

REDOX REACTIVITY OF VANADIUM TOWARD CYTOCHROME C AND OXYGEN

by

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Redox reactivity of vanadium toward cytochrome c  
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## ABSTRACT

The redox reactivity of vanadium and 4 other metal ions toward cytochrome c and oxygen was studied. At pH >3.2 (at which the haem crevice is closed), Vanadate [V(V)] > Cu<sup>2+</sup> > Fe<sup>2+</sup> > Mn<sup>2+</sup> > Al<sup>3+</sup> accelerated the aerobic oxidation of ferrocycytochrome c. Since V(V) oxidized cytochrome c about twice as rapidly as copper at pH 4.0, and since the biological actions (and the toxicity) of vanadium depend on its redox states, we selected vanadium from among these metals for more detailed study.

Vanadium(IV) [V(IV)] and vanadium(III) [V(III)] reduced ferricytochrome c at pH 6.0, 7.0, and 7.4. In contrast, V(V) oxidized ferrocycytochrome c at pH 6.0 anaerobically and aerobically, but not at pH 7.0 and 7.4. In the presence of oxygen, V(IV), and ligand (desferrioxamine, ATP, or EDTA), ferrocycytochrome c became oxidized (initial rate =  $1.1 \times 10^{-3} \mu\text{M s}^{-1}$  in the presence of V(IV) and desferrioxamine at pH 6.0). Vanadate accelerated the reduction of ferricytochrome c induced by desferrioxamine at pH 7.4 (initial rate =  $1.1 \times 10^{-2} \mu\text{M s}^{-1}$  in anaerobic conditions, and  $7.9 \times 10^{-3} \mu\text{M s}^{-1}$  in aerobic conditions).

The addition of ATP, desferrioxamine, and EDTA profoundly accelerated the anaerobic (and aerobic) oxidation of cytochrome c by V(V) (e.g., desferrioxamine increased the rate by 3500 fold). Oxidations of vanadium were in general accelerated by increasing pH, whereas reductions were in general slowed by increasing pH. Clearly, the mechanism of the oxidation of ferrocycytochrome c by vanadium involves two pathways: (i) the direct oxidative action of vanadate complex, and (ii) the pro-oxidant action of reduced species of vanadium [V(IV)] capable of activating oxygen.

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## PREFACE

This thesis consists of a general introduction, four manuscripts, and a final concluding statement. The general introduction gives a brief review of the literature, and outlines the goals and context of the study.

The first manuscript (Chapter II) reports the influences of copper, iron, manganese, aluminum, and V(V) on the autoxidation of ferrocytochrome c in the presence of EDTA, ADP, histidine, or desferrioxamine.

The second manuscript (Chapter III) details the effects of ligand and pH on the redox reactivities of vanadium [V(III), V(IV), V(V)] toward cytochrome c under anaerobic conditions.

The third manuscript (Chapter IV) reports the effects of ligand and pH on the reduction of oxygen by vanadium(III) and vanadium(IV).

In the fourth manuscript (Chapter V), the influences of ligand and pH on the aerobic oxidation and reduction of cytochrome c in the presence of vanadium are investigated in more detail.

A general discussion and concluding statement are provided following Chapter V. The data plotted in the graphs are provided in the appendix in the form of TELLAGRAF plot files.

## CHAPTER I

### GENERAL INTRODUCTION

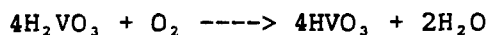
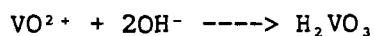
In the past twenty years, the importance of metal ions to the vital functions of living organisms, and concomitantly their health and well-being, has become increasingly evident. Most recently, considerable interest in the biochemistry of vanadium has developed. The interest arises from the multiple effects of vanadium on the biochemical and physiological processes of living cells. One of the most novel aspects of the effect of vanadium is its insulin-like function (Degani, 1981), which makes vanadium potentially useful in the treatment of diabetes. Moreover, as a metal with multiple valence states, vanadium has the potential both to directly oxidize cellular constituents and to mediate their oxidation by molecular oxygen. An understanding of the redox reactivity of vanadium is a prerequisite to an understanding of the biological actions, toxicity and pharmacological use of vanadium (Rubinson, 1981). For the current study we selected cytochrome c as a cellular metabolite to investigate the redox reactivity of vanadium.

#### Redox biochemistry of vanadium

In mammals, vanadium is an "ultratrace" element (Cornelis, 1981). The total body pool of vanadium is less than 100  $\mu\text{g}$  with a daily intake of 10  $\mu\text{g}$  to 60  $\mu\text{g}$  (Byrne, 1978). Most of the vanadium in the body is found inside cells (Versieck, 1980; Simonoff, 1984). It is believed that the main forms of vanadium in the body are vanadate (pentavalent form) and vanadyl (tetravalent form). In plasma, vanadium occurs primarily as the oxidized form, vanadate; while intracellularly it occurs primarily in the reduced form, vanadyl

(Sakurai, 1980). Less than 1% of the intracellular vanadium is free. Most of it is bound to serum albumin, transferrin and other ligands e.g. GSH, ATP, ADP, AMP, etc. (Nechay, et. al., 1986)

The tetravalent state of vanadium occurs in aqueous solution as a blue divalent cation,  $VO^{2+}$  (Nechay, 1986). Vanadyl is stable in acidic conditions, but near neutral pH the vanadyl ion becomes hydroxylated to  $H_2VO_3$ , which is sensitive to oxygen, becoming oxidized to the pentavalent state,  $HVO_3$ .



Although at neutral pH vanadyl is unstable, it can be stabilized by the formation of complexes with ligands, including EDTA, ATP, hemoglobin, and transferrin.

Vanadate is usually colourless, but in aqueous solution between pH 2.5 and 5.5, it can form  $HV_{10}O_{28}^{5-}$  (which is orange in colour), and  $H_2V_{10}O_{28}^{4-}$  (which is yellow) (Chasteen, 1983). Above pH 13, vanadate is  $VO_4^{3-}$  (analogous to  $PO_4^{3-}$ ). Below pH 3.5 vanadate becomes  $VO_2^+$ , while at neutral pH vanadate can be written as  $H_2VO_4^-$ . (Chasteen, 1983). Vanadate also can form complexes with ligands. Desferrioxamine is a very effective chelating agent of vanadate (Fridovich, 1985).

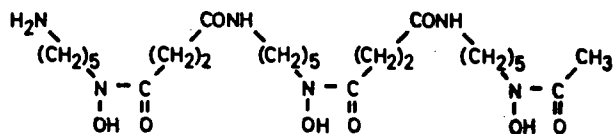


Figure 1. The structure of desferrioxamine

Besides desferrioxamine, ATP, EDTA, histidine, and albumin also are used in this study, since they are either important metabolites or important chelating agents, or both. N-acetyl-L-tyrosine ethyl ester (ATEE) is selected because it is currently used as an analogue of the tyrosine residue of protein.

#### Toxicity of vanadium

The toxicity of vanadium has not been well established. Although some authors claimed that it is not very toxic for humans when given orally (Schroeder, 1963; Nechay, 1986), it should be noted that most of the ingested vanadium is not absorbed (Waters, 1977).

Susic (1986) has reported that vanadium administration induced pulmonary hypertension of rats. Donaldson (1985) reported that orthovanadate induced an increase in lipid peroxidation in kidneys after a single subcutaneous or intraperitoneal injection to rats or mice. Interestingly, the most effective antidote for acute NaVO<sub>3</sub> toxicity is ascorbic acid (Domingo, 1985). In mice,

administration of ascorbic acid prior to acute exposure to vanadium diminished its toxicity (Donaldson, 1985). Therefore, it is possible that the toxicity of vanadium is related to its redox reactivity.

### Redox biochemistry of cytochrome c

Cytochrome c was named and described in the classical work of D. Keilin (1925, 1926), which established the wide spread occurrence of cytochrome c in a variety of cells from mammals to invertebrates and yeast. Thirty years later, Bodo (1955) prepared the first crystalline form of cytochrome c.

Both primary and tertiary structures of eukaryotic and a few prokaryotic cytochromes c are clearly known today. Cytochrome c consists of three main components: polypeptides, protoporphyrin, and iron. The heme moiety of cytochrome c is linked by two thioether linkages to cysteines built into the polypeptide chain of the protein (cysteine 14 and cysteine 17). The heme iron is linked to the haem by four bonds. The fifth and sixth bonds of iron are occupied by amino residues (histidine 18 and methionine 80). This structure confers on cytochrome c a relatively high standard reduction potential.

The main function of cytochrome c in living cells is as an electron carrier in the respiratory chain between cytochrome c<sub>1</sub> and cytochrome oxidase. Cytochrome c also plays an important role in the photosynthetic processes and in the anaerobic dark processes of bacteria such as nitrate reduction and sulphate reduction (Hill, 1951, 1953). It was believed that cytochrome c does not normally react with molecular oxygen under physiological conditions (Keilin, 1930).



The reported effects of metals on the autoxidation of cytochrome c have mainly focused on copper and copper complexes (Yandell, 1981; Augustin and Yandell, 1979; Davison, 1968). In the case of the autoxidation of cytochrome c catalyzed by copper or copper-ligand complexes, the rate determining step was usually the one electron oxidation of the cytochrome c by a cupric ion species, followed by rapid reoxidation of the resulting Cu<sup>+</sup> complexes by molecular oxygen (Augustin and Yandell, 1979). Although it is well established that copper can increase the rate of autoxidation of cytochrome c, there are very few reports about the catalytic effects of other transition metal ions (Wherland and Gray, 1976).

Regarding the pathway of the electron transferring reaction of cytochrome c, most of the work is concerned with the pathway of electron transfer from reductant to ferricytochrome c. There are two pathways for electron transfer in redox reactions of ferricytochrome c (Creutz and Sutin, 1973; Yandell, et. al., 1973). One is an "adjacent attack pathway" requiring the rupture of the iron-sulfur bond or the opening of the heme crevice. Another is a "remote pathway" involving an indirect route, possibly the exposed edge of the porphyrin ring system. However, there is a combined pathway. Wherland (1976) has reported that the reduction of ferricytochrome c by sodium dithionite involved the two pathways at the same time. Wherland and Gray (1976) investigated the reaction of horse heart cytochrome c with some metal complexes (e.g. Fe(EDTA)<sub>2</sub>, Ru(NH<sub>3</sub>)<sub>6</sub>, Fe(CN)<sub>3</sub>, etc.), and concluded that the mechanism of electron transfer involves attack by the small molecule redox reagents near the most exposed region of the heme. This attack is affected by electrostatic interreaction with the positively charged protein, by hydrophobic interactions that permit reagent penetration of the protein surface, and by the availability of  $\pi$ -symmetry ligand (or extended metal)

orbitals that can overlap with the  $\pi$  redox orbitals of the heme group.

Lawrence and Spence (1975) reported the reduction of ferricytochrome c by different forms of molybdenum-cysteine complexes. The reduction by the dioxo-bridged Mo(V)-cysteine complex is relatively slow and its rate is first order with respect to cytochrome c and zero order with respect to di- $\mu$ -oxo-bis-[oxo(1-cysteinato)molybdate(V)]. The reduction by the monoxo-bridged complex  $\mu$ -oxo-bis[oxodihydroxo(1-cysteinato)molybdate(V)] is extremely rapid and its rate is first order with respect to both reactants. Yandell et.al., (1973) reported the direct reduction of cytochrome c by chromium(II).

#### General goals of study

It is uncertain what factors are responsible for the intracellular redox state of vanadium, nor is it known what are the influences of oxygen, ligands, and pH on the redox states of vanadium. Although there is substantial evidence suggesting that the acute toxicity of vanadium is dependent on its redox state, the active species mediating this toxicity have not been unambiguously elucidated. In the current study, We have sought to establish some general principles regarding the roles of oxygen, ligands, and pH in the redox reactivity (including toxicity) of vanadium. Thus we hoped to reveal some of the factors which determine the relative abundance and distribution of the various cellular redox states of vanadium, and to advance the understanding of some aspects of the redox chemistry of oxygen and cytochrome c in relation to transition metal ions.

The results of our investigations are reported in the following 4 chapters. In the first report, we summarize the redox behaviour of cytochrome c in the presence of vanadate and 4 other transition metals. Then, in sequence, we report the anaerobic redox reactivity of vanadium toward cytochrome c, the redox reaction between vanadium and oxygen, and the aerobic redox reactivity of vanadium toward cytochrome c.

Reactivity of ferrocyclochrome c toward molecular oxygen:  
Either a low pH or the presence of an appropriate transition metal complex is  
required.<sup>1</sup>

by

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## CHAPTER II

### REACTIVITY OF FERROCYTOCHROME c TOWARD MOLECULAR OXYGEN

#### Abstract

Effects of metal ions and ligands on the rate of aerobic oxidation of ferrocycytochrome c were greatest at higher pH, where the rate of aerobic oxidation was lowest. Iron ( $\text{Fe}^{2+}$ ), copper ( $\text{Cu}^{2+}$ ), vanadate [ $\text{V(V)}$ ], manganese ( $\text{Mn}^{2+}$ ), and aluminum ( $\text{Al}^{3+}$ ) were tested in combination with EDTA, ADP, histidine or desferrioxamine at pH 2.6, 3.2, and 4.0. At pH 2.6, only vanadate increased the initial rate of the oxidation of ferrocycytochrome c by 6.2-fold, while the other metal complexes had no effect. At pH 4.0, however, all the metals markedly stimulated the oxidation of cytochrome c. The order of effectiveness was:  $\text{V(V)}(\text{desferrioxamine}) > \text{Cu}(\text{ADP}) > \text{Fe}(\text{EDTA}) > \text{Mn}(\text{desferrioxamine}) > \text{Al}(\text{EDTA})$  (where the stated ligand represents the most stimulating one for a given metal). At pH 3.2, the corresponding metal complexes had intermediate effects.

A decrease in pH accelerated the reaction regardless of the nature of the metal complex present, but the greatest pH-induced accelerations occurred in the absence of added metals. At a value of pH for which the heme crevice is closed (pH 4), transition metal ions mediated most if not all of the reduction of oxygen, while at the lowest pH (2.6) transition metal ions were unnecessary and those other than vanadate were ineffective. Vanadate then, was the most active of the metals at all values of pH, and was also the only metal to accelerate the oxidation of ferrocycytochrome c at pH 2.6. However, it was also the only metal for which oxidation of ferrocycytochrome c was not contingent upon aerobic conditions, and was not accompanied by oxygen

consumption. Thus, although vanadate was the most effective of the metal ions tested in promoting the oxidation of ferrocytochrome c, it did not accelerate the autoxidation of ferrocytochrome c. Instead it acted stoichiometrically rather than catalytically, being itself the ultimate electron acceptor.

### Introduction

Although it has been known for many decades that ferrocytochrome c is readily oxidized by molecular oxygen at low pH (1, 2) the mechanism of the reduction of molecular oxygen by ferrocytochrome c is far from clear. At neutral pH, ferrocytochrome c is inert toward oxygen (1, 2). At least two mechanisms are involved, the first residing in the properties of molecular oxygen and the second primarily in the chemistry of cytochrome c.

First, with respect to molecular oxygen, its direct two-electron reduction is spin forbidden. Therefore, oxygen must be activated in order for two-electron oxidation of organic substrates to occur (3, 4, 5). One-electron transfer to oxygen is also slow because of the unfavourable energetics of formation of superoxide in aqueous media. In the case where ferrocytochrome c is the reducing agent, the free energy barrier resides largely in a strongly unfavourable entropy of activation, consistent with a requirement for solvation of the negative charge on the nascent superoxide species (6). Both of these hinderances to the direct reduction of oxygen can be circumvented by participation of transition metal ions. The activation of oxygen in biology involves formation of a complex between a metalloenzyme and either the reductant or oxygen, or formation of a ternary complex involving both oxygen and reductant. In autoxidations or peroxidatic oxidations of many organic species, reduction of oxygen or hydrogen peroxide is achieved by bound

transition metal ions which generate further reactive species which initiate and propagate oxidative damage (7).

Second, with respect to cytochrome c, the heme iron which might otherwise allow direct electron transfer to molecular oxygen, is buried in a hydrophobic crevice in the apoprotein. This hydrophobic environment strongly deters development of negative charge on any oxygen which may reach the heme, since any partly reduced oxygen formed cannot be solvated.

The experimental variables which accelerate the aerobic oxidation of ferrocytochrome c thus include: activation of oxygen e.g. by addition of copper salts and complexes (1, 6, 8, 9), and factors which tend to make the heme crevice more easily opened, such as greatly decreased or elevated pH (1, 2), increased ionic strength (10), halogenated acetic acids (11), halides (12), detergents, or organic solvents (13). A high concentration of urea (> 6M) by opening the heme crevice substantially decreases the redox potential of cytochrome c, and increases the reactivity of ferrocytochrome c toward molecular oxygen (14). Superoxide dismutase reportedly accelerates the aerobic oxidation of cytochrome c and overcomes the inhibitory effect of ferricytochrome c (15). The mechanism of this effect is not clear yet, but it is likely that the superoxide dismutase pre-empts inhibition by the superoxide reaction product.

In general then, any conformational modification which tends to open the heme crevice will render the heme moiety more accessible to molecular oxygen. This in turn will enhance the rate of aerobic oxidation of ferrocytochrome c (12).

The autoxidation of ferrocytochrome c has been extensively studied, but most of these studies concerned copper catalysis (6, 9, 11). First,  $\text{Cu}^{2+}$  oxidizes ferrocytochrome c directly (rate limiting step), then  $\text{Cu}^{1+}$  is reoxidized by the molecular oxygen (8). Kinetic evidence supports participation of a ternary complex (6). Despite early evidence that copper can increase the rate of aerobic oxidation of ferrocytochrome c, information regarding the effects of other transition metals is lacking, perhaps because in the earliest reports of Keilin, iron salts were said to be ineffective (1). In particular with respect to reactivity of metal ions in the autoxidation of cytochrome c, the relationship between catalytic efficiency and presence of ligands remains obscure, although Augustin and Yandell have stated that in the case of copper the addition of *any* ligand decreases catalytic effectiveness (9). In an attempt to uncover the factors which determine the effectiveness of transition metal complexes in catalysing oxidation of ferrocytochrome c and reduction of molecular oxygen, we report the effects of 5 metals (copper, iron, manganese, aluminum and vanadate), and 4 ligands (EDTA, ADP, histidine and desferrioxamine) on the rate of aerobic oxidation of ferrocytochrome c at three different values of pH.

## Materials and Methods

### *Reagents*

Cytochrome c (horse heart type VI), adenosine 5'-diphosphate (ADP) and sodium orthovanadate were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). Aluminum chloride, L(+)-histidine, and glycine were purchased from Matheson, Coleman and Bell (Norwood, Ohio). Cupric acetate and disodium ethylenediaminetetraacetate (EDTA) were purchased from Fischer Scientific



(Fair Lawn, New Jersey). Ferrous sulfate and hydrochloric acid were purchased from Amachem (Portland, Oregon). Manganese sulfate was purchased from Mallinckrodt (Paris, Kentucky). Desferrioxamine (desferal mesylate) was a gift from Ciba Pharmaceuticals (Summit, New Jersey). Water was deionized and distilled.

#### *Preparation of Reduced Cytochrome c*

Reduced cytochrome c was prepared by passing a solution containing 100mg cytochrome c and 5mg sodium dithionite in 1.5ml nitrogen-saturated water through a Sephadex G-15 column. The Sephadex column (d=2.5cm; l=40cm) was filled with Sephadex G-15 that had been soaked in water for 6 hours. Nitrogen-saturated water was used to elute the reduced ferrocytochrome c at a rate of 1 - 1.2 ml/min. The eluate was collected under a nitrogen atmosphere. The concentration of ferrocytochrome c was calculated from the difference in absorbance of the reduced and oxidized forms of ferrocytochrome c at 550 nm, using a molar extinction coefficient of 0.0211 for the difference spectrum (16).

#### *Assay Procedure and Calculations*

A 0.05M glycine-HCl buffer (aerated with water saturated air for 30 minutes at 25°C) along with any other reagents required for the assay, was added to a spectrophotometer cuvette which was then placed in the sample chamber of a Beckman DB-GT spectrophotometer. The reaction was initiated by addition of 20 $\mu$ l ferrocytochrome c to a final concentration of 8  $\mu$ M and the rate of oxidation was monitored by recording absorbance at 550 nm. The pH was measured before and after the recording of the reaction to check for any change of pH during the reaction. The ionic strength of the buffer was not

significantly altered by addition of the metal since the amount of metal added was only 25  $\mu\text{M}$ , and the buffer strength was 50 mM. Each reaction was repeated at least three times. Initial rates were obtained by calculating the slopes of the initial portions of each reaction. The initial rates were compared by one way analysis of variance and Dunnett's procedure (17) for multiple-comparisons with a common control.

## Results

### *Reaction profiles for selected reactions at pH 4*

Progress of several typical reactions are shown in Figure 1. The reaction was very slow at this pH in the absence of added metal ions, so that we merely monitored the initial portion of the reaction. The reaction was however markedly accelerated by the presence of an appropriate transition metal complex.

### *Effects of ligands (in the absence of added metals) at various pH values*

The influences of the ligands on the initial rate of aerobic oxidation of ferrocyanide are shown in Figure 2. At pH 3.2 each of the ligands tested (ADP, EDTA, desferrioxamine, or histidine) caused a slight decrease in the rate of aerobic oxidation of ferrocyanide, but this decrease was statistically significant ( $p < 0.05$ ) only in the cases of EDTA and ADP which decreased the rate by 22% and 19% respectively.

### *Effects of metals and ligands at various values of pH*

Effects of transition metals and ligands on the aerobic oxidation of ferrocyanide depended strongly on the pH. At pH 2.6 only vanadate significantly affected the rate (Figure 3), accelerating the reaction by

**Figure 1. Autoxidation of ferrocytochrome c in the presence of Cu(ADP) or Fe(EDTA).**

The reactions were carried out at 25° C in a 0.05M glycine-HCl buffer at pH 4.0. The final concentration of each ligand was 500μM. Ferrocytochrome c was added to initiate the reaction, with a final concentration of 25μM. The total volume was 2.5ml.

