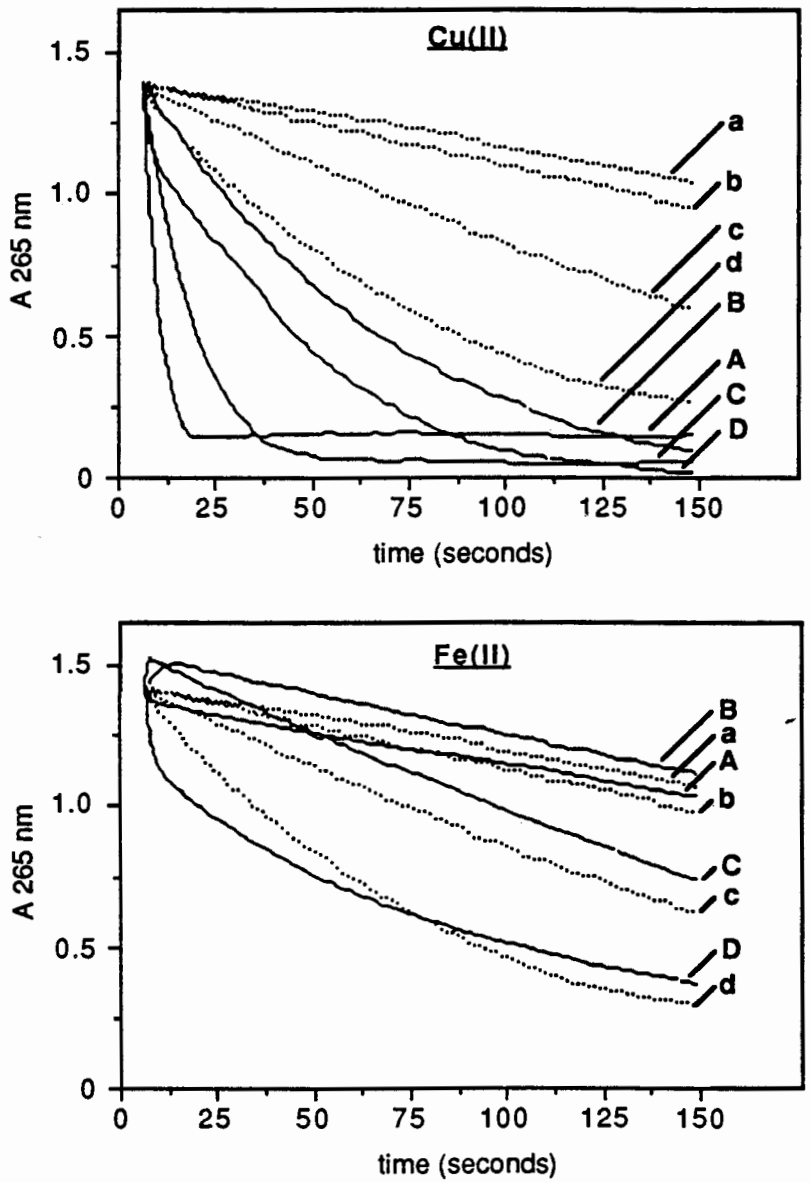
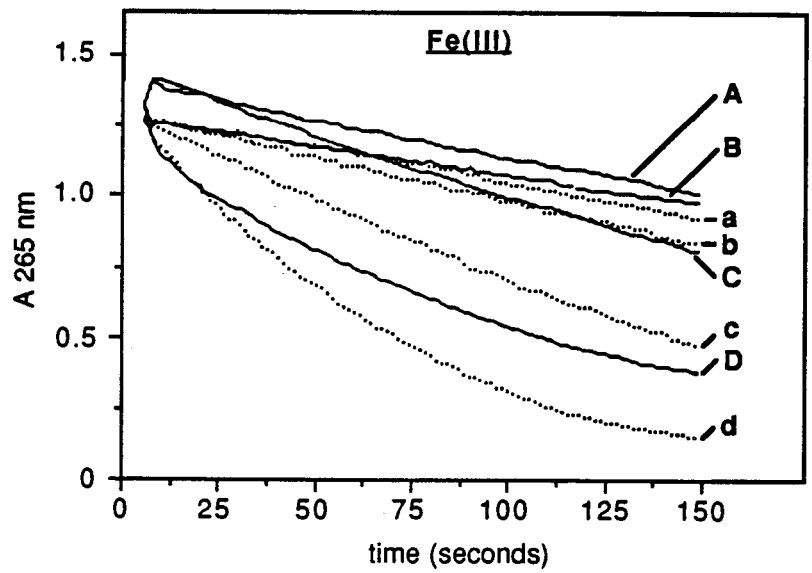


Fig. 12.0

Ascorbate oxidation in the absence and presence of added $AlCl_3$, $CdCl_2$, $CuSO_4$, $FeSO_4$, $FeCl_3$, $HgCl_2$, $MnSO_4$, $PbCl_2$, $VOSO_4$ or $NaVO_3$ and/or hydrogen peroxide: progress of reactions



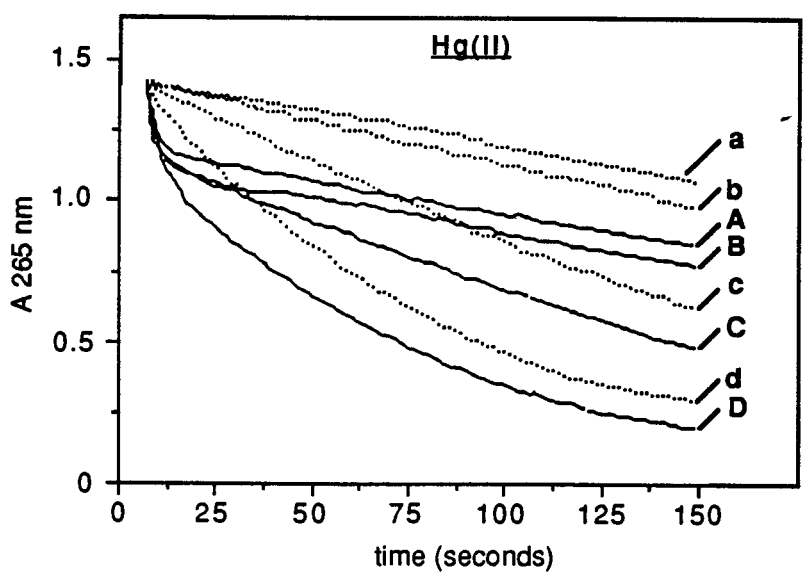
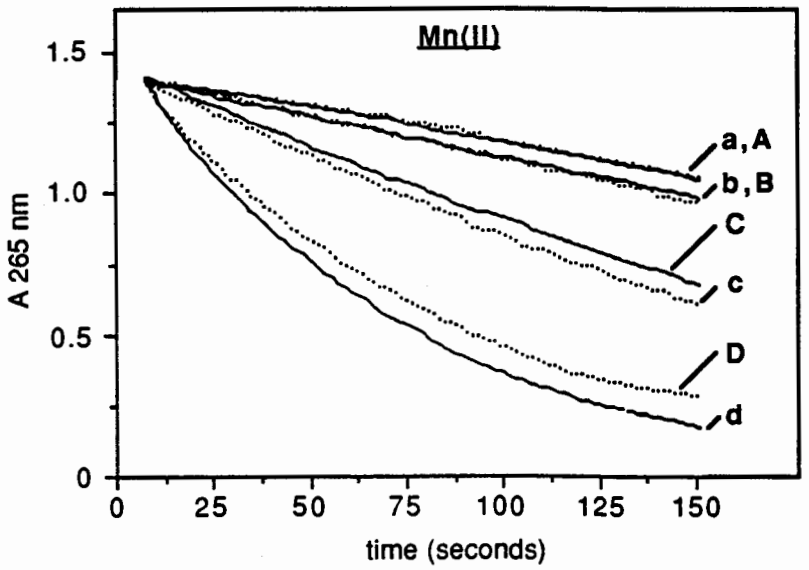