FIGURE 1. AEROBIC OXIDATION OF FERROCYTOCHROME c AT pH 4.0

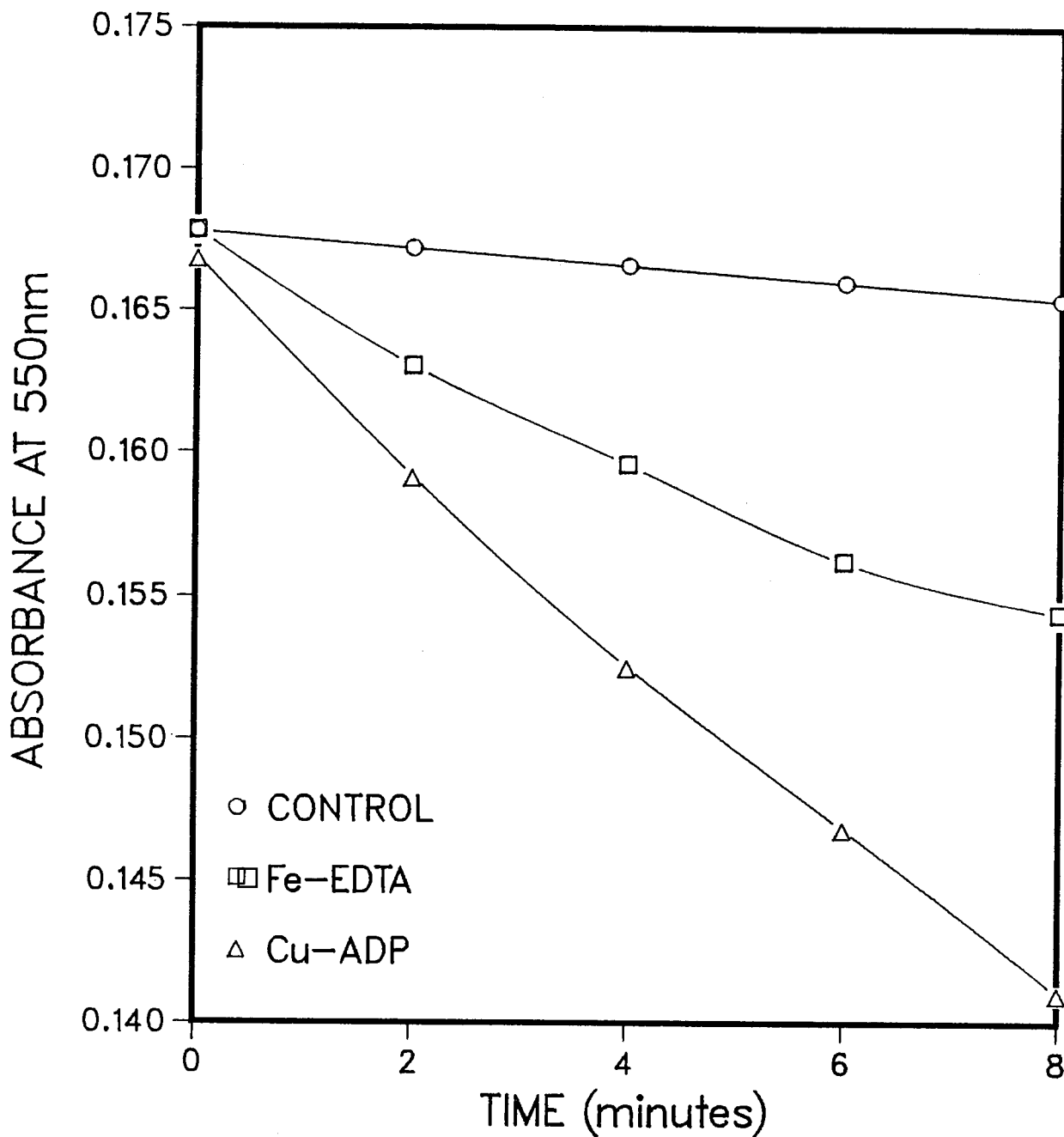
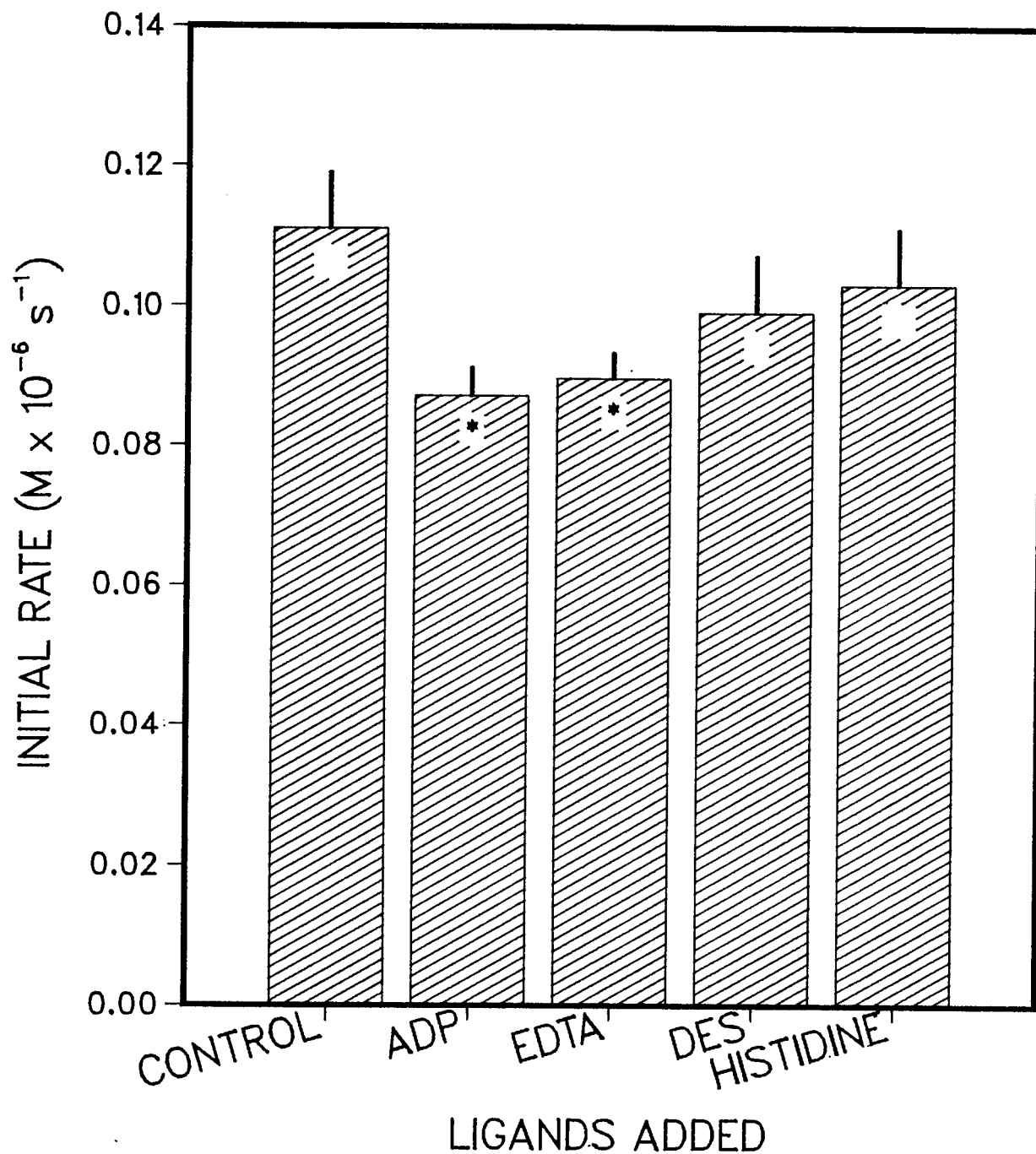


Figure 2. Effects of ligands at pH 3.2 on the initial rate of aerobic oxidation of ferrocyclochrome c.

The reactions were carried out at 25° C in a 0.05 M glycine HCl buffer at pH 3.2. The final concentration of each ligand was 500μM. Other conditions were as in Figure 1. For the control, no metal and ligand were added to the reaction system. The symbol "\*" indicates a value significantly different from control at the  $p < 0.05$  level of significance. The abbreviation of "DES" indicates desferrioxamine.

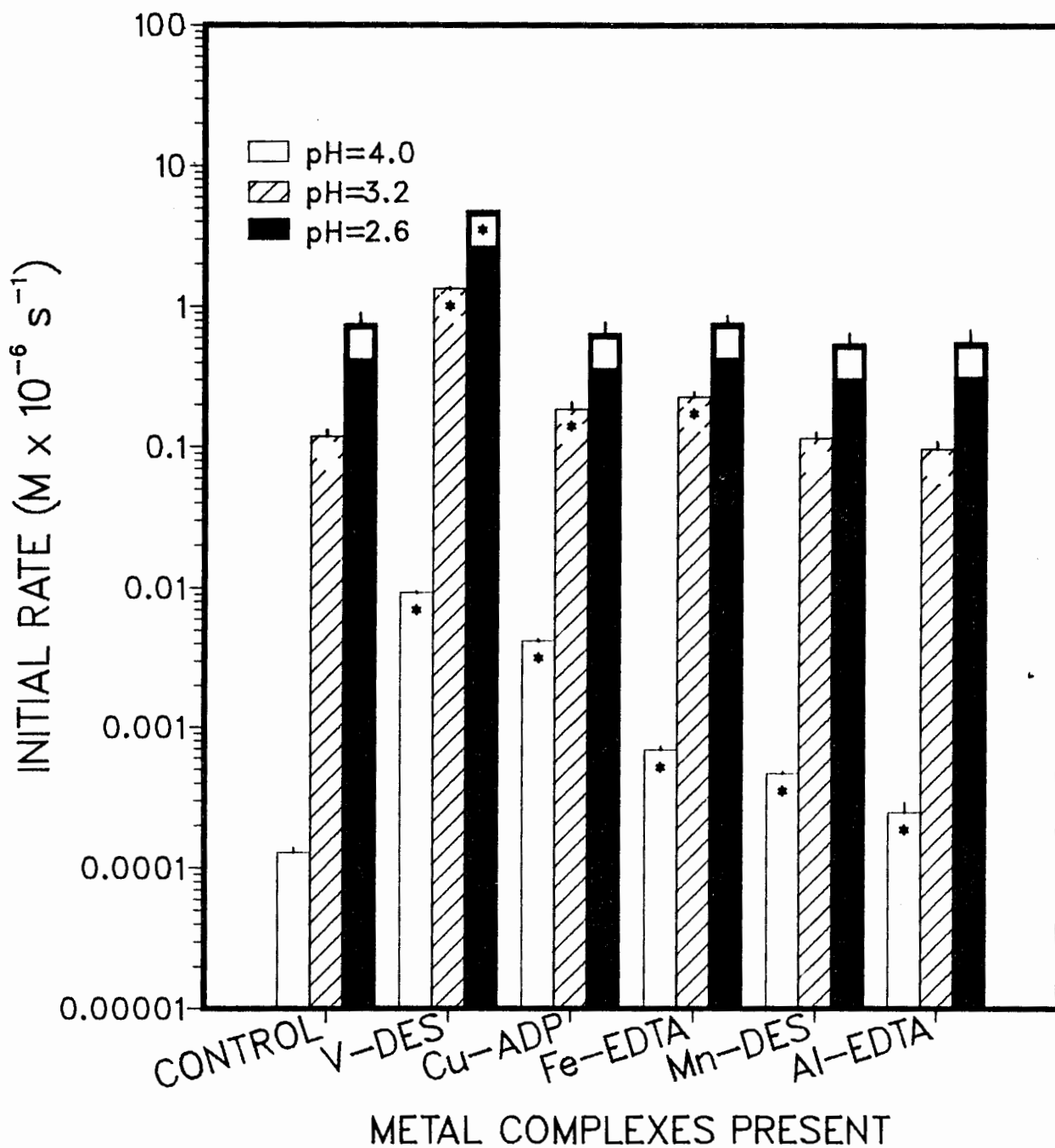
FIG. 2 EFFECTS OF LIGANDS ON THE AUTOXIDATION OF FERROCYTOCHROME C AT pH 3.2



**Figure 3. Effects of pH and ligand on the initial rate of the aerobic oxidation of ferrocyanide  $\text{C}_6\text{H}_5\text{Fe}(\text{CN})_5$ .**

Conditions were as in Figure 1. All the final concentrations of metals tested were  $25\mu\text{M}$ . All the final concentration of ligands used were  $500\mu\text{M}$ . For the controls, no metal or ligand was added to the reaction system. The symbol "\*" indicates a value significantly different from control at the  $p < 0.05$  level of significance. The abbreviation "DES" indicates desferrioxamine.

FIG. 3 EFFECTS OF pH ON AEROBIC OXIDATION OF FERROCYTOCHROME C





500%. Fe(EDTA) increased the initial rate slightly, while Cu(ADP), Al(EDTA) and Mn(desferrioxamine) decreased the initial rate, but these effects did not reach statistical significance. At pH 3.2 the effects of metals (with or without added ligands) were more pronounced, as follows: In the absence of added ligands, vanadate increased the rate 4.4-fold,  $\text{Cu}^{2+}$  accelerated the reaction by 53% ( $p < 0.05$ );  $\text{Fe}^{2+}$  and  $\text{Mn}^{2+}$  slowed the reaction insignificantly and  $\text{Al}^{3+}$  had no effect (Figure 4).

The effects of the most effective metal complexes at the same pH (3.2) were as follows: V(desferrioxamine), Cu(ADP) and Fe(EDTA) accelerated the reaction by 12 fold, 65% and 49% respectively ( $p < 0.05$ ). Al(EDTA) and Mn(desferrioxamine) each slowed the reaction by only 8% ( $p > 0.05$ ). At pH 4.0 all of the metal complexes tested accelerated the reaction: V(desferrioxamine) by 71-fold, Cu(ADP) by 31-fold ( $p < 0.01$ ), Fe(EDTA) by 4.3-fold ( $p < 0.01$ ), Mn(desferrioxamine) by 2.7-fold ( $p < 0.05$ ), Al(EDTA) by 93% ( $p < 0.05$ ) (Figures 3, 4).

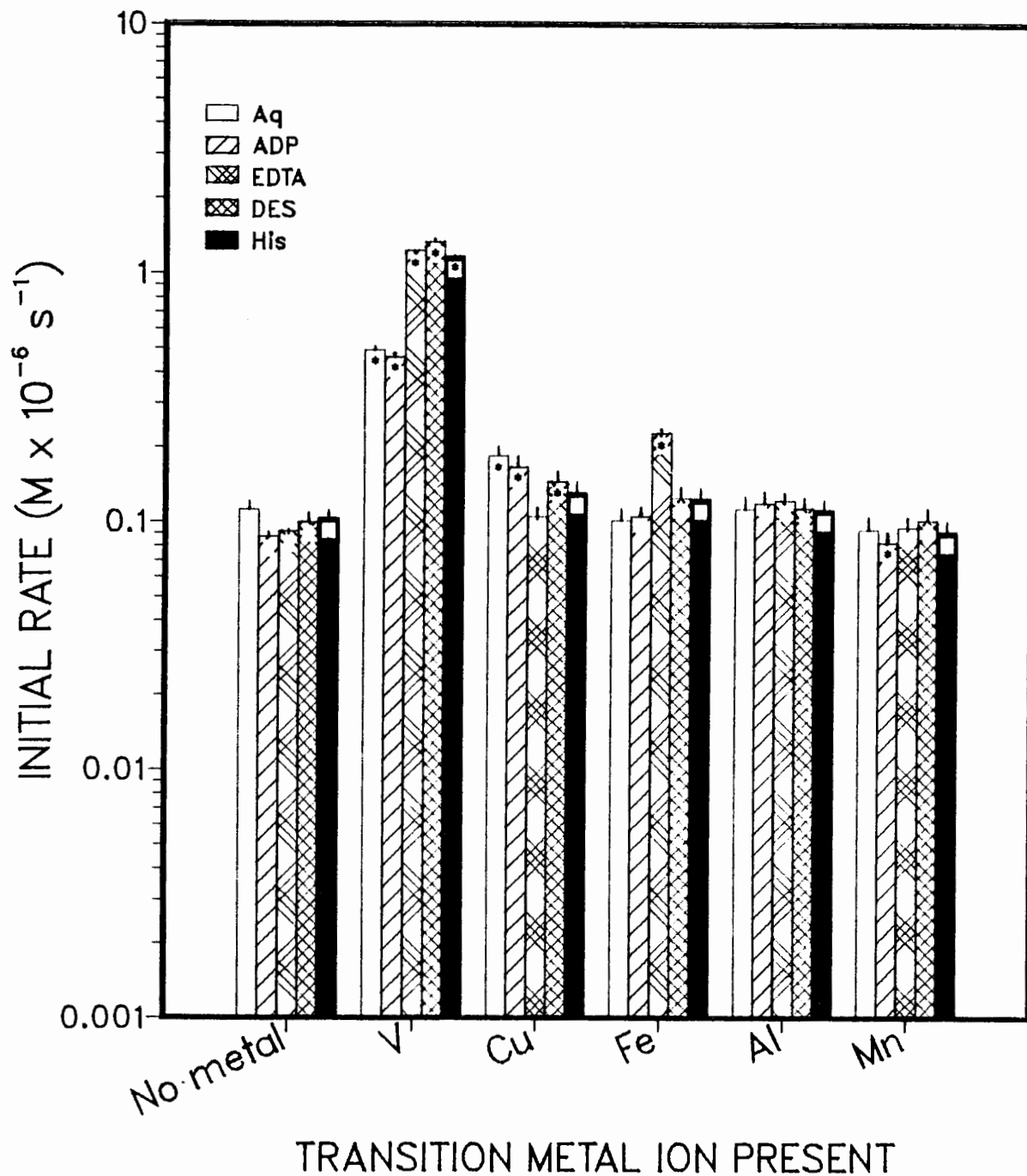
#### *Effect of pH on the aerobic oxidation of ferrocytochrome c*

The aerobic oxidation of ferrocytochrome c accelerated dramatically as pH was decreased. For example, when the pH of the buffer was changed from 4 to 3.2, the initial rate of the aerobic oxidation of ferrocytochrome c increased 930-fold if no metal had been added, 470-fold in the presence of Al(EDTA), 330-fold in the presence of Fe(EDTA), 200-fold in the presence of Mn(desferrioxamine), 140-fold in the presence of V(desferrioxamine) and 40-fold in the presence of Cu(ADP). When the pH was changed from 3.2 to 2.6, the initial rate increased: 6.2-fold (no metal added), 4.3-fold in the presence of Al(EDTA), 3.5-fold in the presence of V(desferrioxamine), 3.3-fold in the presence of Fe(EDTA), 3.4-fold in the presence of Cu(ADP),

**Figure 4. Effects of metal ions and metal complexes on the initial rate of the aerobic oxidation of ferrocyanide at pH 3.2.**

Reactions were carried out in a 0.05M glycine-HCl buffer at pH 3.2. Other conditions were as in Figure 2. The symbol "\*" indicates a value significantly different from control at the  $p < 0.05$  level of significance. The abbreviations "DES" and "His" indicate desferrioxamine and histidine.

Fig. 4 EFFECTS OF METALS AND METAL COMPLEXES ON AEROBIC OXIDATION OF FERROCYTOCHROME c



4.6-fold in the presence of Mn(desferrioxamine) (Figure 3). As can be seen, all of these effects were highly significant ( $p < 0.01$ ).

## Discussion

### *Influence of pH on the aerobic oxidation of ferrocytochrome c*

The current results confirm that in the aerobic oxidation of ferrocytochrome c the influence of pH predominates over other effects (2). This is particularly true in the absence of added metals, simply because the rate at pH 4.0 is much higher in the presence of transition metal ions, while at low pH, all reactions approach the same high reaction velocity.

### *pH-dependent catalytic effects of metal complexes*

Vanadate oxidized ferrocytochrome c at all three values of pH tested. However, no significant oxygen consumption accompanied this oxidation. The conclusion that vanadate directly oxidizes cytochrome c is supported by the observation that oxygen was not required for oxidation of cytochrome c in the presence of vanadate. In contrast, the effects of the other metals were almost totally dependent on the presence of oxygen.

The magnitude of the effects of metal complexes in turn depend on pH. Thus at least one complex of each metal significantly accelerated the aerobic oxidation of ferrocytochrome c at pH 4, but the same metal complexes had no effect at pH 2.6. This is most simply explained on the basis that the weakening of the hydrophobic crevice at low pH (18) augments the reactivity of cytochrome c toward oxygen to the point where extraneous metals become redundant. There are two pathways for electron transfer reactions of cytochrome c (19, 20). The first of these, the "adjacent attack" pathway

requires the rupture of the iron-sulfur bond or the opening of the heme crevice. When the pH is 3.2 or less, weakening of the crevice facilitates breaking of the iron-protein bonds (21) and this in turn allows the adjacent attack to occur. Exposure of heme iron obviates the need for exogenous metals. In this regard, the complete failure of iron, copper or manganese salts to accelerate oxidation at pH 2.6 contrasts strongly with catalytic actions of these metals in many other systems, and indeed in the same system at higher pH. Moreover, the failure of desferrioxamine to inhibit significantly is particularly striking in view of the widely reported inhibition by desferrioxamine of a large number of the reductions of oxygen. It implies that redox cycling of contaminant iron (always present in buffer salts) is not involved under these circumstances.

The second pathway (the "remote pathway") requires mediation by a low molecular weight redox reagent (typically a metal) near the edge of the heme. At pH 4, the heme is unreactive toward CO (1), reflecting the fact that the face of the heme is insufficiently exposed to react with oxygen. Thus the reaction can only occur at the edge of the heme, and exogenous metals are required. That  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Al}^{3+}$  accelerate the aerobic oxidation of ferrocyanide at pH 4 suggests that ferrocyanide may act as an electron donor in Fenton-type or other pro-oxidant processes.

#### *Effectiveness of metals and metal complexes*

Effects of metals and metal complexes at pH 4 extend and are consistent with the earlier reports. Thus the current studies, agree with Keilin (1) that Fe added alone is ineffective. However we found that, while most Fe complexes are similarly ineffective, the Fe(EDTA) complex significantly accelerates oxidation of ferrocyanide. Again, we confirm Keilin's

observation that  $\text{Cu}^{2+}$  is an effective catalyst of the aerobic oxidation of cytochrome c (1), but its effectiveness was diminished by ligands, confirming Augustin and Yandell (9, 22). In the current study aluminum did not profoundly accelerate the aerobic oxidation of cytochrome c as did other transition metals at pH 4.0, from which we conclude that the ability to undergo redox cycling is more important than ability to function as a Lewis acid. Manganese did not accelerate the reaction at pH 3.2, nor was it as effective as iron or copper at pH 4.0, and this may reflect an inappropriate redox potential of all of the complexes used. Alternatively, a lack of  $\pi$ -symmetry ligand-metal orbitals with geometry appropriate to overlap with the  $\pi$  orbitals of the heme group, could account for its lack of reactivity. The distinctive effects of vanadate require further explanation, and to this end we have carried out detailed studies of the reduction of oxygen by vanadium(III)[V(III)] and vanadium(IV)[V(IV)], and of the reactivity of vanadium(III), vanadium(IV) and vanadium(V) toward cytochrome c both anaerobically and aerobically which are currently being prepared for publication. The first of these, the reduction of oxygen by V(III) and V(IV) is considered in an accompanying manuscript.

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**The redox reactivity of vanadium(III), vanadium(IV) and vanadium(V) toward ferri- and ferrocytochrome c anaerobically: Effects of pH and ligands<sup>1</sup>**

by

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**Key Words:** oxidation, reduction, cytochrome c, vanadium, vanadate, vanadyl.

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## CHAPTER III

### THE REDOX REACTIVITY OF VANADIUM TOWARD CYTOCHROME c ANAEROBICALLY: EFFECTS OF PH AND LIGANDS

#### Abstract

Vanadium(V) (vanadate) is an effective oxidizing agent, capable of oxidising ferrocytochrome c at pH 6.0, and also at neutral pH when complexed with some ligands. It is also able to accelerate the reduction of ferricytochrome c at pH 7.4 in the presence of an appropriate ligand, desferrioxamine. Specifically, V(V) oxidized ferrocytochrome c anaerobically at pH 6.0 at a rate of  $1.7 \times 10^{-3} \mu\text{M s}^{-1}$ . Addition of desferrioxamine, histidine, ATP, EDTA and bovine serum albumin increased the initial rate 3500-fold, 18.6-fold, 17.1-fold, 2-fold, and 1.5-fold respectively. Oxidation of ferrocytochrome c by V(V) did not occur at pH 7.0 except in the presence of desferrioxamine, EDTA or ATP (initial rate =  $1.2 \times 10^{-2} \mu\text{M s}^{-1}$  in the presence of desferrioxamine), and did not occur at pH 7.4. Vanadium(IV) (vanadyl) and V(III) were in turn able to reduce ferricytochrome c in the absence of added ligands at pH 6.0, 7.0, and 7.4, the respective rates being  $3.7$ ,  $8.5$ , and  $10.0 \times 10^{-2} \mu\text{M s}^{-1}$  for V(IV). Desferrioxamine also increased the rates of the reduction of ferricytochrome c by V(IV) at all pH values (by 95% at pH 6.0), whereas ATP and EDTA inhibited whether the electron donor was V(III) or V(IV). Surprisingly, at pH 7.4,  $200 \mu\text{M}$  V(V) in the presence of  $1\text{mM}$  desferrioxamine induced complete reduction of ferricytochrome c (initial rate =  $1.1 \times 10^{-2} \mu\text{M s}^{-1}$ ). Since vanadium salts can be reduced by cellular reductants, and can be reoxidised by cytochrome c or molecular oxygen, vanadium has the capacity for redox cycling intracellularly.

## Introduction

Considerable attention has been given to the biochemistry of vanadium [1-3] as a result of the current discoveries of multiple effects of vanadium on the biochemical and physiological processes of living cells [1-2]. Since vanadate has potential in the treatment of diabetes, the possible toxicity of vanadate has begun to attract the attention of biochemists [2-4].

Although the toxicity of orally administered vanadium in humans is reportedly low [5-7], vanadium has been reported toxic in animals [8-10]. For example, rats fed vanadium develop pulmonary hypertension [8], and increased lipid peroxidation in the kidneys is seen after a single subcutaneous or intraperitoneal injection to rats or mice [9]. The most effective antidote for acute vanadate toxicity is reportedly ascorbic acid [10], which, if administered prior to acute exposure to vanadium, diminished the toxicity [9]. It is thus likely that the toxicity of vanadate is at least partly related to its redox reactivity [11]. Furthermore, vanadium exists as V(IV) intracellularly, but it exists as V(V) in plasma [12-15]. This striking distribution of different redox states of vanadium also underlines the importance of redox reactions of vanadium in its metabolism and its biological actions.

Although the inorganic redox chemistry of vanadium has been extensively studied, reports of its redox reactivity in relation to cellular metabolites are rare [16]. Moreover, investigations of effects of ligands on the redox reactivity of vanadium are sporadic and incomplete [17]. It is known that V(V) or V(IV) can form complexes with numerous biological molecules, e.g. ATP, ADP, AMP, creatine phosphate, transferrin, human serum albumin, aspartic acid, glutathione, glutamic acid, ascorbic acid, etc. [6]. Ligands and pH

have critical effects on the redox reactivity of vanadium, since ligand-binding and change of pH may significantly change the reduction potentials of metals [18].

The current study is one of a series intended to define the factors which influence the redox reactivities of vanadium toward oxygen and cellular metabolites, in this case cytochrome c anaerobically. Cytochrome c is an important water soluble component of the respiratory chain, having a reduction potential (at biological standard state, pH 7.0, 25° C) of about 0.25V. This is sufficiently high that substances which fully oxidize ferrocytochrome must be considered as strong oxidising agents relative to others occurring in biological systems. We chose cytochrome c as an initial redox reagent since its structure and redox reactivity have been studied extensively [19-21]. Occasionally, the reduction of ferricytochrome c by transition metals or transition metal-ligand complexes has been reported, e. g., the reduction of ferricytochrome c by cysteine/molybdate complexes [22] or by chromium(II) [23].

## Materials and Methods

### *Reagents*

Cytochrome c (horse heart, type VI), adenosine 5'-triphosphate (ATP), N-acetyl-L-tyrosine ethyl ester (ATEE), Bovine Serum Albumin (BSA), sodium orthovanadate, N-2-hydroxyethylpiperazine -N'-2-ethanesulfonic acid (HEPES) and 2(N-morpholino)ethanesulfonic acid (MES) were purchased from Sigma Chemical Company (St. Louis., Mo., U.S.A.). L(+)-histidine was purchased from Matheson, Coleman and Bell (Norwood, Ohio). Disodium ethylenediaminetetraacetate (EDTA) was purchased from Fischer Scientific Co

(Fair Lawn, New Jersey). Vanadyl sulfate (trihydrate) and vanadium(III) chloride were purchased from Aldrich Chemical Company (Milwaukee, Wis, U.S.A.). Hydrochloric acid was purchased from Amchem (Portland, Oregon). Desferrioxamine (desferal mesylate) was a gift from CIBA Pharmaceuticals (Summit, New Jersey). Water was deionized and distilled.

#### *Preparation of Reduced Cytochrome c.*

Reduced cytochrome c was prepared by passing a solution containing 100mg cytochrome c and 5mg sodium dithionite in 1.5ml nitrogen-saturated water through a Sephadex G-15 column (d=2.5cm; l=40cm) filled with Sephadex G-15 that had been soaked in water for 6 hours. Nitrogen-saturated water was used to elute the reduced ferrocytochrome c at a rate of 1 - 1.2 ml/min. The eluate was collected under a nitrogen atmosphere. The concentration of ferrocytochrome c was calculated from the difference in absorbance of the reduced and oxidized forms of ferrocytochrome c at 550nm, using a molar extinction coefficient of 0.0211 for the difference spectrum [24].

#### *Assay Procedure and Calculations.*

All the experiments were conducted under anaerobic conditions. The anaerobic cuvettes were purchased from TCS-Medical Products Co. (Huntington Valley, U.S.A.). Nitrogen-saturated buffer (0.05M HEPES or MES) along with any other reagents required for the assay, was added to an anaerobic sample cuvette which was sealed and again bubbled with nitrogen for 15 minutes. The Beckman DB-GT spectrophotometer was set to zero with the cell in place. The reaction was initiated by addition of about 35 $\mu$ l cytochrome c to a final concentration of 15  $\mu$ M and the rate of oxidation or reduction was monitored by recording absorbance at 550nm. Each reaction was repeated at least twice.

The data were collected by a digital data acquisition system and simultaneously recorded on a Beckman 10" strip chart recorder. The pH was measured before and after the recording of the reaction to check for any change of pH during the reaction. The ionic strength of the buffer was not significantly altered by addition of the metal since the amount of metal was much less than the buffer strength. The initial rates of the reaction were obtained by calculating the slopes of the initial part of each reaction. In the statistical comparisons of means, we used one way analysis of variance and Dunnett's procedure of multi-comparison [25].

## Results

It is interesting that vanadate could either oxidise ferrocytochrome c or mediate the reduction of ferricytochrome c.

### *Reaction profiles for oxidation of ferrocytochrome c*

Progress of typical oxidations of ferrocytochrome c are shown in Figure 1. In this graph, only the initial portions of each reaction were monitored. The progress shows: although the oxidation of ferrocytochrome c by vanadate was slow, ATP increased the initial rate profoundly.

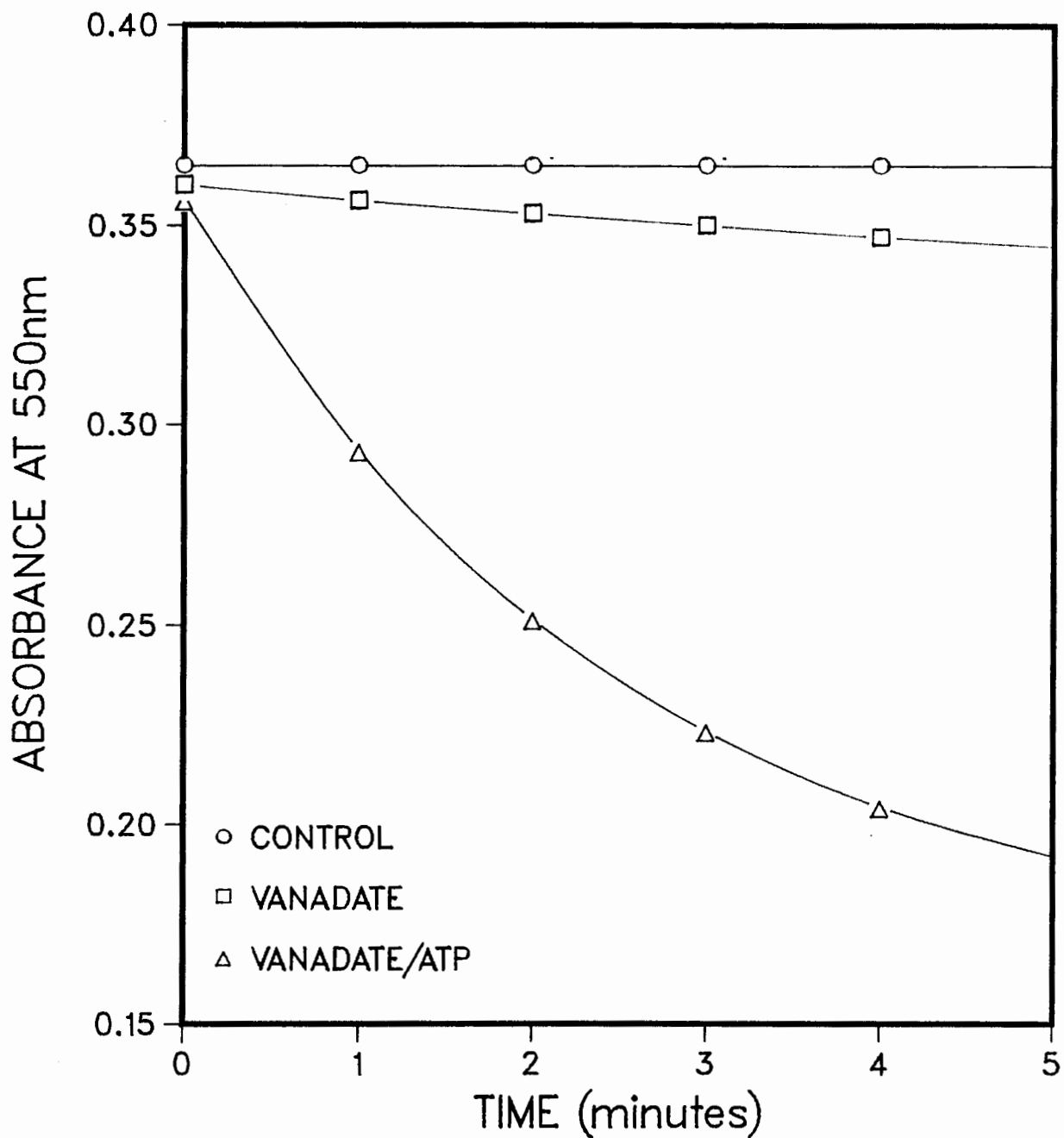
### *The anaerobic oxidation of cytochrome c by vanadate or vanadate complexes*

Under anaerobic conditions vanadate alone did not oxidize ferrocytochrome c significantly at pH 7.0 and 7.4, but at pH 6.0 it did, with a initial rate of  $1.7 \times 10^{-3} \mu\text{M s}^{-1}$ . Figure 2 shows the effects of ligands on the initial rates of these reactions.

**Figure 1. Anaerobic oxidation of ferrocytochrome c by vanadate or vanadate/ATP at pH 6.0.**

The reactions were carried out at pH 6.0, 25°C in a 0.05M MES buffer saturated with nitrogen. Sodium vanadate and ATP were added to the anaerobic cuvette which contained sufficient volume of nitrogen saturated buffer, resulting in a final volume of 2.5 ml. The final concentrations of ATP and vanadate were 1mM. The cuvettes were bubbled with nitrogen for 15 minutes again. To this system, ferrocytochrome c was added to initiate the reaction, yielding a final concentration of ferrocytochrome c 15  $\mu$ M.

FIGURE 1. ANAEROBIC OXIDATION OF FERROCYTOCHROME c BY VANADATE OR VANADATE/ATP COMPLEX AT pH 6.0





At pH 6.0, most of the ligands tested accelerated the oxidation of ferrocytochrome c by vanadate. Addition of 1mM desferrioxamine (final concentration) increased the initial rate by about 3500-fold. Histidine increased the rate by 18.6-fold, ATP increased it 17.1-fold, EDTA increased it 2-fold, and albumin (BSA) increased it 1.5-fold. Among the ligands tested, only ATEE decreased the initial rate (to 58% of control value) (Figure 2).

At pH 7.0, the oxidation of ferrocytochrome c by vanadate alone was not measurable. Desferrioxamine, EDTA and ATP allowed this reaction, but the rates remained 2 to 3 orders of magnitude lower than the corresponding reactions at pH 6.0. In the presence of 1mM (final concentration) desferrioxamine, the initial rate of oxidation of cytochrome c by vanadate was  $1.2 \times 10^{-2} \mu\text{M s}^{-1}$ , i.e. about 7 times that of the control at pH 6.0. In the presence of 1mM EDTA, the initial rate was  $4.4 \times 10^{-4} \mu\text{M s}^{-1}$ , and in the presence of 1mM ATP, the initial rate was  $1.2 \times 10^{-4} \mu\text{M s}^{-1}$ . Other ligands (histidine, ATEE, and albumin) had no significant effect on the oxidation of cytochrome c (Figure 2).

At pH 7.4, none of the ligands allowed measurable oxidation of ferrocytochrome c by 100  $\mu\text{M}$  to 1 mM vanadate.

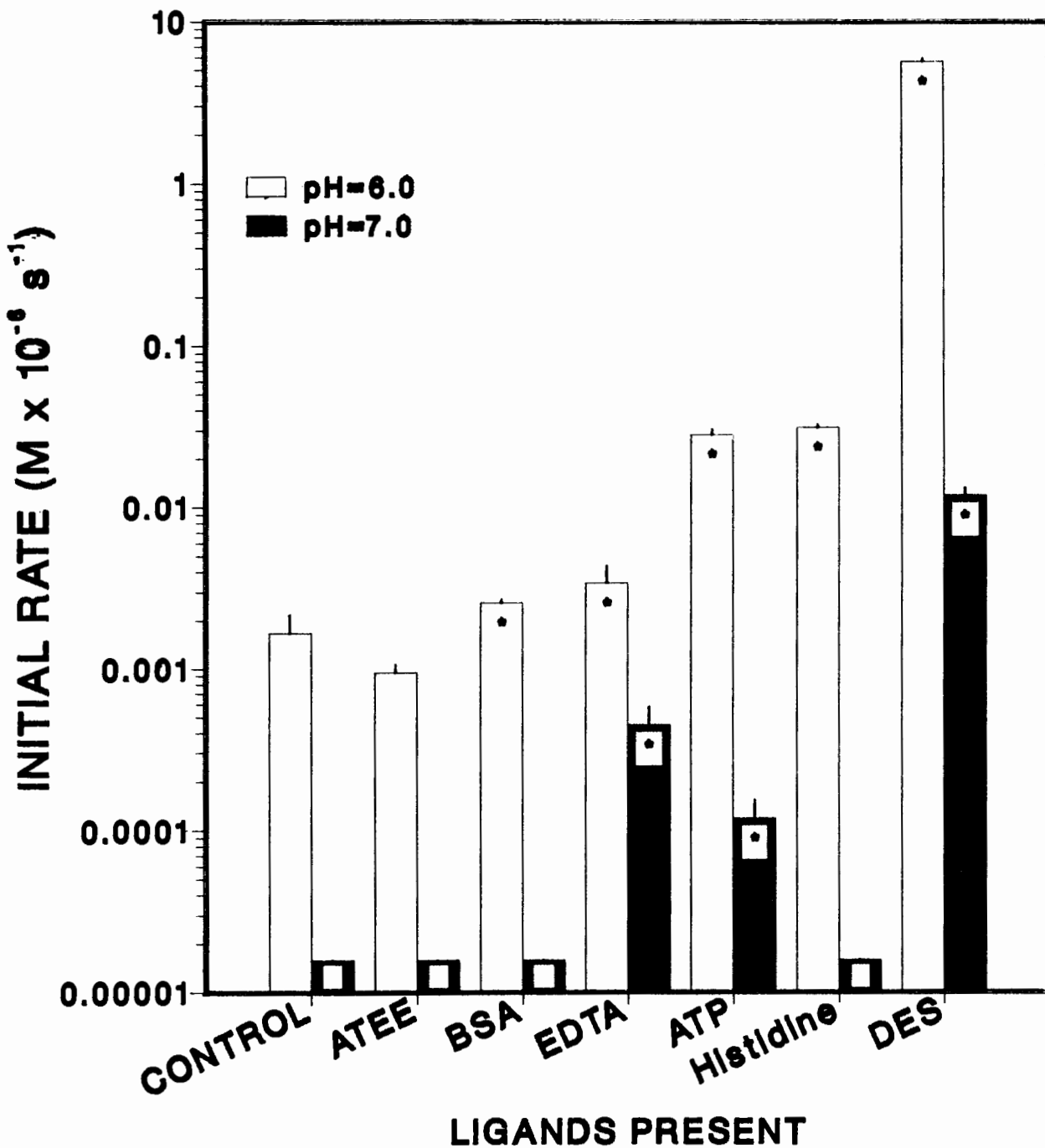
*The reduction of ferricytochrome c by desferrioxamine/vanadate under anaerobic conditions*

Surprisingly, at pH 7.4, the addition of 200 $\mu\text{M}$  vanadate together with 1mM desferrioxamine rapidly reduced ferricytochrome c, with an initial rate of  $1.1 \times 10^{-2} \mu\text{M s}^{-1}$ . Lower or higher concentrations of vanadate were less effective. The progress of this reaction is shown in Figure 3. Other ligands did not reduce ferricytochrome c. Desferrioxamine alone slowly reduced

**Figure 2. Anaerobic oxidation of ferrocyclochrome c by vanadate complexes at pH 6.0 and 7.0: Effects of ligands.**

The reactions were carried out at 25°C in either a 0.05M MES buffer at pH 6.0, or a 0.05M HEPES buffer at pH 7.0. The final concentration of vanadate was 1mM, the final concentrations of ligands were: ATEE 200µM; ATP 1mM ; albumin 600µg/ml; desferrioxamine 1mM; EDTA 1mM; histidine 1mM. Other conditions were as Figure 1. The final concentration of ferrocyclochrome c was 15 µM. The symbol "\*" indicates a value significantly different from control at the p<0.05 level of significance. The abbreviation "DES" indicates desferrioxamine.

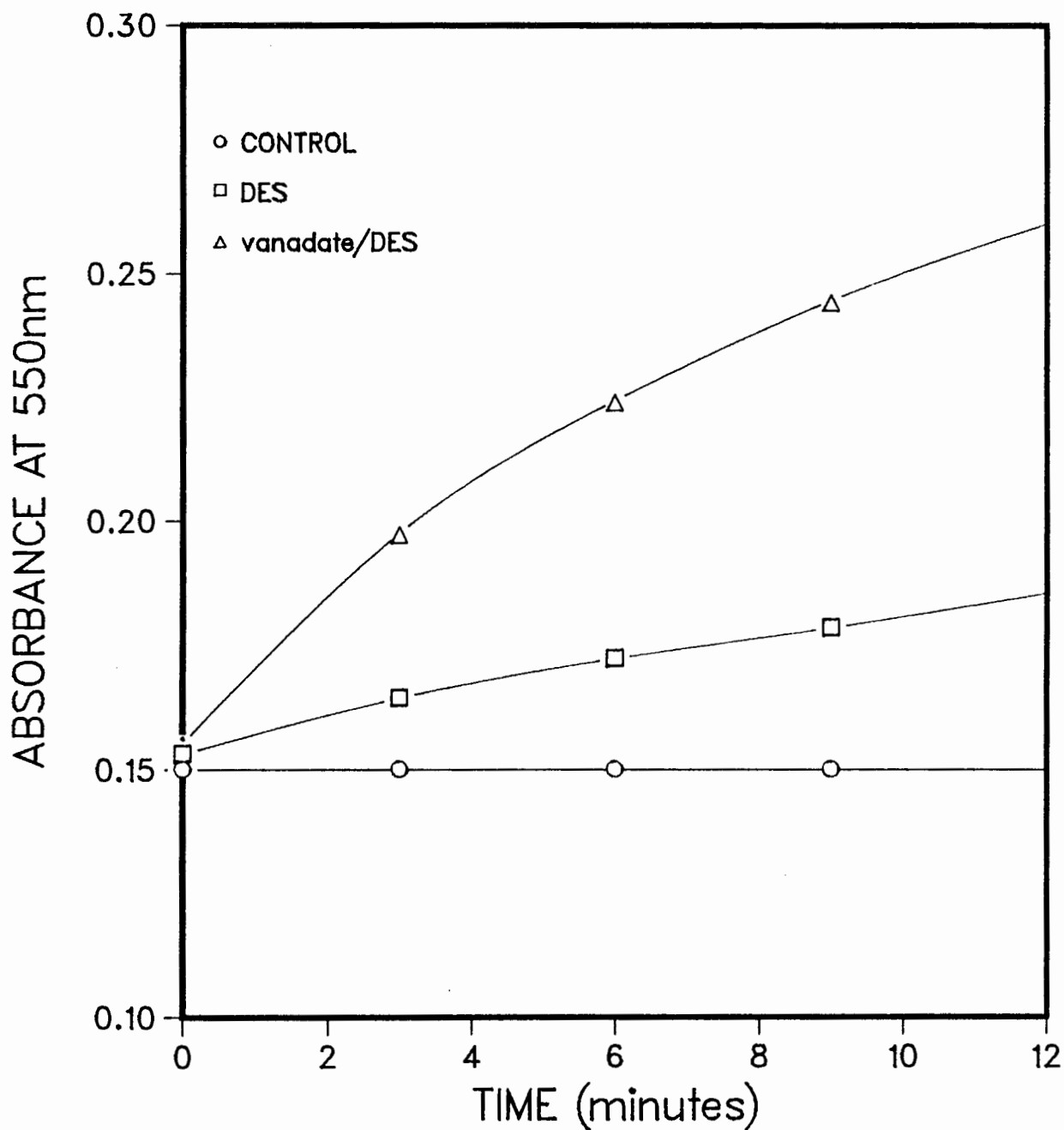
**FIGURE 2. ANAEROBIC OXIDATION OF FERROCYTOCHROME c BY VANADATE COMPLEXES: EFFECTS OF LIGANDS**



**Figure 3. Anaerobic reduction of ferricytochrome c by vanadate desferrioxamine complex.**

The reactions were carried out at pH 7.4, 25°C in a 0.05M HEPES buffer saturated with nitrogen. The final concentrations of vanadate and desferrioxamine were 200 $\mu$ M and 1mM respectively. Other conditions were as Figure 1. The abbreviation "DES" indicates desferrioxamine.