Fig. 12.0

Ascorbate oxidation in the absence and presence of added $AlCl_3$, $CdCl_2$, $CuSO_4$, $FeSO_4$, $FeCl_3$, $HgCl_2$, $MnSO_4$,$PbCl_2$, $VOSO_4$ or $NaVO_3$ and/or hydrogen peroxide: progress of reactions



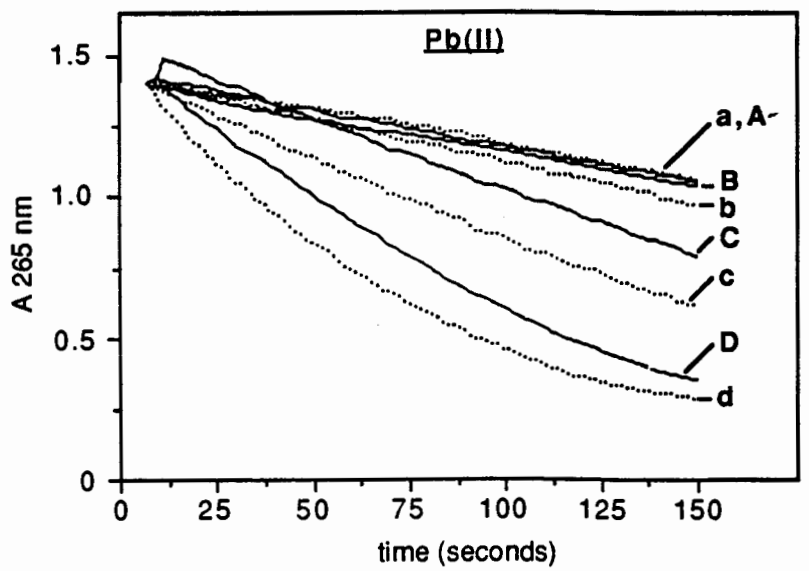
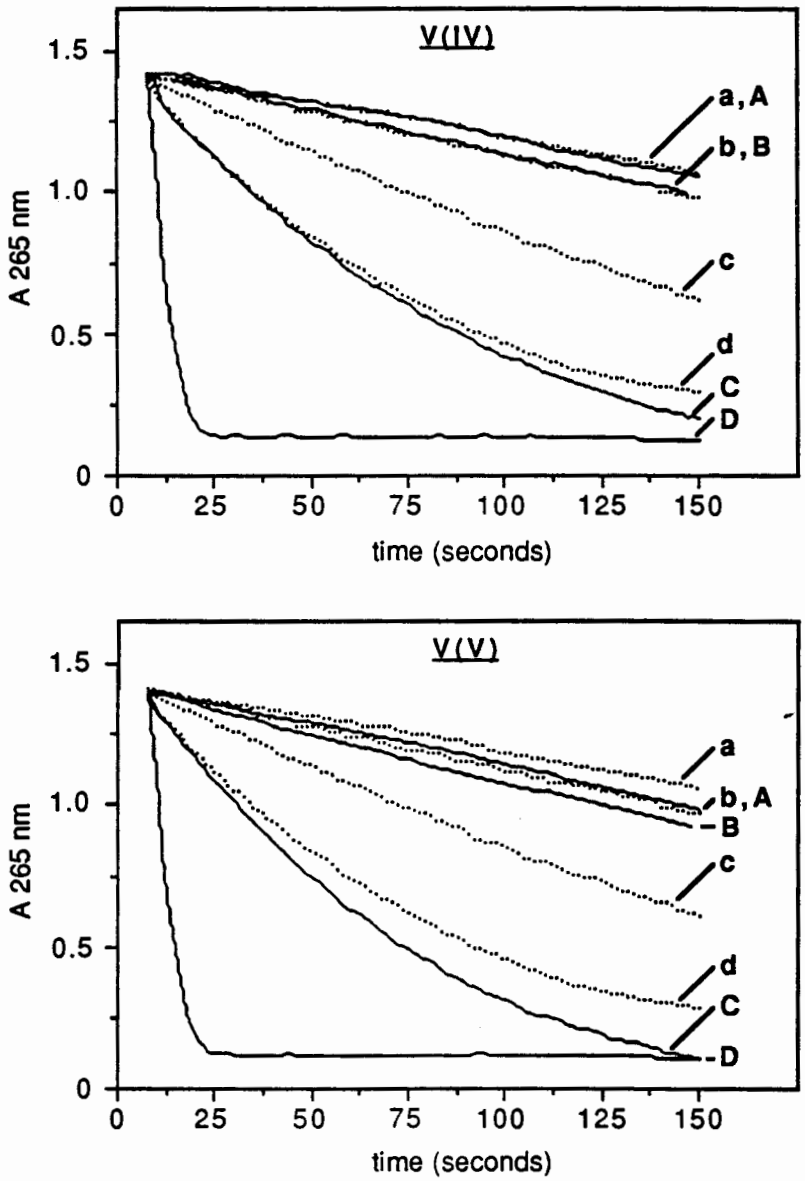