FIGURE 3 ANAEROBIC REDUCTION OF FERRICYTOCHROME c BY DESFERRIOXAMINE OR VANADATE/DESFERRIOXAMINE COMPLEX



ferricytochrome c anaerobically with an initial rate of about 25% of the rate of reduction by vanadate/desferrioxamine.

*Anaerobic reduction of ferricytochrome c by vanadium(IV) and its complexes*

Vanadium(IV) reduced ferricytochrome c anaerobically, the initial rate of reduction of ferricytochrome c being  $3.7 \times 10^{-2} \mu\text{M s}^{-1}$  at pH 6.0,  $8.5 \times 10^{-2} \mu\text{M s}^{-1}$  at pH 7.0 and  $1.0 \times 10^{-1} \mu\text{M s}^{-1}$  at pH 7.4. These rates were dramatically affected by addition of ligands shown in Figures 4-6.

At pH 6.0, ATP decreased the rate of the reduction of ferricytochrome c by 95%, EDTA decreased the rate by 75% in comparison with control values. However, desferrioxamine and albumin increased the rate of the reduction of ferricytochrome c by 95% and 90% in comparison with the control values ( $p < 0.05$ ). Both ATEE and histidine slightly accelerated the reduction of ferricytochrome c, but these changes did not reach statistical significance (Figure 4).

At pH 7.0, ATP decreased initial rate by 93%, and EDTA decreased it by 99%, in comparison with the control values. Desferrioxamine accelerated the reduction, but it was not statistically significant. In contrast, histidine increased the initial rate by 40% of the control value ( $p < 0.05$ ) (Figure 4).

At pH 7.4, histidine increased the anaerobic reduction of ferricytochrome c by 80% of the control value ( $p < 0.05$ ). ATP was less inhibitory than at pH 7.0, decreasing the initial rate by 76% of the control value ( $p < 0.05$ ), while EDTA still inhibited by nearly 99% (Figure 5).

**Figure 4. Anaerobic reduction of ferricytochrome c by vanadium(III) or vanadium(IV) complexes at pH 6.0: Effects of ligands.**

The reactions were carried out at pH 6.0. A final concentration of 200 $\mu$ M of vanadium(III) or 200 $\mu$ M of vanadyl was used. Other conditions described in Figure 2. The symbol "\*" indicates a value significantly different from control at the  $p < 0.05$  level of significance. The abbreviations "DES" and "HIS" indicate desferrioxamine and histidine.

FIGURE 4. ANAEROBIC REDUCTION OF FERRICYTOCHROME *c* BY VANADIUM(III) OR VANADYL(IV) COMPLEXES. AT pH 6.0: EFFECTS OF LIGAND

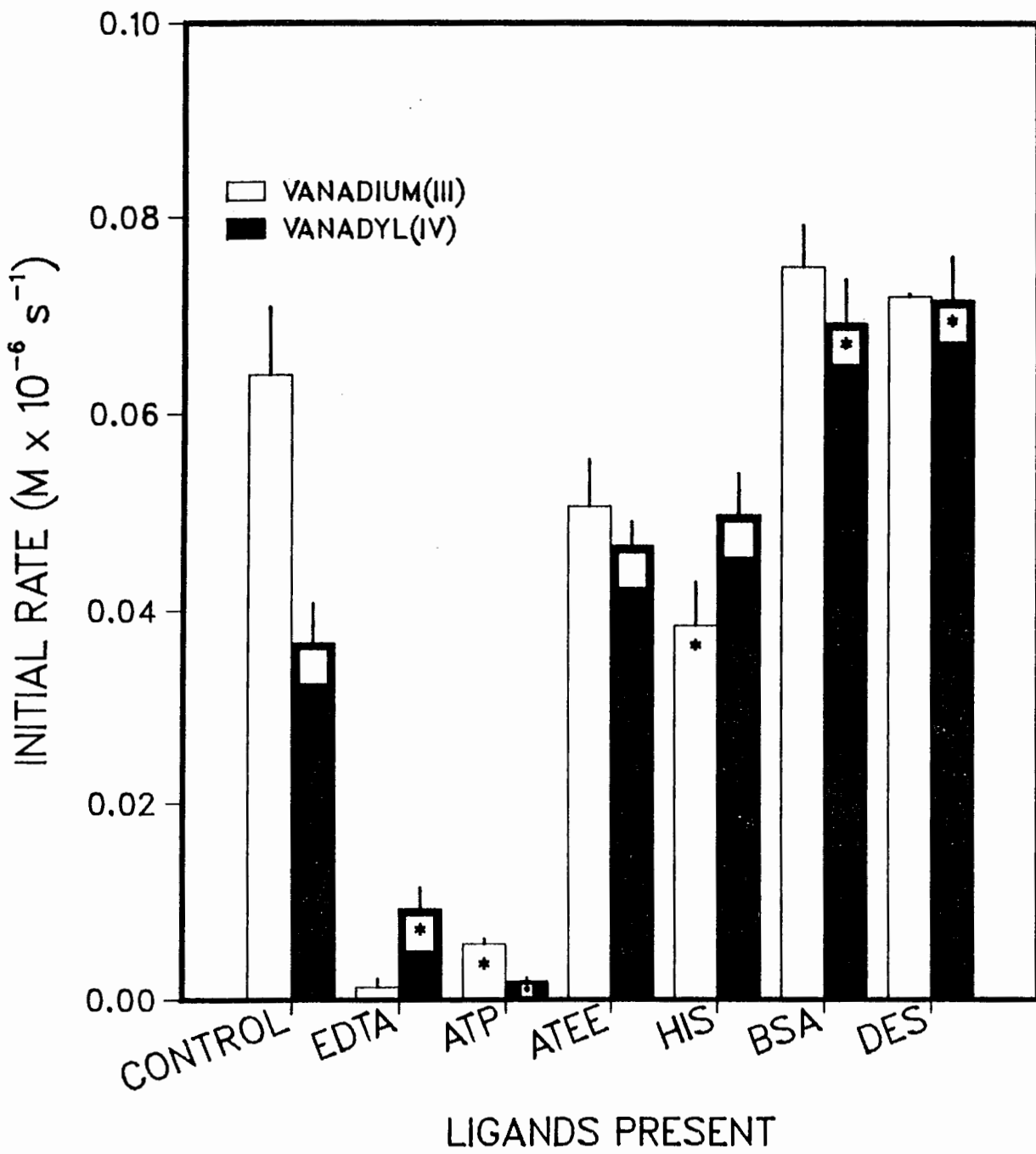
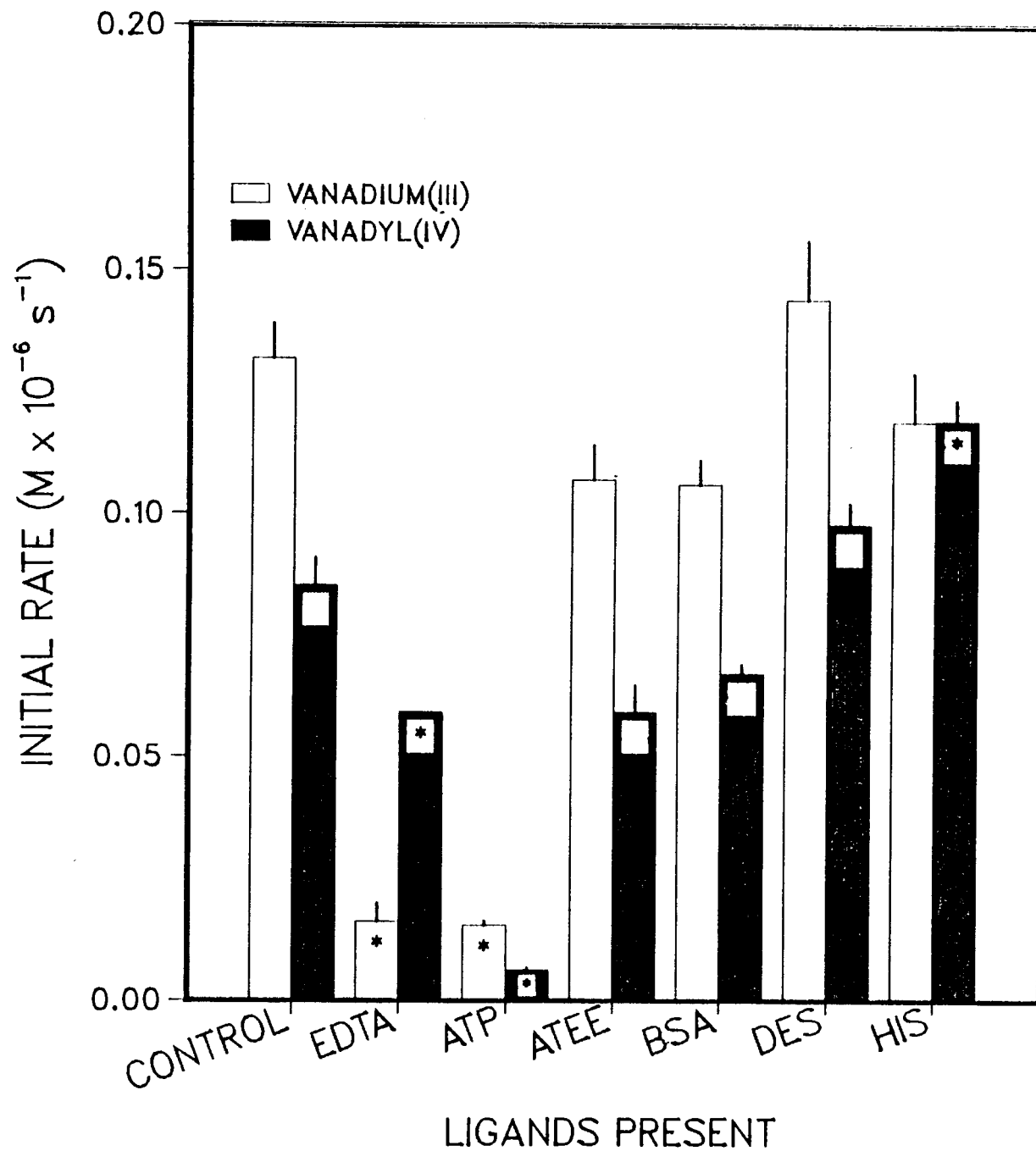




Figure 5. Anaerobic reduction of ferricytochrome c by vanadium(III) or vanadium(IV) complexes at pH 7.0: Effects of ligands.

The reactions were carried out at pH 7.0. Other conditions described as in Figure 2. The symbol "\*" indicates a value significantly different from control at the  $p < 0.05$  level of significance. The abbreviations "DES" and "HIS" indicate desferrioxamine and histidine.

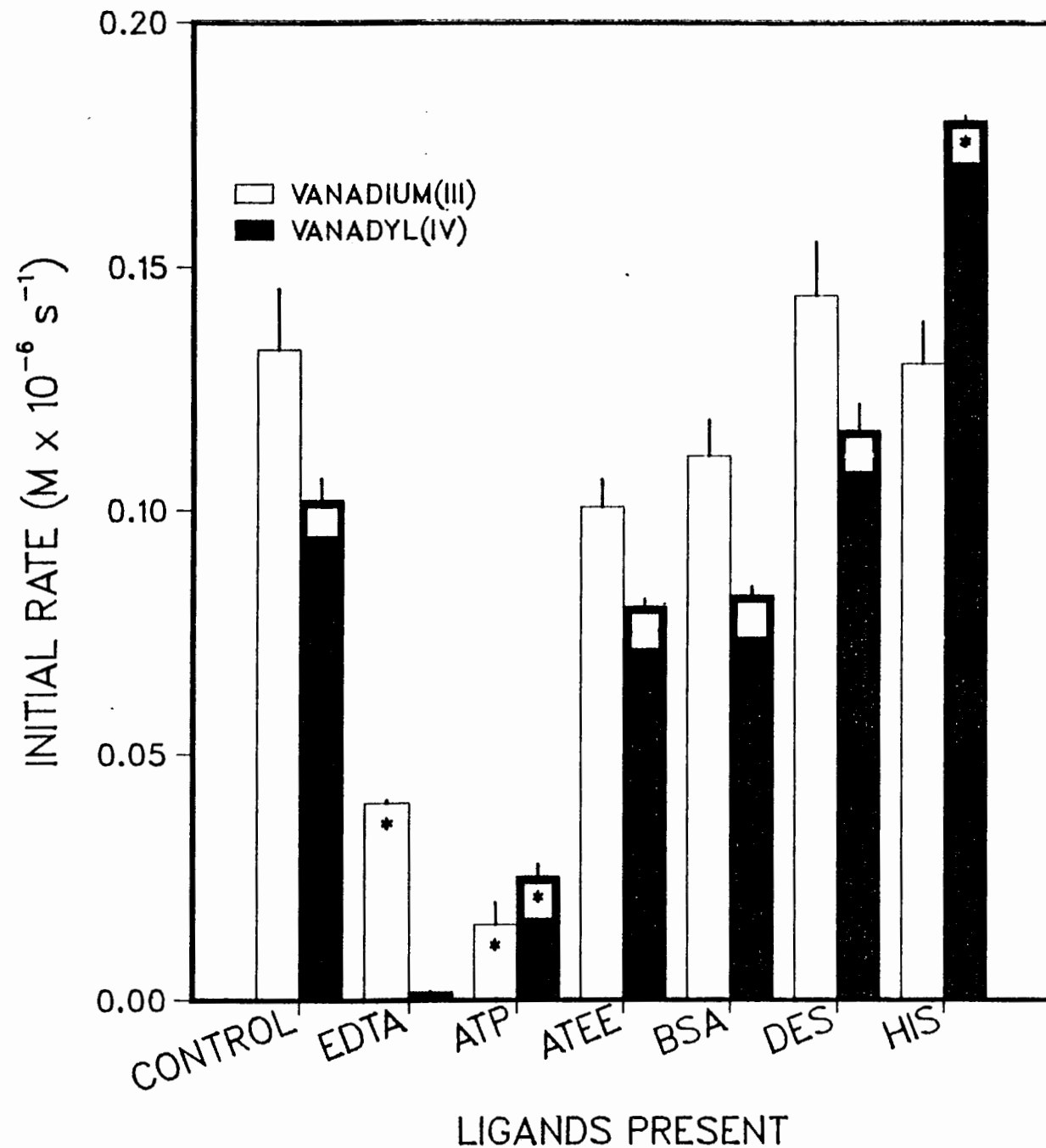
FIGURE 5. ANAEROBIC REDUCTION OF FERRICYTOCHROME *c* BY VANADIUM(III) OR VANADYL(IV) COMPLEXES AT pH 7.0: EFFECTS OF LIGAND



**Figure 6. Anaerobic reduction of ferricytochrome c by vanadium(III) or vanadium(IV) complexes at pH 7.4: Effects of ligands.**

The reactions were carried out at pH 7.4. Other conditions were the same as Figure 2. The symbol "\*" indicates a value significantly different from control at the  $p < 0.05$  level of significance. The abbreviations "Des" and "HIS" indicate desferrioxamine and histidine.

FIGURE 6. ANAEROBIC REDUCTION OF FERRICYTOCHROME  $c$  BY VANADIUM(III) OR VANADYL(IV) COMPLEXES AT pH 7.4: EFFECTS OF LIGAND



*The effects of ligands on anaerobic reduction of ferricytochrome c by vanadium(III)*

Under anaerobic conditions, vanadium(III) reduced ferricytochrome c at all three pH levels tested. The initial rate of the reduction of ferricytochrome c by vanadium(III) was larger than that of vanadyl (Figures 4, 5, 6). The reduction of ferricytochrome c by vanadium(III) was also pH dependant. At pH 6.0, 7.0, and 7.4, the initial rate was  $6.4 \times 10^{-2} \mu\text{M s}^{-1}$ ,  $1.32 \times 10^{-1} \mu\text{M s}^{-1}$ , and  $1.33 \times 10^{-1} \mu\text{M s}^{-1}$  respectively. Addition of most ligands slowed the reduction of ferricytochrome c (Figures 4, 5, 6)

At all three pHs tested, EDTA (1 mM) or ATP (1 mM) significantly inhibited the reduction of ferricytochrome c by V(III). At pH 6.0, 7.0 and 7.4, EDTA decreased the initial rates of reduction of ferricytochrome c by 98%, 88%, and 70% in comparison with control values. At the same three values of pH, ATP decreased the initial rates by 91%, 88%, and 88% respectively. In contrast to its effects on the reactivity of V(V), or V(IV), desferrioxamine had no significant effect on the anaerobic reduction of ferricytochrome c by vanadium(III) at any of the values of pH tested. Albumin accelerated the anaerobic reduction of ferricytochrome c by vanadium(III) at pH 6.0, but this effect was not statistically significant. Other ligands (histidine and ATEE) had almost no effect on the anaerobic reductions by vanadium(III).

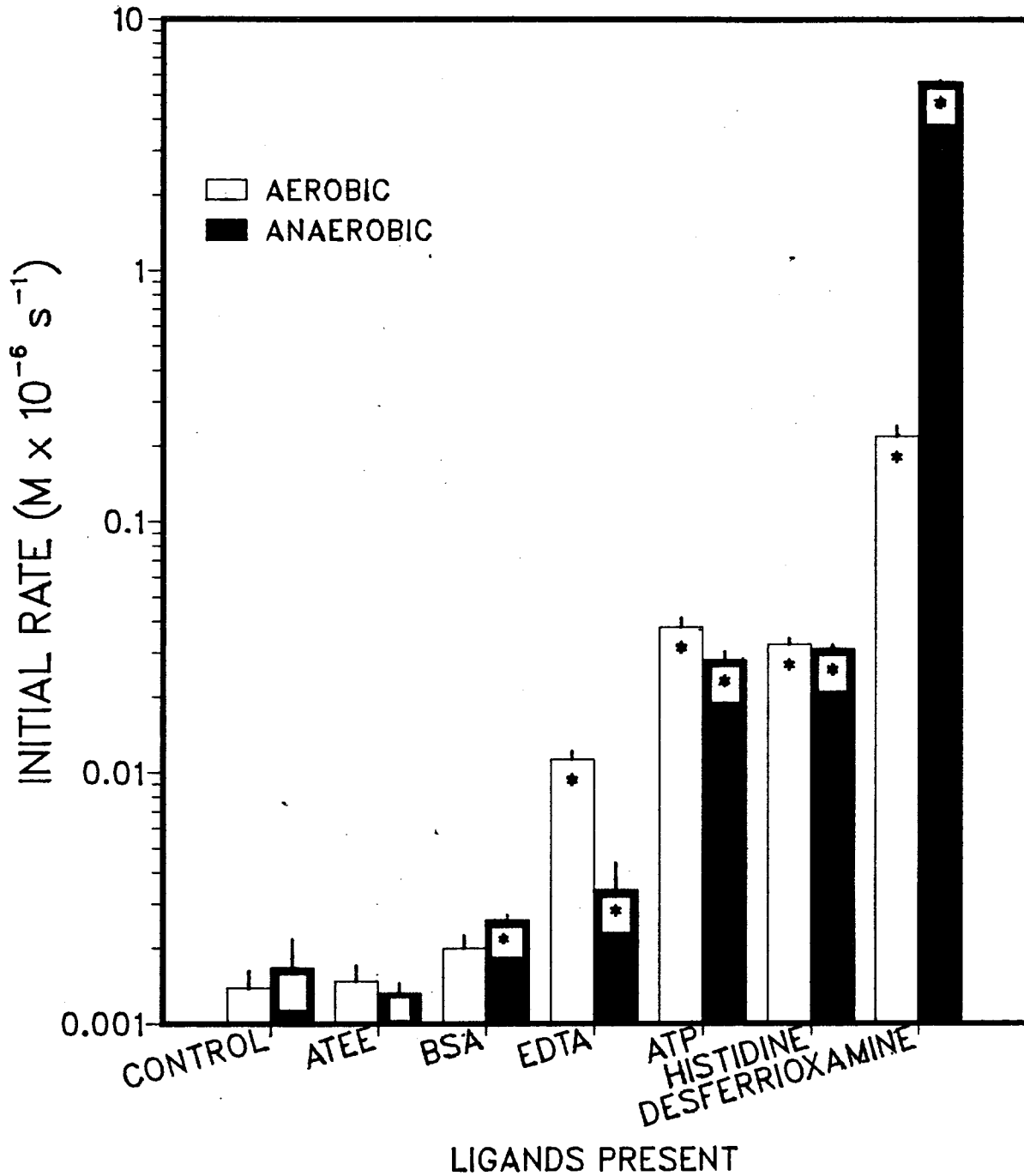
*Comparisons of aerobic and anaerobic conditions*

For most of the vanadate complexes tested (ATP, albumin, and histidine), the rates of oxidation of ferrocytochrome c were not very different anaerobically or aerobically as can be seen in Figure 7. Clearly, except for vanadate/EDTA complex, the presence of molecular oxygen did not facilitate the

**Figure 7. Comparison of the initial rates of oxidation of ferrocyanide c under aerobic and anaerobic conditions**

Reactions were carried out in 0.05M MES air saturated or nitrogen saturated buffer at pH 6.0. The concentrations of ligands and other conditions were as Figure 2. The symbol "\*" indicates a value significantly different from the control at the  $p < 0.05$  level of significance. The abbreviation of "DES" indicates desferrioxamine.

FIGURE 7. COMPARISON OF INITIAL RATE OF OXIDATION OF FERROCYTOCHROME *c* BETWEEN AEROBIC AND ANAEROBIC CONDITIONS



oxidation of ferrocyclochrome  $c$  by either vanadate or vanadate complexes at pH 6.0. The most effective ligand in both aerobic or anaerobic conditions was desferrioxamine. Surprisingly, (at pH 6.0) under aerobic conditions, its effect was less than under anaerobic conditions. Under anaerobic conditions, addition of desferrioxamine increased the initial rate of the oxidation of ferrocyclochrome  $c$  3500-fold, while under aerobic conditions, it only increased the rate 160-fold. Thus, the presence of molecular oxygen diminished the effectiveness of desferrioxamine in accelerating the oxidation of ferrocyclochrome  $c$  by vanadate. Since most other complexes of vanadate were equally effective aerobically and anaerobically, it is likely that the inhibitory effect of oxygen comes from an interaction between desferrioxamine (or desferrioxamine-vanadate complex) with oxygen.

#### *Absorption spectrum of a vanadate desferrioxamine complex*

As can be seen in Figure 8, when vanadate and desferrioxamine were both present in the reaction system, a yellow complex (perhaps a charge transfer complex) was formed, having an absorption maximum at about 332 nm. This absorption peak is located between the ultraviolet and visible spectrum.