Fig. 12.0

Ascorbate oxidation in the absence and presence of added $AlCl_3$, $CdCl_2$, $CuSO_4$, $FeSO_4$, $FeCl_3$, $HgCl_2$, $MnSO_4$,$PbCl_2$, $VOSO_4$ or $NaVO_3$ and/or hydrogen peroxide: progress of reactions



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CHAPTER 5

GENERAL CONCLUSION

1.0.0 THE REDOX BEHAVIOR OF VANADIUM TOWARDS O_2, REDUCED O_2 SPECIES AND REDUCING AGENTS IS UNIQUE

The general aim of this thesis was to examine and compare the behavior of vanadium with that of a variety of physiologically and/or toxicologically significant metal ions in biologically relevant free radical reactions. On the basis of the results reported herein, we conclude that the redox behavior of vanadium towards O_2, reduced O_2 species and reductants is unique since: 1) the major questions we sought to address in the present thesis included the following: 1) the catalytic effectiveness of vanadium compounds towards biological reducing agents in general differs greatly from that of the other transition and non-transition metals tested, 2) the mechanisms of action of vanadium species are quite dissimilar to those of these other metal ions, in large part because of the presence of bound O_2 molecules on vanadium, as well as its multiple oxidation states, 3) although the specific roles of O_2 in vanadium-catalyzed oxidations appear to be similar to those for reactions catalyzed by other metal ions , the roles of H_2O_2 are not, primarily because of the catalatic actions of V (ie. the formation and decomposition of V(V) peroxo complexes), 4) vanadium-catalyzed oxidations of biological reducing agents are more dependent upon reduced O_2 species than are oxidations catalyzed by other metals, 5) the catalytic effects of vanadium are modulated in different ways by pH and ligands than those of other metals and 6) the yields of potentially toxic free radical species from vanadium-catalyzed oxidations differ substantially from those of reactions catalyzed by other metals.

2.0.0 REDOX REACTIONS OF METAL IONS IN BIOLOGICALLY-RELEVANT FREE RADICAL REACTIONS: A NEW PERSPECTIVE ON METAL TOXICOLOGY

On the basis of these results, we propose that similar, comprehensive experimental approaches detailing redox reactions of metal ions in biologically-relevant free radical reactions may prov ide a basis for a new perspective on metal toxicology. Metal ions are ubiquitous components of our environment. The health hazards posed by metals have been recognized since the earliest human civilizations. However, the concept of 'toxicity' is particularly difficult to define with regard to

metals. Indeed, toxicity is only one part of an entire spectrum of effects metals may produce. For example, toxicity does not at all mitigate against potential essentiality, and each essential trace mineral element is toxic in excess. Indeed, no biologically relevant classification system for metals currently exists. Further, the the mechanisms of toxicity of specific metals at the molecular level remain largely unknown. Few truly effective therapeutic regimens exist. In many instances, therapeutic intervention is accompanied by uncertain and often hazardous side effects.

2.1.0 Traditionally metal toxicity has been discussed in terms of the binding of metals to tissue components

Traditionally, studies of metal toxicity have usually focussed on nonessential heavy metals such as Pb, Hg and Cd. The mechanisms of toxicity of these metals have been discussed mainly in terms of their ability to bind and essential biologic macromolecules including membranes, enzymes, receptor structures and DNA. The intrinsic or inherent toxicity of a metal has been measured in terms of its ability to interfere with essential physiologic functions by binding irreversibly to tissue components. Further, the interactions between toxic metals and susceptible systems in biologic tissues have usually been viewed as being complex, but relatively non-specific Attempts have been made to correlate the toxicity of metals has been associated with specific physicochemical properties including: 1) electrochemical character and oxidation state, 2) particle size, 3) solubility and stability of compounds in biologic fluids and degree of hydration of ions formed, 4) the extent of hydrolysis and subsequent olation in tissues and subsequent reactivity of these products and 5) the relative tendencies of different metals to exist in colloidal or particulate forms in the tissues.

2.2.0 The toxicity of metals depends both qualitatively and quantitatively on their redox properties

Yet it is reasonable to assume that the binding of metals to tissue components represents only a part of any given mechanism of toxicity. Any attempt to understand the fundamental processes underlying the mechanisms of toxicity of metals in living organisms must incorporate an

understanding of normal control systems. The uptake, transport, metabolism and excretion of metals are subject to highly efficient and integrated control systems. Dynamic, interrelated systems for metal uptake, transport, sequestration, elimination exist at every level of the metabolic system. Although, accumulation in tissues does not necessarily imply occurrence of a toxic effect, since inactive complexes or storage depots may be formed, those metals having the greatest potential for causing damage are those which accumulate in the body, bypassing these control mechanisms.

When a metal is introduced into a biological system, each formal valence state of that metal is available for chemical or biochemical reactions. The toxicity of each metal depends both qualitatively and quantitatively on its redox properties. Indeed, a metal is an element which under biologically significant conditions may react by losing one or more electrons to form a cation. Metabolic conversion or processing of metals depends on the available metal concentration, the nature of the biochemical mechanisms involved, the pH, temperature, redox potential and synergistic or antagonistic effects of other metabolic or chemical processes. Redox reactions of metals with biological oxidants (O_2, H_2O_2), or reducing agents (ascorbate, thiols, catecholamines, sugars, NAD(P)H) are of particular interest in this regard.

2.3.0 Reactions of metals with biological oxidants or reducing agents are highly regulated in living organisms

Reactions of metals with biological oxidants or reducing agents are highly regulated in living organisms because of their inherent tendency to interact under physiological conditions. Oxygen is unique as a source of biological energy in that it is abundant, available, has a high energy potential and reacts slowly. However, the tremendous energetic advantages provided by aerobic metabolism are offset by concomitant production of free radical species. Metal ions are the best redox agents available to living organisms, being essential components of electron transfer pathways and in the capture and reduction of molecular oxygen. Only trace quantities exist as free metal ions, the vast majority being bound to proteins and other biological macromolecules. The roles of metals in living

organisms are extremely varied and complex. Where metals have been incorporated into complex gene products, the metals selected have unique properties which make them particularly suitable for the structural or functional roles they fulfill. Only the rigorous control of the relative presence of metals and oxygen by sophisticated gene products allows derivation of the potential benefits to be gained from these interactions. Thus, metal control systems form one part of integrated antioxidant defense system. Unabashedly opportunistic, living organisms have capitalized on the functional flexibility of complex protein synthetic systems to modulate the inherent redox properties of a number of metals including Fe, Cu, Zn, Mn, Mo and Se. It is random interactions occurring outside of these control systems that are of primary concern. In order to characterize these reactions in detail, there is a need for basic information concerning the redox behavior of metals of physiological or toxicological significance

3.0.0 DIRECTIONS FOR FUTURE RESEARCH: A COMPREHENSIVE STRATEGY FOR INVESTIGATING THE REDOX PROPERTIES OF METALS

This basic information can only come from research based upon a comprehensive strategy for investigating the redox properties of metals. The research approach we have taken in the present thesis, in which we attempted to characterize reactions of vanadium with biological oxidants and reducing agents represents a small step in this direction, however much more detailed work will be required to extend and expand the present results so as to accomplish this difficult task. Thus, we can only hope that we have contributed in a positive way to these directions for future research.