#### Discussion

##### *The reactivity of vanadium and the effects of ligands*

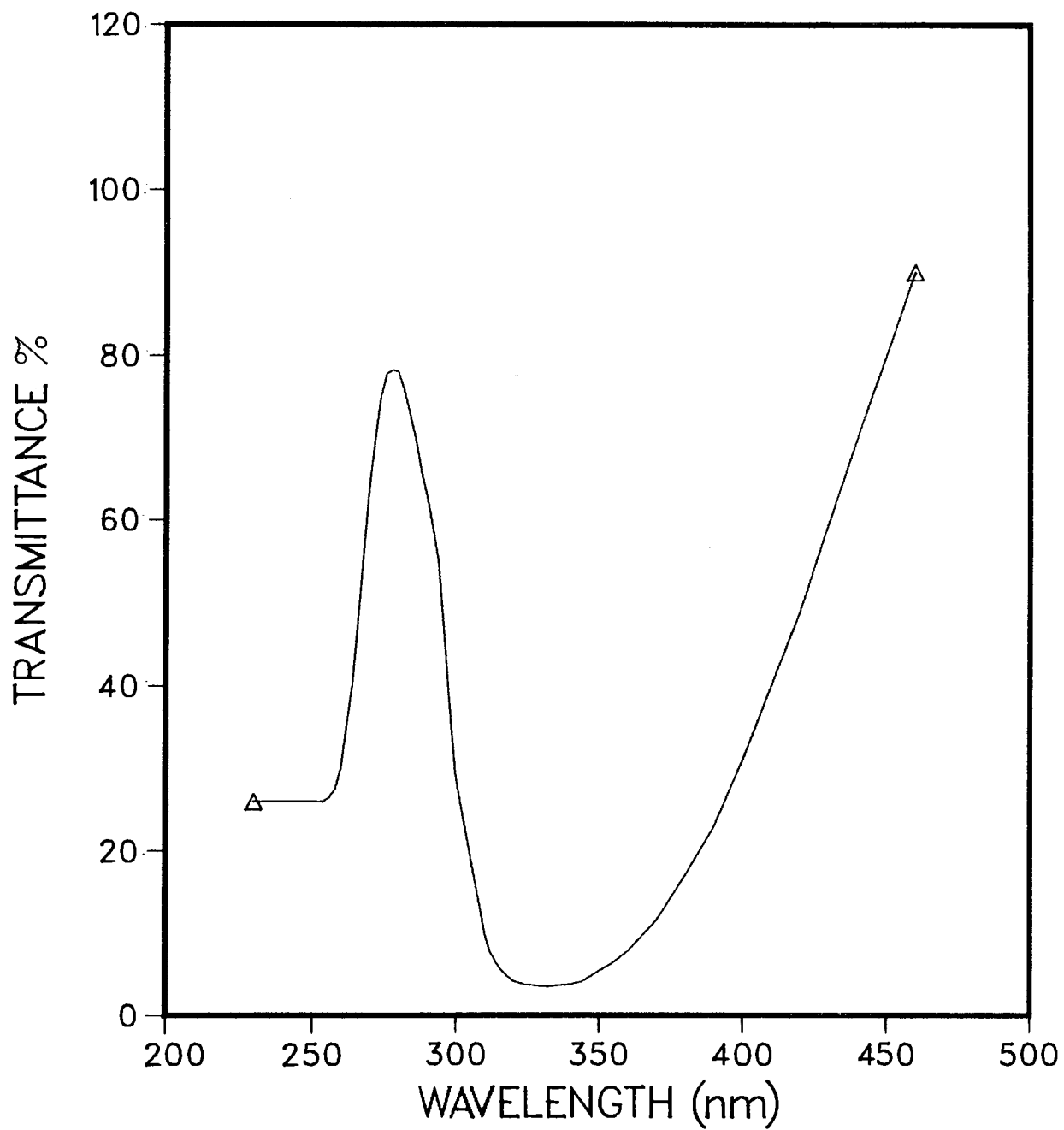
As was seen in Figures 2-7 ligands dramatically modify redox reactions of vanadium (especially vanadium(V) and vanadium(IV)) with cytochrome  $c$ , reflecting changes in the free energies of activation. Since ATP and EDTA increased the rate of the oxidation of cytochrome  $c$  by vanadium(V) and decreased the rate of the reduction by vanadium(IV), the presence of EDTA or



**Figure 8. Absorption spectrum of a vanadate desferrioxamine complex**

The complex was recorded in 0.05M air saturated HEPES buffer at pH 7.0.  
The concentrations of vanadate and desferrioxamine both were 1mM.

FIGURE 8 SPECTRUM OF VANADATE DESFERRIOXAMINE COMPLEX.



ATP presumably increases the reduction potential of vanadium(V). On this basis, changes in the free energy of activation and thus changes in reaction rate, merely follow the changes in free energy level of the reactants.

*The special reactivity of vanadium desferrioxamine complexes*

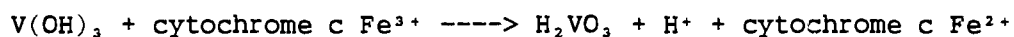
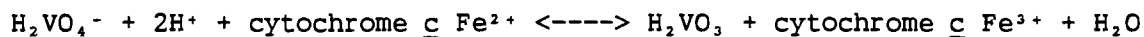
Among the six ligands tested, desferrioxamine was by far the most effective, accelerating some reactions by over 3 orders of magnitude. Effects of desferrioxamine in relation to other metals has involved decreased accessibility of the metal, and also increased reduction potential of the complexed metal consistent with accelerated reduction of electron acceptors, but a concomitant slowing of oxidations. However, in relation to reactions between vanadium(V) and cytochrome c, desferrioxamine accelerates both oxidations and reductions. Evidently the vanadium(V) desferrioxamine complex forms a particularly stable transition state with cytochrome c.

Some of the specific interactions between vanadium(V) and desferrioxamine are reflected in formation of a yellow complex, having an absorption maximum at 332nm. No such colour reaction was seen with any of the other ligands, nor with complexes of desferrioxamine with Fe, Cu, Mn, or Al. The altered optical properties reflect altered electronic distribution arising from conjugation of electron orbitals between vanadium(V) and desferrioxamine, perhaps as a charge transfer complex. On this basis then, the increased facility of both reduction and oxidation of cytochrome c by vanadium(V) resides the ability of newly available  $\pi$ -symmetry orbitals of the desferrioxamine-vanadium(V) complex to overlap with the  $\pi$ -orbitals of the exposed edge of the heme group of cytochrome c [26].

Since desferrioxamine does not significantly alter the rate of autoxidation of cytochrome c either when it is added alone, or in the presence of other transition metals (Cu, Fe, Mn, Al) [27], it follows that neither oxygen nor other transition metal ion and oxygen form complexes with desferrioxamine capable of to facilitating redox reactions with cytochrome c.

#### *The effect of pH*

The most obvious influence of pH is the participation of H<sup>+</sup> as a reactant in the reduction or oxidation of cytochrome c by different forms of vanadium.



That both of these reactions involve H<sup>+</sup> explains why decreased pH facilitates reduction of vanadium(V) by ferrocytochrome c.

Another aspect of the influence of pH on the redox reactivities of vanadium complexes involves alteration of the stability of vanadium complexes by protonation. In transition metal complexes, all ligands act as Lewis bases (i.e. electron pair donors), and the metal ions can act as Lewis acids. Protonation of a ligand decreases its basicity thus decreasing the affinity between metal and ligands [17]. In some cases, different metal-ligand complexes are formed at different values of pH. At pH 6.0, vanadium(V) desferrioxamine rapidly oxidizes ferrocytochrome c, but at pH 7.4, it is a particularly effective reducing agent, capable of fully reducing ferricytochrome c. The optimum molar ratio for this reducing action is close to vanadate:desferrioxamine = 1:5. At this ratio, the above-mentioned obvious

visible absorption spectrum (332nm) was markedly decreased.

The same 1:5 molar ratio of vanadium(V):desferrioxamine, was optimum for reduction of ferricytochrome c, while a 1:1 ratio of vanadium(V):desferrioxamine failed to support *any reduction* of ferricytochrome c. These results are consistent with formation of different vanadium(V) desferrioxamine complexes at different values of pH and at different relative proportions of vanadium(V) and desferrioxamine. Specifically, one of the vanadium(V)/desferrioxamine complexes has an absorption peak at 332nm, and can be formed at all three values of pH tested. Its molar absorptivity increases with the concentration of vanadium(V). It readily oxidises ferrocyanide c at pH 6.0 and 7.0, but cannot oxidise ferrocyanide at pH 7.4. Another complex can be formed at pH not lower than 7.4 at a molar ratio of 1:5) and it is a strong reducing agent at pH 7.4.

The reduction of ferricytochrome c by the vanadate desferrioxamine complex is paradoxical, since redox states of vanadium higher than vanadium(V) in aqueous media are implausible. Desferrioxamine itself provides a more plausible alternative source of the reducing power, and this view is supported by the observation that desferrioxamine alone can induce reduction of ferricytochrome c (albeit at a much lower rate). It follows that vanadium(V) acts catalytically, accelerating reduction of ferricytochrome c by desferrioxamine.

Desferrioxamine is routinely used in the study of redox reactions, often on the assumption that it will pre-empt participation of metals, iron in particular. It therefore seems important to ask to what extent desferrioxamine participates directly in other redox reactions, whether binding to any other metals releases its latent redox reactivity, and whether

there are any biological ligands which interact with vanadium(V) in a way which induces a similar dramatic increase in its reducing power, and whether such interactions play any part in its biological actions or its toxicity. These issues, then, deserve further study.

In conclusion then, vanadium(V) is an effective oxidizing reagent, at pH 6.0, and also at neutral pH when complexed with some ligands. Since the kidney is a relatively acidic site, this pH dependence may explain the peroxidative damage observed in the kidney after administration of vanadium(V) [9]. Moreover, since vanadium can be reduced by cellular reductants and can also be reoxidized by oxygen or cytochrome c it has the capacity to undergo redox cycling intracellularly. The intracellular ratio of vanadium(IV) to vanadium(V) thus represents a dynamic steady state, rather than an equilibrium. The intracellular ratio of vanadium(IV) to vanadium(V) is thus capable of rapid and dramatic changes as a result of changes in the availability of ligands, concentrations of reducing or oxidising agents (including oxygen itself), or intracellular pH. Among the factors accounting for the intracellular occurrence of vanadium largely in the reduced form (vanadium(IV)) [6], we must include 1) the intracellular availability of ATP and related ligands which accelerate its reduction, 2) the diminished concentration of oxygen intracellularly, 3) the relatively low intracellular pH.

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**The reduction of oxygen by vanadyl and vanadium(III) in the presence of  
different ligands <sup>1</sup>**

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## CHAPTER IV

### THE REDUCTION OF OXYGEN BY VANADYL AND VANADIUM(III) IN THE PRESENCE OF DIFFERENT LIGANDS

#### Abstract

Vanadium(IV) [V(IV)] reduced oxygen 1.6 to 4 times as rapidly as did vanadium(III) [V(III)] over the pH range 6.0, 7.0, and 7.4. The highest rates (but the smallest differences between V(III) and V(IV)) occurred at pH 7.4 where the respective initial rates of reduction of oxygen were 0.10 and 0.16  $\mu\text{M s}^{-1}$ . Although N-acetyl-L-tyrosine ethyl ester (ATEE) had no measurable effects on the reaction rate at any pH, the rates of reduction of oxygen were significantly affected by the addition of other ligands, specifically desferrioxamine, EDTA, ATP, histidine, or albumin. Moreover, the effects of these ligands were altered by changes in pH. For example, desferrioxamine accelerated the reduction of oxygen by V(III) at pH 6.0 (by 2.5-fold). However, this stimulatory action disappeared at pH 7.0 (no significant effect), and changed to an inhibitory action (21% inhibition) at pH 7.4. Desferrioxamine accelerated the reduction of oxygen by V(IV) even more dramatically. Thus at pH 6.0 the rate of reduction of oxygen was increased 6.7-fold, while at pH 7 and 7.4, it did not increase the initial rate significantly. Histidine also accelerated reduction of oxygen by V(IV) at pH 7.0 and 7.4 (by 130% and 50% respectively). The other ligands, if they had any effect, were inhibitory. Specifically: ATP inhibited reduction of oxygen by both V(III) and V(IV) by 50-60% at pH 6.0, and by 70-80% at pH 7.0, and 7.4; albumin inhibited reduction by V(III) at pH 6.0 by 55%; while EDTA over the whole pH range, inhibited reductions of oxygen by V(III) by 15-30%, and

by V(IV) by 80-90%. Other combinations of ligand and pH had no statistically significant effects ( $p < 0.05$ ). Catalase decreased the initial rate of the reduction of oxygen by vanadyl by 77% at pH 7.4. Superoxide dismutase had no significant effect on this reaction.

Clearly both V(III) and V(IV) are readily oxidized by oxygen at physiological pH, thus accounting for the preponderance of the oxidised V(V) state in plasma. At lower pH, or in the presence of ATP, these autoxidations are slower, thus accounting for the preponderance of reduced forms intracellularly. Since V(III) reacts substantially more slowly than V(IV) with molecular oxygen, bioaccumulation of V(III) may be favoured kinetically under conditions where substantial reduction and oxidation of vanadium are occurring simultaneously (thermodynamic considerations notwithstanding).

### Introduction

Among biochemical investigations of the transition metal ions, vanadate is of particular current research interest (1). In large measure this is because there are now several lines of evidence which suggest that vanadium at very low concentrations imitates the actions of several cellular growth factors (2). For example, vanadium is known to: (i) accelerate the growth of laboratory animals (3), (ii) to decrease the insulin requirements of diabetic rats (4), and (iii) to stimulate proliferation of cultured cells (5).

A common explanation for these growth promoting actions emerges from the observation that vanadate can spontaneously esterify phenolic compounds, presumably including exposed tyrosine residues on proteins (6). These esters can promote growth factor like effects, since many cellular growth factors

(the "insulin-like growth factors" and insulin itself) produce their biological actions by stimulating esterification of tyrosine residues. They do so by activating tyrosine specific protein kinases on the inner surfaces of cell membranes (7).

Due to thermodynamic constraints, the amounts of tyrosine ester formed by vanadate would be insignificant, were it not that the protein kinase system involves amplification through autophosphorylation of the target proteins after their initial esterification (2). Thus formation of even a small amount of esterified protein triggers an autocatalytic conversion of most of the remainder. These events are central to the mechanisms of carcinogenesis, since tyrosine kinase activities are inherent in several of the products and mechanisms of the major oncogenes.

Reportedly the biologically accessible redox states of vanadium are small amounts of V(III), with the major part being metavanadate extracellularly, and vanadyl ions intracellularly (8). The oxidation state of the vanadium is crucial to its growth promoting activities, since the only known effective form of vanadium for tyrosine esterification is vanadate (the pentavalent form). However, the form which predominates intracellularly is the quadrivalent form (V(IV) or vanadyl). This is because binding of vanadium by the main cellular ligands (protein and phosphate) results in an equilibrium which overwhelmingly favours the V(IV) state (7), and also because of the lower intracellular oxygen concentrations (9). The growth factor-like actions of vanadium thus result from the small amounts of V(V) arising intracellularly. On this basis then, attainment of an effective concentration of vanadate for esterification of tyrosine depends on the operation of factors which displace the redox steady-state toward the

pentavalent state. Factors influencing the intracellular concentration of V(V) include the presence of ligands, oxidants and reductants, specifically on a preponderance of species (including oxygen and its active metabolites) which favour oxidation, over species which favour reduction.

With proposals that vanadium administration might be beneficial to patients with diabetes (4) or sickle cell anaemia (10), a systematic and detailed knowledge of the effect of complex formation on its redox reactivity and biological actions becomes increasingly important (7). Vanadate or vanadyl form complexes with a variety of biological ligands (proteins, adenine nucleotides, apotransferrin) (9), and also with itself e.g.  $HV_{10}O_{28}^{5-}$  and  $H_2V_{10}O_{28}^{4-}$  (7). Formation of such complexes may alter the redox state from a preponderance of V(IV) to a preponderance of V(V). It is therefore surprising that while the redox chemistry of vanadium has been extensively studied, the influence of ligands (especially biologically relevant ligands) on the redox reactivity of vanadium toward oxygen is virtually unknown, although a start has very recently been made (9).

Our interest in the reactivity of vanadium with oxygen and with biological reductants arises from findings that among a range of transition metal complexes which accelerate the aerobic oxidation of cytochrome c, vanadate is the most effective (11). We have therefore undertaken a series of studies of the effects of ligands on the redox properties and biological reactivity of vanadate, vanadyl and vanadium(III). Among the major findings are: (1) that vanadate and its complexes with desferrioxamine, ATP, EDTA or histidine dramatically accelerate the oxidation of ferrocytochrome c both aerobically and anaerobically, and (2) that vanadyl and its complexes, with desferrioxamine, ATP, or EDTA, efficiently mediate the aerobic, but not

anaerobic oxidation of ferrocytochrome c. In order to study the role of oxygen in determining the biological state of complexes of vanadium, we have undertaken in the current study, to investigate more systematically the reactions of vanadyl (or vanadium(III)) with oxygen, and the effects of ligands and pH on the rates of reduction of oxygen.

## Materials and Methods

### *Reagents*

Adenosine 5'-triphosphate (ATP), N-acetyl-L-tyrosine ethyl ester (ATEE), bovine serum albumin, N-2-hydroxyethylpiperazine -N'-2-ethanesulfonic acid (HEPES), 2(N-morpholino)ethanesulfonic acid (MES) and superoxide dismutase were purchased from Sigma Chemical Company (St. Louis., Mo., U.S.A.). Catalase was purchased from Boehringer Mannheim (West Germany). L(+) histidine was purchased from Matheson, Coleman and Bell (Norwood, Ohio). Disodium ethylenediaminetetraacetate (EDTA) was purchased from Fischer Scientific (Fair Lawn, New Jersey). Vanadyl sulfate (trihydrate) and vanadium(III) chloride were purchased from Aldrich Chemical Company (Milwaukee, Wis., USA). Hydrochloric acid, was purchased from Amachem (Portland, Oregon). Desferrioxamine (desferal mesylate) was a gift from CIBA Pharmaceuticals (Summit, New Jersey). Water was deionized and distilled.

### *Assay Procedure and Calculations.*

Oxygen consumption was followed by YSI Model 53 Biological Oxygen Monitor which was calibrated daily and the oxygen probe membrane was replaced as necessary. Air-saturated buffer (0.05M HEPES or MES) which was prepared by bubbling with air for 30 minutes at 25° C was used in these experiments as

well as to test the oxygen probe (YSI 5331). Three ml of air-saturated buffer together with other reagent (ligands) and a magnetic stirring bar were placed into the sample tube of the monitor. Six minutes were allowed for the stabilization of recording trace and temperature equilibrium. Vanadium (III or IV) was added to the sample tube to initiate the reaction and oxygen consumption. The reaction was monitored on a Beckman 10" strip chart recorder, with a data acquisition system connected to the monitor. The pH was measured before and after the recording of the reaction to check for any change of pH during the reaction. The ionic strength of the buffer was not significantly altered by addition of the metal since the amount of metal is much less than the buffer strength. The initial rates of the reaction were obtained by calculating the slopes of the initial part of each reaction. In the statistical comparisons of means, we used one way analysis of variance and multi-comparison (12).

## Results

### *Reaction profiles for selected reactions*

Progress of typical reductions of oxygen by vanadyl complexes at pH 7.4 are shown in Figure 1, while a comparison of the rates of the reduction of oxygen by vanadium(III) with that by vanadyl is given in Figures 2 and 3. Clearly the reduction of oxygen by V(IV) is markedly faster than reduction by V(III), the respective rates being 0.16 and 0.10  $\mu\text{M s}^{-1}$ . In each case the reaction was profoundly accelerated by the addition of desferrioxamine. The differences between the reducing effectiveness of V(III) and V(IV) diminished with increasing pH. Thus whereas the reduction of oxygen by V(IV) was 400% faster at pH 6.0, the rates were only 200% and 160% greater at pH 7.0 and 7.4



respectively.

#### *Reduction of oxygen by vanadium(III) and its complexes*

As shown in Figure 2, Vanadium(III) reduced molecular oxygen more rapidly as pH increased. It was  $2.0 \times 10^{-2} \mu\text{M s}^{-1}$ ; at pH 6.0,  $6.0 \times 10^{-2} \mu\text{M s}^{-1}$  at pH 7.0, and  $1.0 \times 10^{-1} \mu\text{M s}^{-1}$  at pH 7.4.

Although N-acetyl-L-tyrosine ethyl ester and histidine had no measurable effects at any pH, the rates of reduction were clearly affected by the addition of the other ligands: desferrioxamine, EDTA, ATP, or albumin. Moreover, the effects of these ligands were altered by changes in pH. For example, desferrioxamine dramatically accelerated the reduction of oxygen by V(III) at pH 6.0 by 2.5-fold. However, this stimulatory action disappeared at pH 7.0 (no significant effect), and changed to an inhibitory action (21% inhibition) at pH 7.4. The other ligands, if they had any effect, were inhibitory. Specifically, ATP inhibited reduction of oxygen by 52%, 71% and 81% at pH 6.0, 7.0 and 7.4. EDTA over the same pH range, inhibited reduction of oxygen by 31%, 26%, and 15%. Other combinations of ligand and pH had no effects which reached statistical significance (at  $p < 0.05$ ).

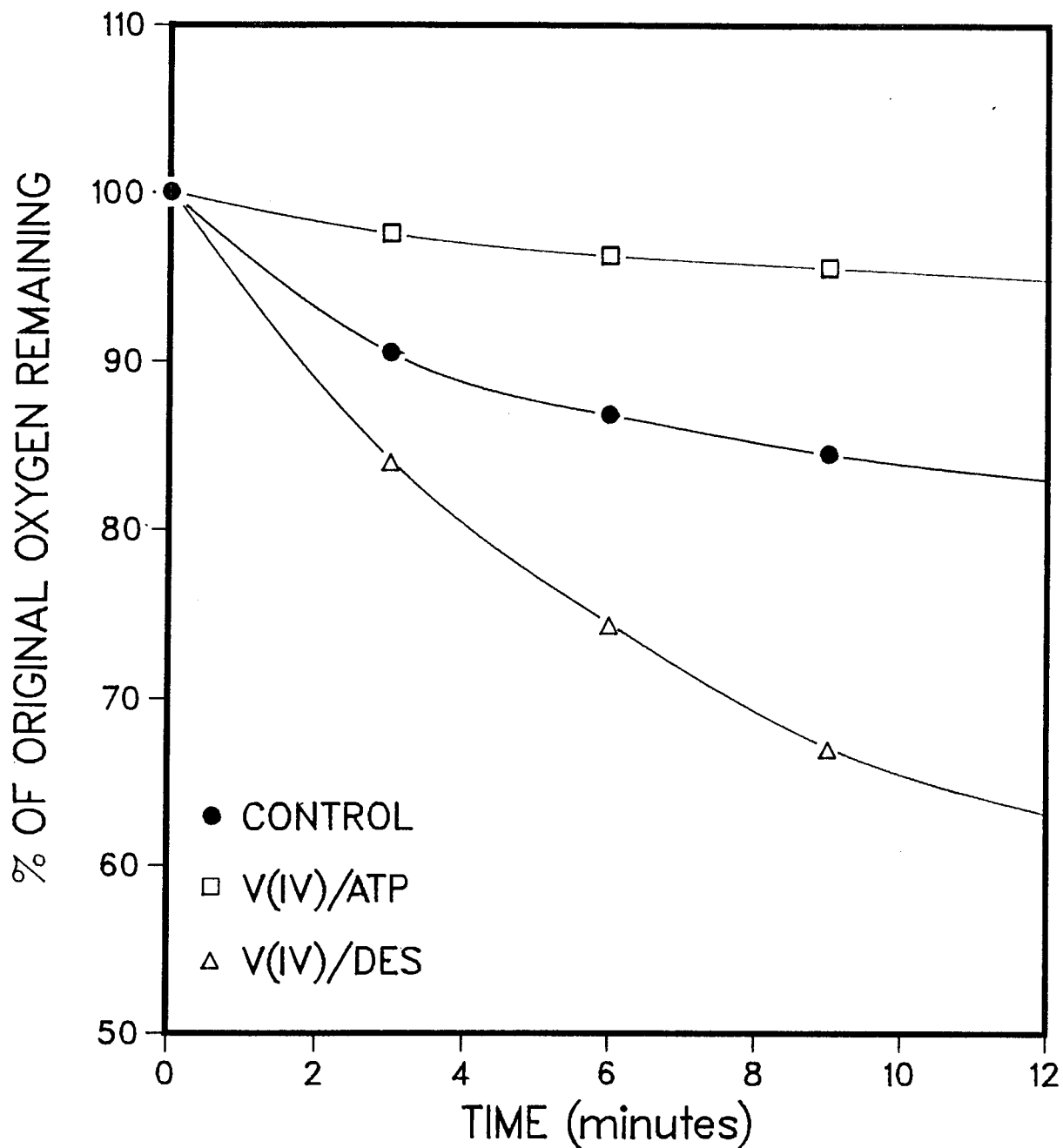
#### *Reduction of oxygen by vanadium(IV) and its complexes*

Rates of reduction of oxygen by vanadium(IV) are summarised in Figure 3 from which it can be seen that vanadyl(IV) reduced oxygen even more rapidly than did V(III), at all three values of pH. The initial rates of the reduction of oxygen again increased with increasing pH, so that at pH 6.0 the initial rate was  $7.9 \times 10^{-2} \mu\text{M s}^{-1}$ , at pH 7.0 it was  $1.3 \times 10^{-1} \mu\text{M s}^{-1}$ ; and at pH 7.4, it was  $1.6 \times 10^{-1} \mu\text{M s}^{-1}$ .

**Figure 1. Reduction of oxygen by vanadyl complexes at pH 7.4.**

Reactions were carried out at 25°C, pH 7.4 in an air-saturated HEPES buffer. The final concentrations of ligands used were 1 mM for ATP and desferrioxamine. The total volume was 3 ml with a final concentration 1mM of vanadyl. The abbreviation of "DES" indicates desferrioxamine.

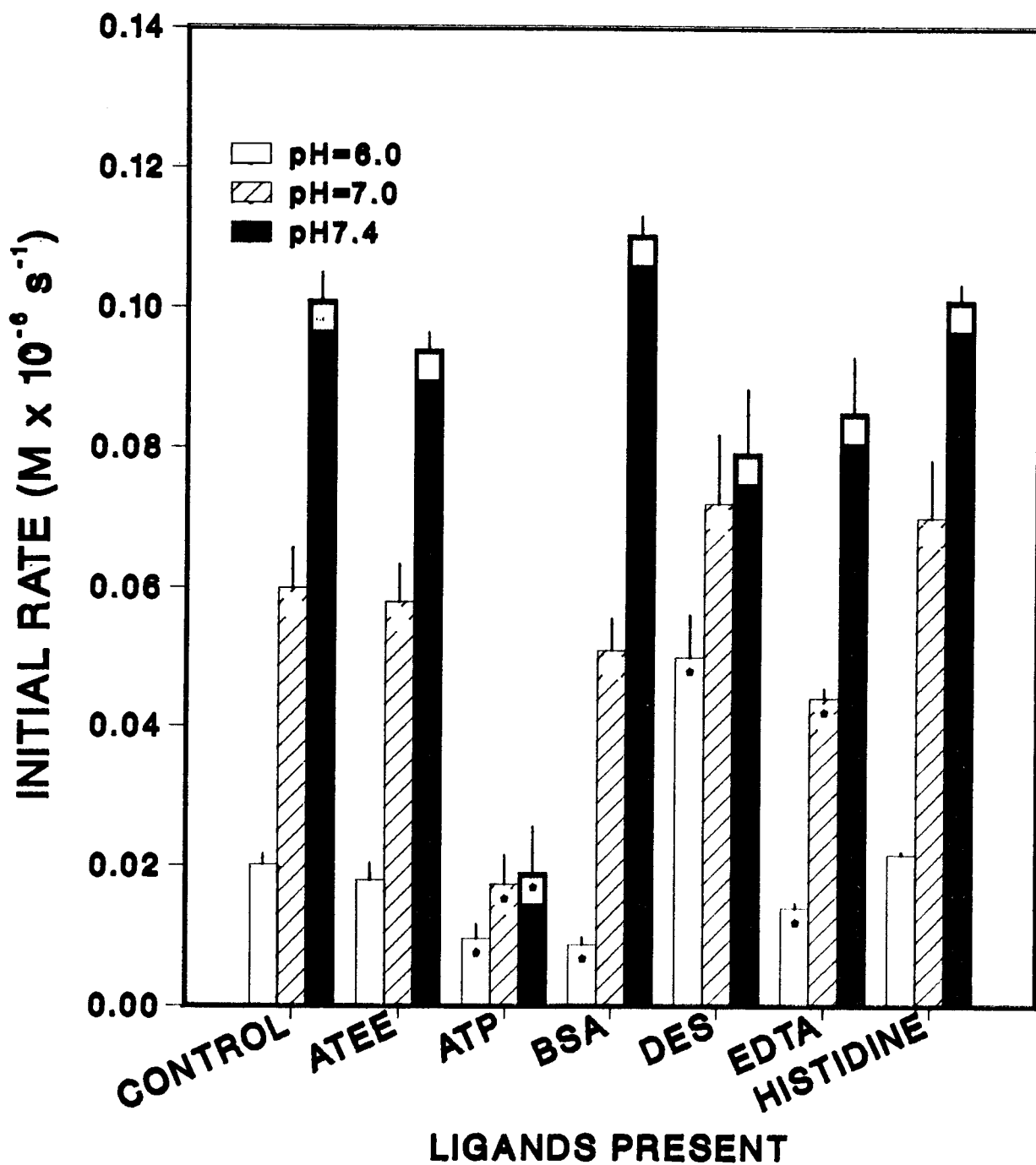
FIGURE 1. REDUCTION OF OXYGEN BY VANADYL AND VANADYL COMPLEXES AT pH 7.4



**Figure 2. Reduction of oxygen by vanadium(III) complexes: Effects of ligands.**

Reactions were carried out at 25°C, in a 0.05M MES buffer at pH 6.0 or a 0.05 HEPES buffer at pH 7.0 and 7.4. All the buffers were air-saturated. The final concentration of ligands used were ATEE, 200µM; ATP, 1mM; BSA, 600µg/ml; desferrioxamine, 1mM; EDTA, 1mM ; histidine, 1mM. The total volume was 3 ml with a final concentration of 1mM vanadium(III). The symbol "\*" indicates a value significantly different from control at the  $p < 0.05$  level of significance. The abbreviation of "DES" indicates desferrioxamine.

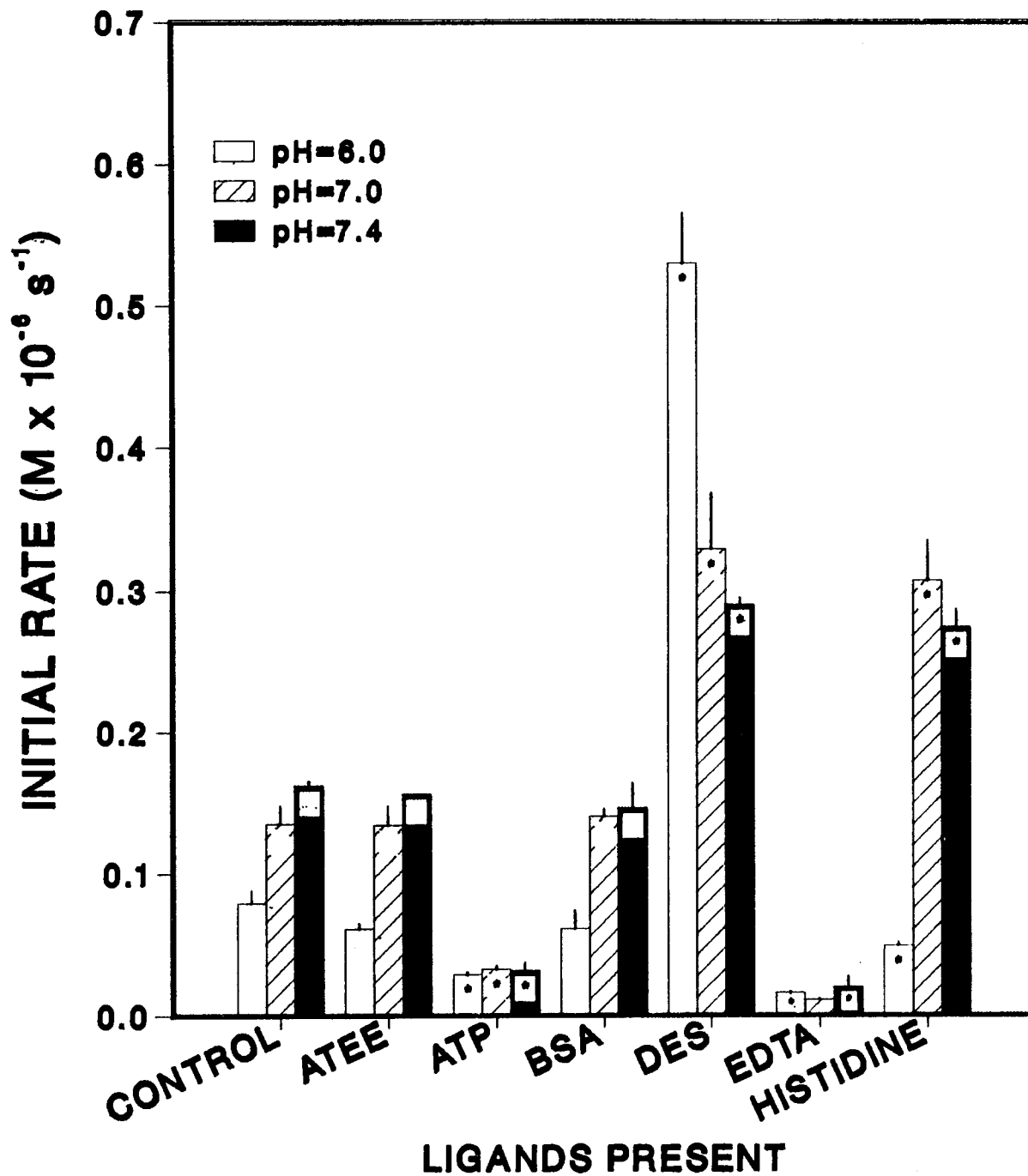
FIGURE 2. REDUCTION OF OXYGEN BY VANADIUM(III) COMPLEXES



**Figure 3. Reduction of oxygen by vanadyl complexes: Effects of ligands.**

The conditions were as Figure 2. The final concentration of vanadyl was 1mM. The symbol "\*" indicates a value significantly different from control at the  $p < 0.05$  level of significance. The abbreviation of "DES" indicates desferrioxamine.

FIGURE 3. REDUCTION OF OXYGEN BY VANADYL COMPLEXES



Although N-acetyl-L-tyrosine ethyl ester and albumin had no measurable effects at any pH, the rates of reduction were affected by the addition of the other ligands: desferrioxamine, EDTA, ATP, or histidine. Once again, the magnitude of the effects of these ligands were altered by changes in pH. Desferrioxamine accelerated the reduction of oxygen by V(IV) even more powerfully than in the case of V(III). Thus at pH 6.0 the rate of reduction of oxygen was increased 6.7 fold. At pH 7 and 7.4 stimulatory actions of desferrioxamine on reduction of oxygen by V(IV) were less marked, diminishing progressively to 120% and 80% respectively. Histidine also accelerated reduction of oxygen by V(IV) at pH 7.0 and 7.4 (by 130% and 50% respectively). The other metal complexes, if they had any effect, were (as in the case of V(III)) inhibitory. Specifically, ATP inhibited reduction of oxygen by V(IV) by 63%, 76%, and 80% at pH 6.0, 7.0, and 7.4. EDTA over the whole pH range, uniformly inhibited reductions of oxygen by 80-90%. Other combinations of ligand and pH had no effects which reached statistical significance (at  $p < 0.05$ ).

*Effects of catalase on the oxidation of vanadyl by molecular oxygen*

Table 1 shows the effects of a range of concentrations of catalase (33 to 132 units/ml) or superoxide dismutase on the rates of reduction of oxygen by vanadium(IV) at pH 7.4.



Table 1. The effect of catalase and superoxide dismutase on the oxidation of vanadyl by oxygen \*

units of scavenger	initial rate (R) $\mu\text{M s}^{-1}$	S.D.	p
control	0.22	0.01	
catalase			
32 U/ml	0.17	0.01	<0.05
64 U/ml	0.18	0.01	<0.05
132 U/ml	0.17	0.01	<0.05
superoxide dismutase			
32 U/ml	0.22	0.02	
64 U/ml	0.21	0.02	
132 U/ml	0.23	0.02	

\* Experiments were conducted in a 0.05M HEPES buffer at 25° C, pH 7.4. The final concentration of vanadyl was 1mM. Albumin (final concentration 1.2mg/ml) or boiled superoxide dismutase (originally 66 units/ml) were used as controls.

All concentrations of catalase decreased the initial rate of oxidation of vanadyl by molecular oxygen by 18 to 23% of the control value ( $p < 0.05$ ). Addition of superoxide dismutase had no significant effect on the reduction of oxygen by vanadyl, over the concentration range.

## Discussion

The current data support earlier reports (1, 9) that both vanadium(IV) and vanadium(III) are readily oxidized under aerobic conditions. The observed rates emphasize that the intracellular distribution of vanadium among its redox states reflects not an equilibrium but a steady state.

Ligands and pH significantly influence the position of the steady state by modifying the redox reactivity of both species. Most ligands inhibited reduction of oxygen by V(III) and V(IV), confirming a report that apoferritin inhibited autoxidation of V(IV), and consistent with their conclusion that oxidation of V(IV) probably occurs via the "free metal ion" (1). In sharp contrast however is the current observation that desferrioxamine accelerated the reduction of oxygen by V(III) at pH 6.0 and by V(IV) at pH 6.0, 7.0 and 7.4.

Lower values of pH such as those occurring intracellularly significantly slow the oxidation of V(III) and V(IV) by oxygen, while values close to that of plasma facilitate oxidation (Figures 2 and 3). In contrast to the other ligands, the presence of desferrioxamine caused an inverted pH dependence in the case of V(IV), reflected in a 40% *decrease* in reaction rate as pH changed from 6.0 to 7.4. The differences in the effects of pH presumably arise in part from the different stabilities and states of hydration of the respective complexes which the ligand forms with oxidized and reduced forms of vanadium.

The reason for the anomalous effects of desferrioxamine are not yet clear. However we have obtained evidence in other studies (submitted to BBA) that vanadate forms a complex with desferrioxamine, having a broad peak at 332nm. Formation of such a complex in the autoxidation of vanadium(IV) in the

current studies was confirmed by development of an absorption peak at 332nm which increased as oxygen was consumed. Formation of a stable vanadate/desferrioxamine complex may explain, at least in part, the stimulation by desferrioxamine of the reduction of oxygen by vanadyl, since formation of this complex can shift the equilibrium of the reaction to the right. The nature of this complex is not yet known, but one or more of the three hydroxamate residues in desferrioxamine are candidates for esterification by vanadate (6). To the extent that vanadyl/desferrioxamine complexes form, they may also be involved in the observed effects on redox reactivity. Certainly formation of vanadyl/ATP or vanadyl/EDTA complexes which are relatively inert to molecular oxygen have been reported (7), thus explaining the observation that ATP and EDTA inhibited the oxidation of vanadyl by oxygen.

The inhibition of the autoxidation of vanadyl by catalase implicates hydrogen peroxide in the reaction mechanism. Probably the inhibitory effect of catalase is attributable in part to inhibition of the oxidation of vanadyl by hydrogen peroxide, and in part to release of oxygen by the action of catalase on the  $H_2O_2$ , thus mitigating oxygen consumption (13, 14). Such activation of oxygen to peroxide and subsequent Fenton type reactions may contribute to the renal lipid peroxidation observed in rats treated with vanadate (15).

The preponderance of V(IV) intracellularly, in contrast to the preponderance of V(V) in the plasma has hitherto been explained primarily on the basis of thermodynamic considerations (7). 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 recent results of Chasteen, et. al., (1) require that kinetic considerations be given at least equal weight. The current data emphasize that the intracellular levels of V(IV) reflect not an equilibrium but a steady state. Intracellular reducing agents contribute to the reduction of vanadium to both V(IV) and perhaps V(III) (1, 9), while oxygen and perhaps hydrogen peroxide contribute to the reoxidation.

The resultant V(III) and V(IV) are more readily oxidized by oxygen at the plasma pH of 7.4 than at intracellular pH, thus contributing to the preponderance of the oxidized V(V) state in plasma. In addition, the lower pH and the presence of ATP, slow these autoxidations intracellularly, favouring the intracellular preponderance of reduced forms. Biological concentrations of V(III) are not easily determined, since V(III) is silent to EPR measurements (1). However, although it might be argued on thermodynamic grounds that V(III) has too low an oxidation potential to accumulate biologically, this view requires reexamination in the light of the current results. Since V(III) reacts so much more slowly with molecular oxygen than does V(IV), bioaccumulation of V(III) may be favoured kinetically under conditions where substantial reduction and aerobic oxidation of vanadium are occurring simultaneously (thermodynamic considerations notwithstanding).

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**Redox reactions of vanadium(IV) and vanadium(V) with cytochrome c in the presence of oxygen<sup>1</sup>**

by

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## CHAPTER V

### REDOX REACTIONS OF VANADIUM(IV) AND VANADIUM(V) WITH CYTOCHROME c IN THE PRESENCE OF OXYGEN

#### Abstract

Addition of vanadium(V) (vanadate) (1mM) at pH 6.0, or vanadium(IV) (vanadyl) (200 $\mu$ M, at pH 6.0, 7.0, or 7.4) significantly accelerated the aerobic oxidation of ferrocytochrome c (15 $\mu$ M). The initial rate of the oxidation of ferrocytochrome c in the presence of V(IV)/desferrioxamine at pH 6.0 was  $1.1 \times 10^{-3} \mu\text{M s}^{-1}$ , in comparison with a negligibly small control rate. At pH 6.0 in the presence of V(V), desferrioxamine, histidine, ATP, EDTA and albumin increased the initial rate by 160-fold, 23-fold, 27-fold, 8-fold, and 1.4-fold respectively. Moreover, although no acceleration of the oxidation of ferrocytochrome c was induced by V(V) alone at pH 7.0, the presence of desferrioxamine, EDTA or ATP, allowed V(V) to oxidize ferrocytochrome c at this pH at initial rates of 73, 5.5, or  $7.1 \times 10^{-4} \mu\text{M s}^{-1}$ , respectively. At pH 7.4, however, no oxidation of ferrocytochrome c was induced by V(V) or any of its complexes. Instead, as discussed hereunder, V(V)-desferrioxamine becomes a *reductant* of cytochrome c at this pH. Surprisingly, V(IV), the more reduced species, was a more effective stimulant of the oxidation of ferrocytochrome c than V(V). Addition of V(IV) to ferricytochrome c at pH 6.0, 7.0, or 7.4 caused almost complete reduction followed by complete reoxidation. Paradoxically, in the presence of desferrioxamine at pH 7.4, the oxidized species, V(V) caused *reduction* of ferricytochrome c (15 $\mu$ M) by desferrioxamine, at an initial rate of  $7.9 \times 10^{-3} \mu\text{M s}^{-1}$ . An explanation for this may reside in the observation that the V(IV)



stimulated oxidations were prevented if the oxygen in the system was replaced by nitrogen. However oxygen was not required for the V(V) stimulated oxidations. Since desferrioxamine alone caused a much slower reduction of ferricytochrome  $c$ , it is presumably the desferrioxamine itself which is the reductant, and V(V) is a catalyst.

## Introduction

Effects of vanadium on the oxidation of biochemical substrates have become apparent only recently, with reports by Liochev and Fridovich [1] of enhancement of NADH oxidation by vanadate. Concomitantly, Chasteen et al. [2] have reported the oxidation of vanadyl by molecular oxygen. Since vanadium(IV) (vanadyl) or vanadium(III) can directly reduce oxygen, it is of interest to ask whether vanadium resembles the many transition metal ions which have pro-oxidant actions [3, 4], in that they mediate the oxidation of biological substrates by molecular oxygen. If it does so, it can be further asked whether such effects involve redox cycling of the metal. Moreover, since the biological actions of vanadium (particularly its ability to esterify tyrosine [5]) are dependent on its redox state [6-10], it has become important to explore the effects of oxygen, ligands and cellular reductants on the redox state and redox reactions of vanadium. The toxicity and biochemical side effects of vanadium have assumed greater importance as a result of the finding that vanadium has potential therapeutic applications in diabetes mellitus [11] and sickle cell anaemia [12].

There is however no clear evidence as to whether intracellular vanadium is fixed in the vanadium(IV) redox state, as suggested by Nechay [13], or whether it is undergoes redox cycling with the preponderance in the reduced

state reflecting a net excess of reduction over reoxidation.

In a study of the effectiveness of various metal complexes in accelerating the aerobic oxidation of ferricytochrome c (submitted for publication) vanadate was (at higher values of pH) by far the most effective of the metals tested [14]. Consequently, we sought to investigate the aerobic redox reactivities of vanadyl and vanadate toward molecular oxygen and cytochrome c in greater detail, playing particular attention to the effects of pH and selected ligands. The current account of vanadium-mediated reduction or oxidation of cytochrome c forms part of the series of studies of the redox behaviour of vanadate. These studies include investigations of the reduction of oxygen by vanadium(IV) and (III), and the anaerobic reduction and oxidation of cytochrome c by vanadium complexes.

In contrast to the paucity of studies on the redox reactivity of vanadium complexes, the redox reactions of ferrocycytochrome c have been extensively studied [15]. Most studies of transition metal ion catalysis of the autoxidation of ferrocycytochrome c have primarily emphasized copper catalysis [15-18]. The rate determining step is the one electron oxidation of ferrocycytochrome c by  $\text{Cu}^{2+}$ . The resulting  $\text{Cu}^{1+}$  is then reoxidized by molecular oxygen [16]. Kinetic evidence suggests that these steps take place within a ternary cytochrome c metal oxygen complex [17]. Occasionally, reduction of ferricytochrome c by transition metals or transition metal complexes has been reported, e.g. the reduction of ferricytochrome c by cysteine/molybdate complexes [19] or by chromium(II) [20, 21].

## Materials and Methods

### *Reagents*

Cytochrome c (horse heart, type VI), adenosine 5'-triphosphate (ATP), N-acetyl-L-tyrosine ethyl ester (ATEE), bovine serum albumin (BSA), sodium orthovanadate, N-2-hydroxyethylpiperazine -N'-2-ethanesulfonic acid (HEPES) and 2(N-morpholino)ethanesulfonic acid (MES) were purchased from Sigma Chemical Company (St. Louis, Mo., U.S.A.). L(+) histidine was purchased from Matheson, Coleman and Bell (Norwood, Ohio). Disodium ethylenediaminetetraacetate (EDTA) was purchased from Fischer Scientific (Fair Lawn, New Jersey). Vanadyl sulfate (trihydrate) and vanadium(III) chloride were purchased from Aldrich Chemical Company (Milwaukee, Wis., U.S.A.). Hydrochloric acid was purchased from Amachem (Portland, Oregon). Desferrioxamine (desferal mesylate) was a gift from CIBA Pharmaceuticals (Summit, New Jersey). Water was deionized and distilled.

### *Preparation of Reduced Cytochrome c*

Reduced cytochrome c was prepared by passing a solution containing 100mg cytochrome c and 5mg sodium dithionite in 1.5ml nitrogen-saturated water through a column (d=2.5cm; l=40cm) filled with Sephadex G-15 that had been soaked in water for 6 hours. Nitrogen-saturated water was used to elute the reduced ferrocytochrome c at a rate of 1 - 1.2 ml/min and the eluate was collected under a nitrogen atmosphere. The concentration of ferrocytochrome c was calculated from the difference in absorbance between the reduced and oxidized forms of cytochrome c at 550 nm, using a molar extinction coefficient of 0.0211 for the difference spectrum [22].

## *Assay Procedure and Calculations*

### 1. Measurement of oxidation of ferrocyclochrome c

A 0.05M air-saturated buffer (MES or HEPES) which was prepared by bubbling with air for 30 minutes at 25° C, along with any other reagents required for the assay, was added to a sample cuvette. A DB-GT Beckman spectrophotometer was set to zero with the cell in place. The reaction was initiated by addition of about 35 $\mu$ l cytochrome c to a final concentration of 15  $\mu$ M and the rate of oxidation or reduction was monitored by recording absorbance at 550 nm.

### 2. Measurement of consumption of oxygen

Oxygen consumption was followed polarographically, using a YSI Model 53 Biological Oxygen Monitor. The oxygen probe was calibrated daily, and the membrane was replaced as necessary. Three ml of 0.05M air-saturated buffer (MES or HEPES) together with other reagents (and any ligands) were placed into the sample tube of the monitor. Six minutes were allowed for temperature equilibrium and stabilization of the recording trace. Vanadium (vanadium(III) or (IV)) was then added to the sample tube to initiate the reaction. The reaction was monitored on a Beckman 10" strip chart recorder, with a data acquisition system connected to the monitor. The pH was measured before and after the recording of the reaction to check for any change of pH during the reaction. Each reaction was repeated at least twice. Initial rates were calculated by regression to the logarithm of the initial portions of recorded absorbance values as a function of time. The initial rates were compared by one way analysis of variance using Dunnett's procedure [23] for multiple-comparisons with a common control. The level of probability to be

required for rejection of the null hypothesis (of no differences between means) was arbitrarily set in advance at  $p < 0.05$ .

## Results

### *Reaction profiles for selected reactions*

Progress of several typical reactions are shown in Figures 1 and 2. Figure 1 compares the progress of a control reaction with the accelerated reactions occurring in the presence of added vanadium(V) or vanadium(IV) aerobically and anaerobically. It can be seen that at pH 7.4, the vanadium(V)/desferrioxamine complex did not oxidize ferrocyclochrome c significantly. In contrast, vanadium(IV) catalysed the oxidation; however oxygen is required. Figure 2 compares the progress of the reduction of ferricycyclochrome c by vanadium(IV) alone with that induced by desferrioxamine alone, and that induced by desferrioxamine plus vanadium(V), or desferrioxamine plus vanadium(IV). Clearly vanadium(IV) is a good reductant of ferricycyclochrome c but vanadium(V) is not. However desferrioxamine alone slowly reduces ferricycyclochrome c, and this reduction is dramatically accelerated by the simultaneous presence of vanadium(V).

### *Oxidation of ferrocyclochrome c by vanadium in the presence of ligands*

Vanadium(IV) and vanadium(V) effectively accelerated the aerobic oxidation of ferrocyclochrome c under certain conditions.

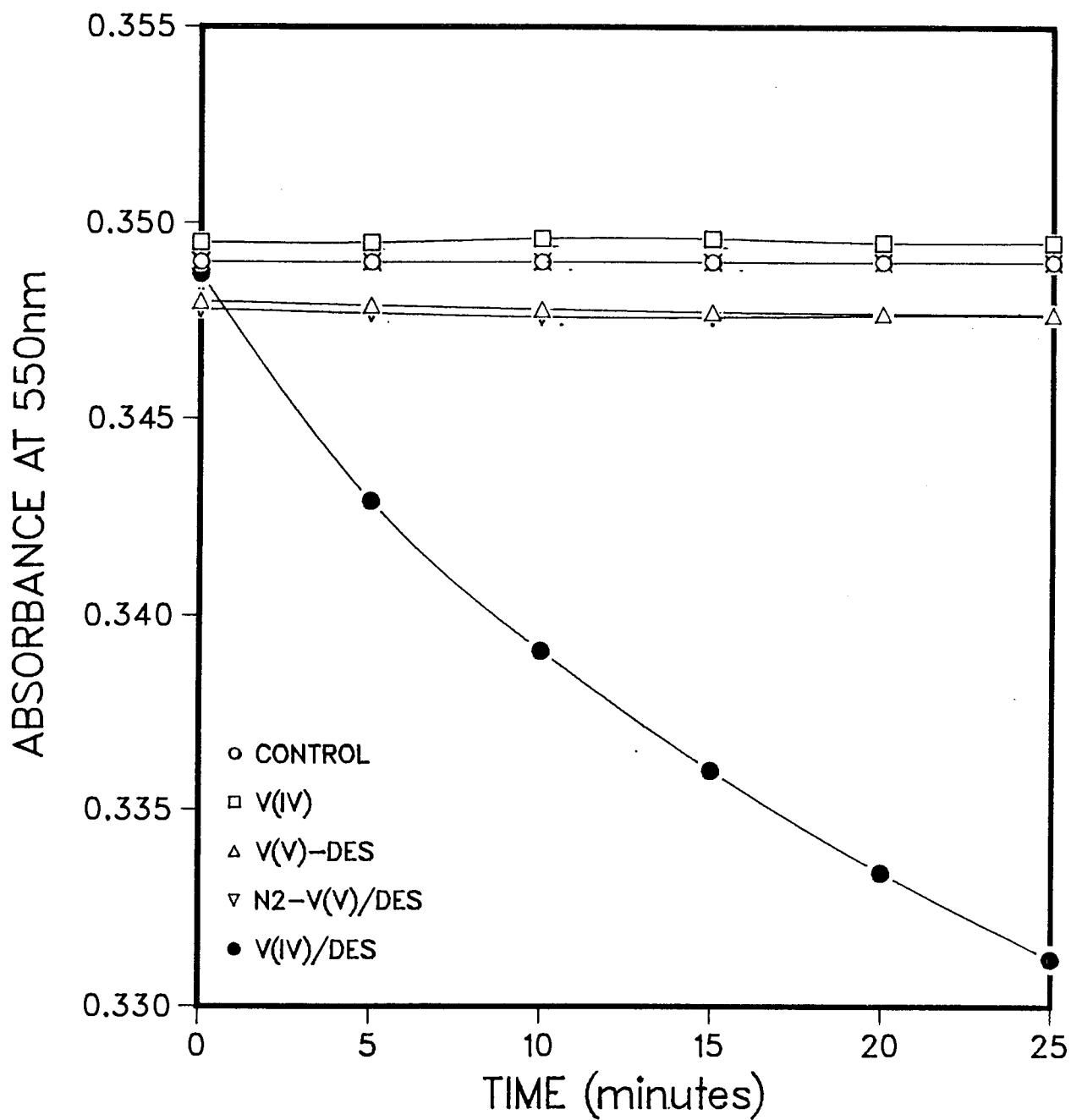
Vanadium(V) oxidized ferrocyclochrome c (at an initial rate of  $1.4 \times 10^{-3} \mu\text{M s}^{-1}$ ) at pH 6.0, but no oxidation of ferrocyclochrome c by vanadate was measurable at pH 7.0 or 7.4 (Figure 3).

**Figure 1. Oxidation of ferrocyclochrome c in the presence of vanadate or vanadyl complexes**

The experiments were conducted in a 0.05M air saturated HEPES buffer at pH 7.4. The final concentrations were: vanadyl, 200  $\mu$ M; vanadate, 1mM; ATP, 1mM; EDTA, 1mM; desferrioxamine 1mM. The abbreviation "DES" indicates desferrioxamine.

"N2" indicates anaerobic conditions.

FIGURE 1. OXIDATION OF FERROCYTOCHROME c

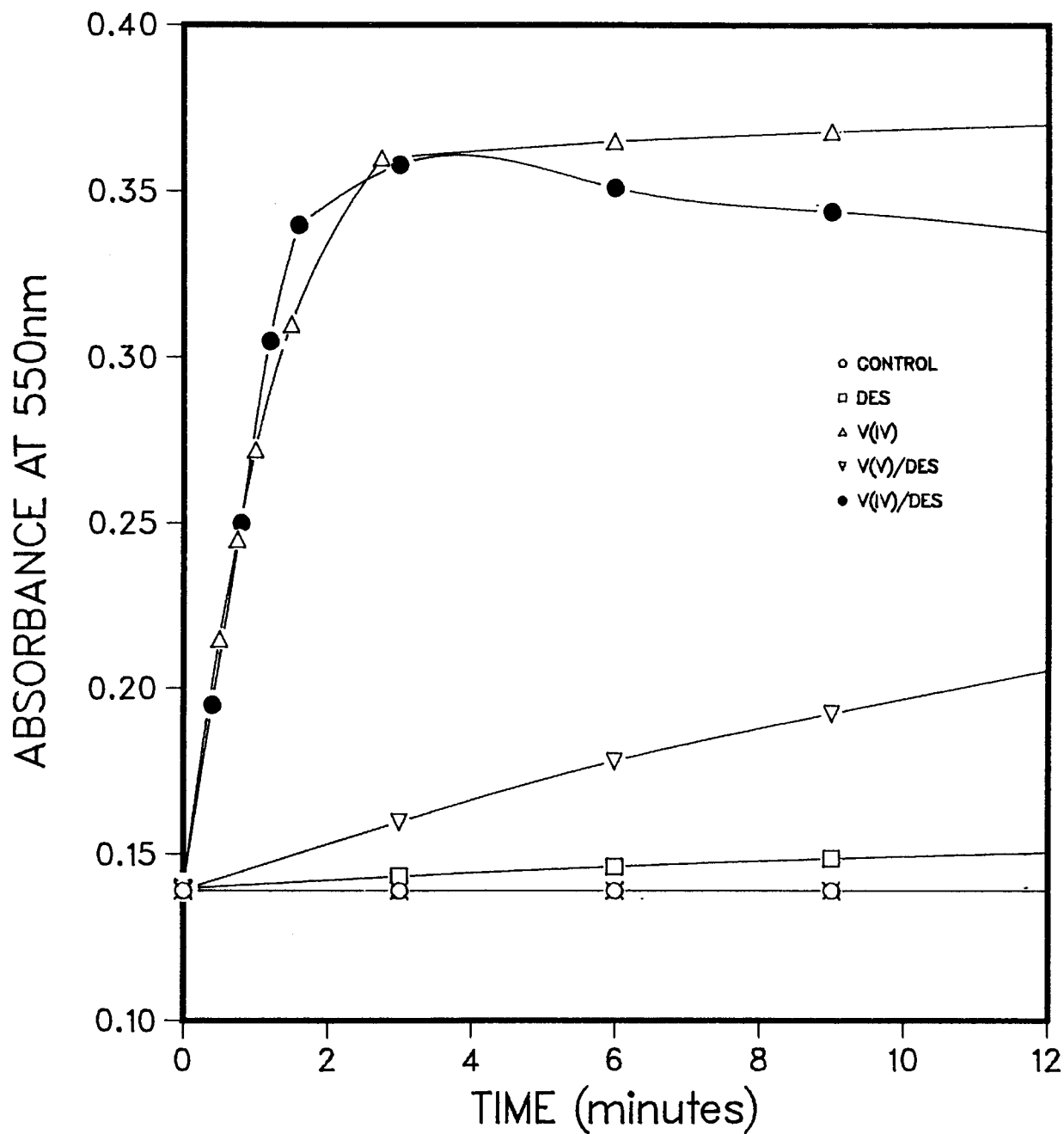


**Figure 2. Reduction of ferricytochrome c by V(IV), desferrioxamine, and V(V)/desferrioxamine.**

The experiments were conducted under aerobic conditions in a 0.05M air saturated HEPES buffer at pH 7.4. The final concentration of vanadate and desferrioxamine were 200 $\mu$ M and 1mM respectively. The abbreviation "DES" indicates desferrioxamine.



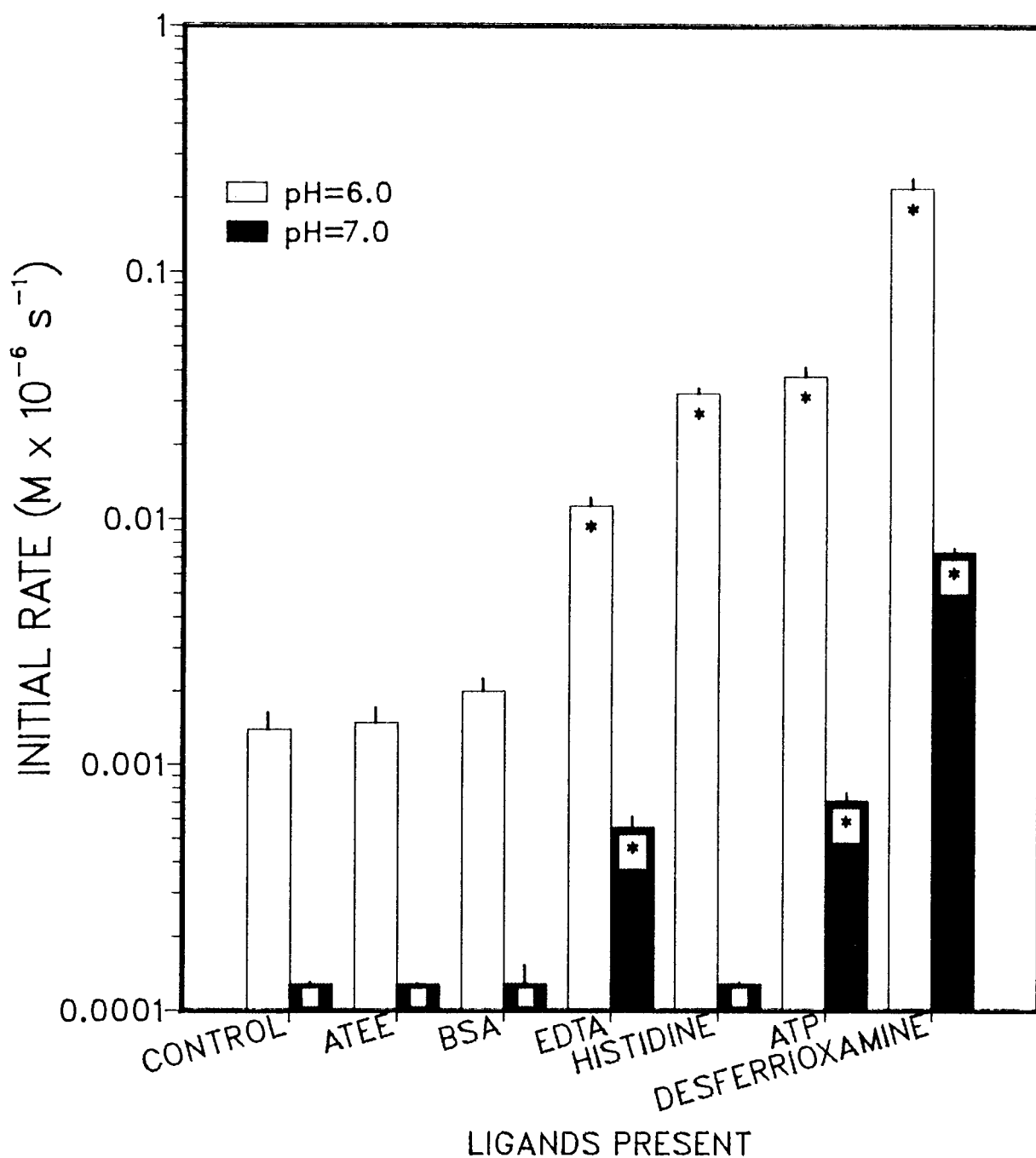
FIGURE 2. REDUCTION OF FERRICYTOCHROME C



**Figure 3. Effects of ligands on the aerobic oxidation of ferrocyclochrome c by vanadate.**

The reactions were carried out at 25°C in a 0.05M MES buffer at pH 6.0, or in a 0.05M HEPES buffer at pH 7.0. Both of these buffers were air-saturated. The final concentration of vanadate was 1mM. The total volume was 2.5 ml, with a final concentration of ferrocyclochrome c of 15 μM. The final concentration of ligands added were: ATEE, 200μM; ATP, 1mM; albumin, 600μg/ml; desferrioxamine, 1mM; EDTA, 1mM; histidine, 1mM. The control was without any ligands (other conditions the same). The air pressure was 733-737 mm Hg during the period of experiment. The symbol "\*" indicates a value significantly different from control at the p < 0.05 level of significance. The abbreviation "DES" indicates desferrioxamine.

FIGURE 3. EFFECTS OF LIGANDS ON AEROBIC OXIDATION OF FERROCYTOCHROME *c* BY VANADATE



At pH 6.0, among the ligands tested, ATP, albumin, desferrioxamine, EDTA and histidine increased the rate of oxidation of ferrocytochrome c by vanadium(V). Among these, desferrioxamine was the most effective, increasing the rate 160-fold. Other ligands' effects are as follows: histidine increased the initial rate 23-fold; ATP increased it 27-fold; EDTA increased it by 8-fold. Albumin increased the initial rate by 1.4-fold, but this was not statistically significant ( $p > 0.05$ ). Similarly ATEE (at about 106% of the control value) had no significant effect on the initial rate.

At pH 7.0 only desferrioxamine, ATP and EDTA allowed measurable oxidation of ferrocytochrome c. Of these, desferrioxamine was the most effective, inducing an initial rate of oxidation of cytochrome c as high as  $7.3 \times 10^{-3} \mu\text{M s}^{-1}$ . In the presence of EDTA, the initial rate of oxidation of ferrocytochrome c was  $5.5 \times 10^{-4} \mu\text{M s}^{-1}$ . ATP increased the initial rate to  $7.1 \times 10^{-4} \mu\text{M s}^{-1}$ . Other ligands (histidine, ATEE, and albumin) had no significant effects on the initial rate of the oxidation of cytochrome c by vanadate (Figure 3).

At pH 7.4, no measurable oxidation of ferrocytochrome c was induced by vanadate or any of the vanadate complexes, over the range of vanadate concentrations from  $100 \mu\text{M}$  to  $1 \text{mM}$ . Unexpectedly, the vanadate/desferrioxamine complex became a *reducing* agent toward ferricytochrome c at pH 7.4 as described in the following section.

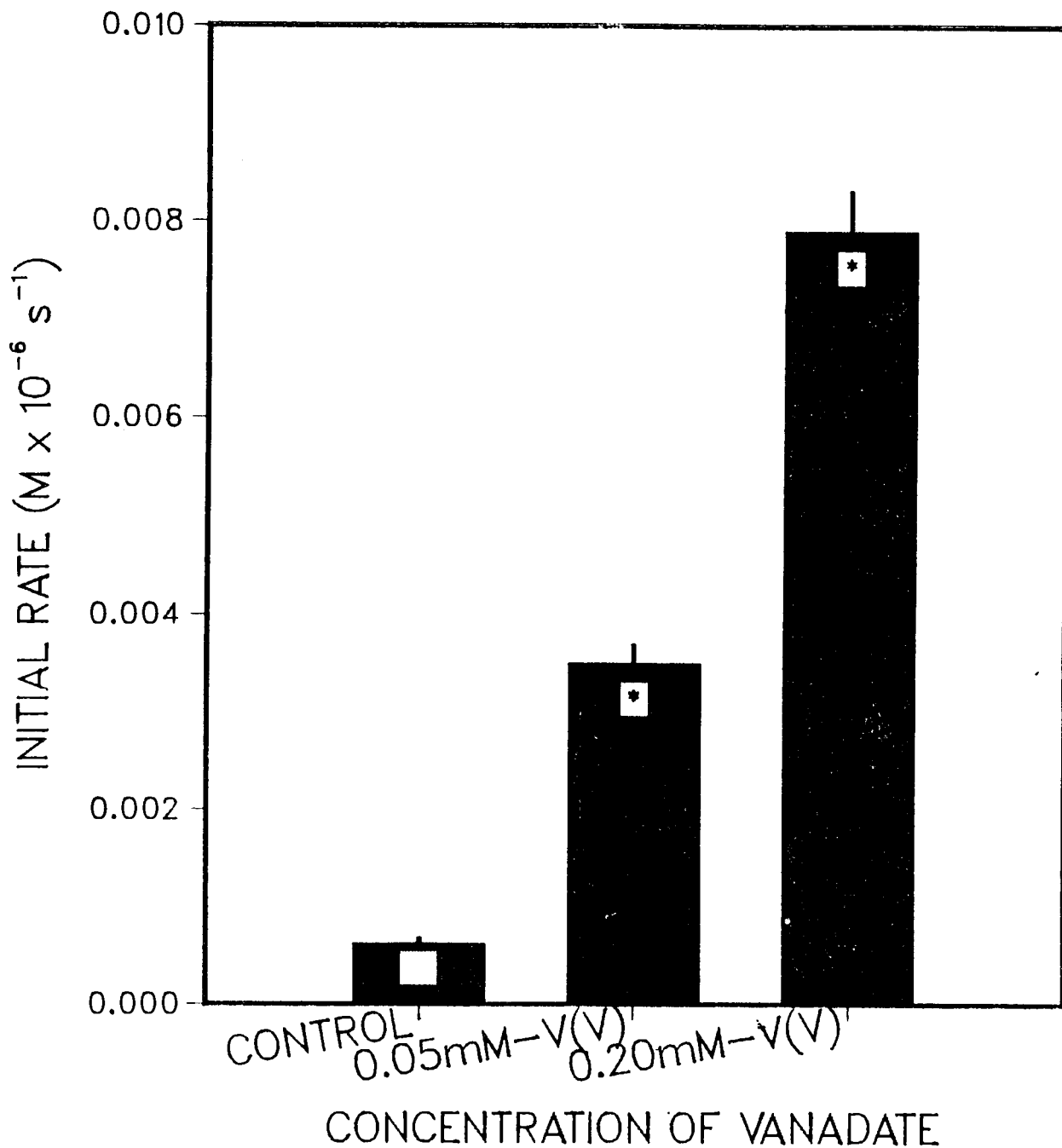
#### *Reduction of ferricytochrome c by vanadate-desferrioxamine*

Paradoxically, ferricytochrome c underwent substantial reduction in the presence of desferrioxamine and vanadate (Figures 2 and 4). A final concentration of  $200 \mu\text{M}$  vanadate, together with  $1 \text{mM}$  desferrioxamine, fully

**Figure 4. Reduction of ferricytochrome  $c$  by vanadate/desferrioxamine at pH 7.4.**

The experiments were conducted in the presence of 1mM desferrioxamine and a certain amount of vanadate. 1mM desferrioxamine was used as the control. The final concentration of vanadate used is indicated on the graph. Other conditions were as Figure 3. The symbol "\*" indicates a value significantly different from the control at the  $p < 0.05$  level of significance.

FIGURE 4. REDUCTION OF FERRICYTOCHROME  $c$   
BY VANADATE/DEFERRIOXAMINE AT pH 7.4



reduced ferricytochrome c at pH 7.4, at an initial rate of  $7.9 \times 10^{-3} \mu\text{M s}^{-1}$ . Lower or higher concentrations of vanadate diminished the rate. In the presence of 1mM vanadate and 1mM desferrioxamine the initial rate of the reduction of ferricytochrome c fell to only  $3.5 \times 10^{-3} \mu\text{M s}^{-1}$ . Desferrioxamine alone also reduced ferricytochrome c, although at  $6.2 \times 10^{-4} \mu\text{M s}^{-1}$  this was only 18% as fast as when accompanied by vanadate. Furthermore, unless accompanied by vanadate, desferrioxamine alone was not able to reduce ferricytochrome c fully. No other ligands or vanadate complexes induced any measurable reduction of ferricytochrome c.

#### *Oxidations and reductions of ferrocytochrome c mediated by vanadyl complexes*

Vanadium(IV) (alone) was capable of either oxidizing or reducing cytochrome c depending on the circumstances. Thus, if ferrocytochrome c was added in the presence of desferrioxamine and vanadyl, it became fully oxidized at an initial rate of  $1.1 \times 10^{-3} \mu\text{M s}^{-1}$  (Figures 1, 5). However, under the same reaction conditions (and also at pH 7.0 or 7.4) vanadium(IV) could also reduce ferricytochrome c. Moreover this was not merely progress toward an equilibrium position. Instead the reaction occurred in two distinct phases. First, ferricytochrome c rapidly became 90% reduced, and then, reoxidation commenced and continued until the cytochrome c was once again fully oxidized (Figure 2).

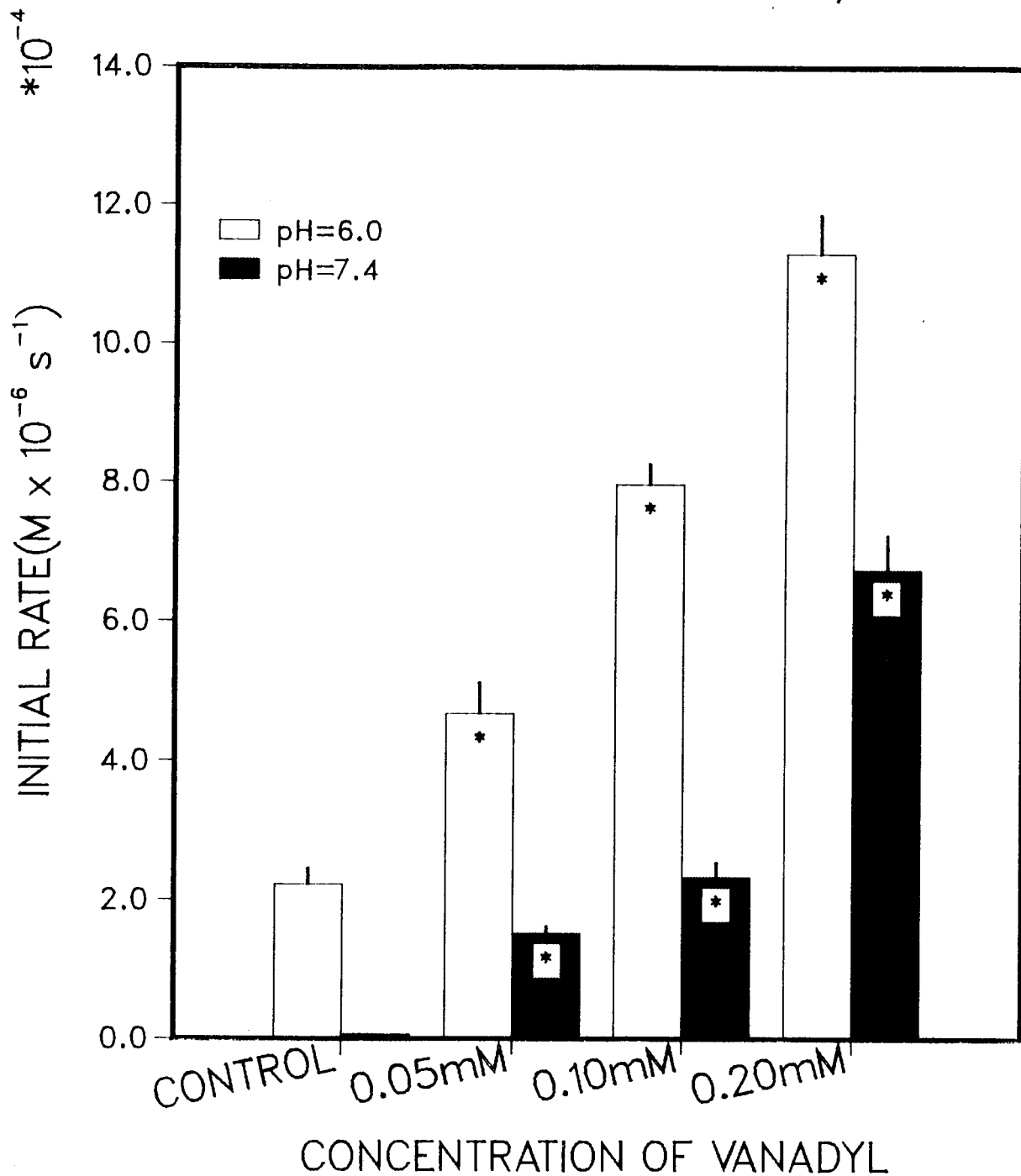
As pH changed over the range 6.0, 7.0, to 7.4, the rate of oxidation of ferrocytochrome c stimulated by vanadium(IV) (200  $\mu\text{M}$ ) in the presence of desferrioxamine (1mM) decreased by 100% (Table 1). At pH 7.4, the initial rate of the aerobic oxidation of ferrocytochrome c was about  $5.4 \times 10^{-4} \mu\text{M s}^{-1}$ . ATP and EDTA also substantially enhanced the aerobic oxidation of ferrocytochrome c induced by oxygen and vanadyl at pH 7.4 (Table 1). A

**Figure 5. Initial rate of the aerobic oxidation of ferrocytochrome c mediated by desferrioxamine/vanadyl complexes.**

The experiments were conducted either in a 0.05M MES buffer at pH 6.0 or in a 0.05M HEPES buffer at pH 7.4. The final concentrations of vanadyl are shown in the graph. The final concentration of desferrioxamine was 1mM. The symbol "\*" indicates a value significantly different from the control at the  $p < 0.05$  level of significance.



FIGURE 5. INITIAL RATE OF THE AEROBIC OXIDATION OF FERROCYTOCHROME c MEDIATED BY VANADYL/DEFERRIOXAMINE



comparison of the effects of desferrioxamine/vanadyl complexes on the autoxidation of ferrocytochrome c is shown in Figure 5.

Table 1. Initial rates of oxidation of ferrocytochrome c in the presence of V(IV)

ligand	pH	initial rate R x 10 <sup>-4</sup> μM s <sup>-1</sup>	S.D. (10 <sup>-4</sup> )
desferrioxamine	6.0	11	1.0
desferrioxamine	7.0	6.6	0.5
desferrioxamine	7.4	5.4	0.6
EDTA	7.4	3.6	0.1
ATP	7.4	3.2	0.4

\* The experiments were conducted in an air saturated 0.05M HEPES buffer at 25° C. The concentration of ATP, EDTA, and desferrioxamine was 1 mM. The final concentration of vanadyl was 200μM.

## Discussion

### *Oxidation of ferrocytochrome c by vanadium and its complexes*

Since the presence of molecular oxygen did not facilitate significantly and was not required for oxidation of ferrocytochrome c by vanadium(V) or its complexes, it is clear that vanadium(V) is merely a stoichiometric oxidant of ferrocytochrome c, and not a catalyst of its reactions with oxygen. The observation therefore that vanadium(V) desferrioxamine stimulates oxidation of ferrocytochrome c more effectively than the most effective complexes of Fe, Cu, or Mn [14] does not mean that vanadium(V) is a more effective redox catalyst, merely that it is a more effective oxidising agent.

Vanadium(IV), the more reduced species, is more effective than vanadium(V) in inducing oxidation of ferrocytochrome c. This paradox is accentuated by the observation that oxidations of ferrocytochrome c induced by vanadium(IV) were oxygen dependent. Here again redox cycling of vanadium seems improbable, because in the presence of desferrioxamine at pH 7.4 where the vanadyl induced oxidation still occurred at a substantial rate, vanadate did not oxidize ferrocytochrome c measurably. Certainly the rate of this step is too slow to allow rereduction of any vanadate produced in reactions between vanadium(IV) and oxygen at pH 7.4. Consequently vanadium is locked exclusively in oxidized forms at this pH. Why then is the vanadium(IV) induced reaction oxygen dependent? The explanation that H<sub>2</sub>O<sub>2</sub>, produced in the reaction of vanadium(IV) with oxygen is the actual reducing agent is negated by the observation that the reaction is inhibited by catalase only to the extent of about 23% (our lab data). The most plausible explanation is that a complex of oxygen with the vanadium(IV) is the actual oxidant for the ferrocytochrome c. At pH 7.0 and even more so at pH 6.0, the reduction of vanadium(V) by ferricytochrome c is sufficient to support redox cycling of the vanadium.

*Special roles of desferrioxamine in the redox reactivity of cytochrome c and vanadium*

That vanadium(IV) reduces ferricytochrome c aerobically is not as surprising as the observation that ferricytochrome c is reduced by vanadium(V)/desferrioxamine. This phenomenon is surprising, since electron transfer from vanadium(V) to ferricytochrome c is thermodynamically implausible. Since desferrioxamine on its own reduces ferricytochrome c it is

presumably the desferrioxamine itself which is the electron donor, and vanadate acts to catalyse the electron transfer from desferrioxamine.

The most effective ligand in catalysing oxidations of ferrocytochrome c was desferrioxamine, the same ligand which is most effective in catalysing reductions of ferricytochrome c. Obviously, the desferrioxamine cannot be acting in both instances just to modify the redox potential, since changes which would favour one reaction would retard the other. It follows then that the presence of desferrioxamine facilitates electron transfer between vanadium and cytochrome c in *both* directions. Presumably electron orbitals of the complex are capable of specific overlap with orbitals at the electron transfer site of the cytochrome c molecule [24].

#### *Effects of pH and ligands on the aerobic oxidation of ferrocytochrome c*

Decreasing pH slows the oxidation of vanadium(IV) by oxygen, but it accelerates the anaerobic oxidation of ferrocytochrome c by vanadate (our lab data), just as it accelerates the aerobic vanadate induced oxidation of ferrocytochrome c. The addition of ATP or EDTA strongly stimulated the aerobic oxidation of ferrocytochrome c induced by vanadium(V). In this respect, the system resembles the anaerobic oxidation of ferrocytochrome c by vanadium(V), rather than the reaction of vanadium(IV) with oxygen which is strongly inhibited by these same ligands. Clearly the rate limiting step where redox cycling is occurring, is the reduction of vanadium(V) by ferrocytochrome c, and thus the effects of pH and ligands on the overall reaction are those of their action on this first, rate determining step. In part this reflects the high concentrations of both oxygen and cytochrome c, which are much greater than can be envisaged intracellularly.

### *Comparison of aerobic and anaerobic reactions*

For similar reasons and due to the location of the rate determining step prior to the participation of oxygen, there is no consistent inhibition when aerobic conditions are replaced by anaerobic. The only case in which the reaction was slower anaerobically was in the presence of EDTA where the oxidation was slowed by 50% by the removal of oxygen. Surprisingly, under anaerobic conditions the oxidation of ferrocytochrome c induced by vanadate in the presence of desferrioxamine at pH 7.4 was accelerated by 60%. Removal of oxygen also accelerated (by 24%) the net reduction of ferricytochrome c by vanadate in the presence of desferrioxamine at pH 6.0, presumably by slowing reoxidation.

The most effective ligand in catalysing oxidations of cytochrome c was desferrioxamine. At pH 6.0, its effectiveness under aerobic conditions was less than under anaerobic conditions. Under anaerobic conditions the initial rate increased 3500-fold, while under aerobic conditions, it only increased 160-fold.

### *Physiological implications of the redox reactivity of vanadium*

The current data confirm that vanadate is an effective cellular oxidant at pH 6.0, or in the presence of an appropriate ligand at neutral pH. This is consistent with the intracellular occurrence almost entirely as vanadyl, since intracellular ligands (ATP, ADP etc.) at neutral pH accelerated the reduction of vanadate, displacing the equilibrium in favour of the reduced form [13]. In addition to the abovementioned thermodynamic considerations, a number of kinetic constraints contribute to the steady state level of reduction of vanadium intracellularly, which explain why it exists primarily

in the vanadium(IV) state:

1. There are a number of effective reducing agents intracellularly which are absent from the plasma. These include components of the electron transport chain, for example cytochrome c as demonstrated in the current study.
2. Oxygen concentrations are lower intracellularly.
3. The lower values of intracellular pH both retard oxidations of vanadium(IV) by oxygen and simultaneously accelerate reductions of vanadium(V) by at least one intracellular reductant.
4. Intracellular ligands like ATP retard aerobic oxidation of vanadium(IV).

Since the biological activities attributed to vanadium are thought to be due to the intracellular actions of vanadium(V), the above mechanisms should be considered protective against, for example, the tyrosine esterifying actions of vanadium(V). The redox reactivity of vanadium suggests a possible mechanism for the formation of lipid peroxides as a result of vanadium toxicity., i.e., the aerobic oxidation of cellular metabolites mediated by vanadyl complexes. The cellular concentrations of even toxic amounts of vanadium are likely to be much lower than those used in the current study. Consequently such reactions are likely to be contingent on redox cycling of the metal and if they occur at all, they should be more evident in regions of low pH or relatively high oxygen concentration. This is consistent with reports that oxidative damage caused by the intake of vanadate, is seen particularly in the kidney (an acidic site) [7] and the lung, an oxygen rich site [8].

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## GENERAL DISCUSSION AND CONCLUSION

### *Influence of ligands on the redox reactivity of vanadium*

Chapters 2 to 5 demonstrate clearly the profound influence that ligands have on the redox reactivity of vanadium, attributable at least in part to the formation of vanadium complexes. Of these, the vanadate/desferrioxamine complex, having an absorption peak at 332nm, is the most reactive. Probably vanadate forms a cyclic complex with desferrioxamine through the hydroxamate and carbonyl groups. It is possible that vanadate also esterifies the hydroxyl group of desferrioxamine to some extent. A spectrally visible complex formed by vanadate, but not by vanadyl or vanadium(III), has measurable absorbance when less than 20 $\mu$ M vanadate is present, and thus may prove useful as a simple method for detection of this redox species of vanadium.

### *Influence of pH on the redox reactivity of vanadium*

Changes in pH profoundly influence the redox reactivity of ligand-vanadium complexes toward cytochrome c, by at least three mechanisms: (1) by altering the stability and reduction potential of the complexes; (2) by involvement of H<sup>+</sup> as one of the reactants in the reactions; (3) by changing the conformation and thus the redox reactivity of cytochrome c.

### *The possible toxicity of different species of vanadium*

Vanadium has been shown to cause oxidative damage to kidney of rats. This may result from: (i) the direct oxidative action of a vanadate complex, or, (ii) from pro-oxidant actions of reduced species of vanadium [V(IV)] capable of activating oxygen. Both of these are more likely to take place

inside the cell rather than in the plasma. First, the pH is relatively low, and a wide range of ligands is available inside the cell. Second, intracellular vanadium exists predominantly in the form of vanadyl. Intracellular reducing agents convert vanadate to vanadyl inside the cell, and make it possible for redox cycling and consequent amplification of pro-oxidant damage. The direct oxidative damage caused by vanadate becomes more serious in acidic environments, such as the kidney in which vanadate is expelled from plasma to the urine (its pH may be as low as 4.5). Peroxidation of lipids of the kidney has been reported in rats and mice after vanadium exposure (Donaldson, 1985).

#### **Future directions**

In order to further reveal the biological actions of vanadium, we suggest some further studies:

1. Kinetic studies of the reduction of vanadate complexes (especially vanadate/desferrioxamine) by ferrocyclochrome c
2. Stoichiometric studies of the interaction between vanadyl (or vanadium(III)) and active oxygen species.
3. Stoichiometric studies of the redox reaction between vanadium and another cellular reductant (e.g. ascorbate or glutathione).
4. NMR and ESR studies of the various vanadium desferrioxamine complexes.
5. Kinetic and stoichiometric studies of the reduction of ferricytochrome c by other reductants (e.g. ascorbate or glutathione) in the presence of vanadate.

From the current data we conclude that:

1. Vanadate functions as a pH dependent cellular oxidant, in that at pH 6.0, it oxidizes ferrocytochrome c . However, at neutral pH (pH 7.0 and 7.4), the oxidation of ferrocytochrome c by vanadate is not measurable.
2. Ligands profoundly influence both the reduction of vanadate(V) and the oxidation of vanadyl(IV) or vanadium(III). Generally speaking, the influences of ligands on the oxidation of vanadium(III) are smaller than their influences on the redox reactivities of the other two.
3. pH is a critical factor in determining the redox reactivity of vanadium complexes.
4. Desferrioxamine slowly reduces ferricytochrome c; vanadate mediates this reaction.
5. Vanadyl complexes mediate the oxidation of cytochrome c by oxygen at pH 6.0, 7.0, and 7.4. At pH 7.4, the mechanism involved probably is not the redox cycling of vanadyl and vanadate.
6. Since heme iron is located in the crevice of the apoprotein of cytochrome c , transition metals are required as catalysts of the autoxidation of cytochrome c only when the crevice is closed. Decreasing pH to < 3.2 diminishes the need for external transition metals.

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## APPENDIX

### PLOTFILE

#### Chapter-2

Figure 2-1

```
CASE IS ASCII. EVERY COLOR IS BLACK . LAYOUT LRH . GENERATE A PLOT. WINDOW
DESTINATION 1 10 1 8. x numbered. x page 11, y page 11. X AXIS LENGTH=6.5 . Y
AXIS LENGTH=7.5 . curve 1 symbol type=16 . curve 2 symbol type=17 . curve 3
symbol type=18 . curve 4 symbol type=19 . curve 5 symbol type=2 . curve 6
symbol type=6 EVERY CURVE COLOR=BLACK . EVERY CURVE SYMBOL BLANKING ON .
every curve legend line existence off . every curve solid GRACE 0 . frame.
LEGEND HEIGHT .2 LEGEND FRAME=OFF . LEGEND TEXT ' ' . every curve symbol
count=1. LEGEND UNITS PLOT% . LEGEND BOX 2.5 35 2.5 35. LEGEND ' ' . AXIS
FRAME . TITLE IS 'FIGURE 1. AEROBIC OXIDATION' 'OF FERROCYTOCHROME C AT pH
4.0'. X AXIS LABEL TEXT IS 'TIME (minute)' . Y AXIS LABEL TEXT IS 'ABSORBANCE
(550nm)' . y min is 0.14. Y MAX IS 0.17. INPUT DATA . 'CONTROL' 0, 0.1678 2,
0.1672 4 , 0.1666 6 , 0.1660 8 , 0.1654 'CONTROL' 0, 0.1678 2, 0.1672 4 ,
0.1666 6 , 0.1660 8 , 0.1654 'Fe-EDTA' 0, 0.1678 2, 0.1630 4 , 0.15956 6 ,
0.1562 8 , 0.1544 'Fe-EDTA' 0, 0.1678 2, 0.1630 4 , 0.15956 6 , 0.1562 8 ,
0.1544 'Cu-ADP' 0, 0.1668 2, 0.1591 4 , 0.1525 6 , 0.1468 8 , 0.1410 'Cu-ADP'
0, 0.1668 2, 0.1591 4 , 0.1525 6 , 0.1468 8 , 0.1410 END OF DATA. CURVE 1
SCATTERED. CURVE 2 legend entry existence off. CURVE 2 SMOOTH. CURVE 3
SCATTERED. CURVE 4 legend entry existence off. CURVE 4 SMOOTH. CURVE 5
SCATTERED. CURVE 6 legend entry existence off. CURVE 6 SMOOTH. SEND.
```

Figure 2-2

```
CASE IS ASCII. generate a bar. window destination 1 10 1 8. TITLE HEIGHT IS
0.25 . X PAGE 11, Y PAGE 11 . LEGEND FRAME OFF . LENGTH X=6.5, Y=7.5. AXIS
FRAME . LEGEND UNITS PLOT% . LEGEND FRAME=OFF. LEGEND BOX 2.5 35 85 97.5.
LEGEND ' ' . layout=lrh. y axis label is "INITIAL RATE (M x 10<EX.5)-6<EXHX)
s<EX.5)-1<EXHX))". x axis label is "LIGANDS ADDED". independent division
labels are-"CONTROL"- "ADP"- "EDTA"-- "DESFERIOXAMINE"- "HISTIDINE". sequence
data. " " 0.111 0.0871 0.0896 0.0991 0.103 " " 0.008 0.0041 0.0038 0.0081
0.0081 end data. frame the plot. bar root=0.00000. Title is "FIG. 2 EFFECTS
OF LIGANDS ON THE AUTOXIDATION" "OF FERROCYTOCHROME C AT pH 3.2". all
distribution color is black. dist 1 shade pattern is 30140 dist documentation
content = user-text. dist 1 doc text " " "*" "*" " " " ". DIST 2 WIDTH 2 .
DIST 2 LEGEND ENTRY EXISTENCE OFF . DIST 2 STACKED . SEND.
```

Figure 2-3

```
CASE IS ASCII. generate a bar. window destination 1 10 1 8. layout=lrh. TITLE
HEIGHT IS 0.25 . X PAGE 11, Y PAGE 11 . LEGEND FRAME OFF . LENGTH X=6.5,
Y=7.5. AXIS FRAME . LEGEND UNITS PLOT% . LEGEND FRAME=OFF. LEGEND BOX 2.5 35
75 97.5. LEGEND ' ' . y axis label is "INITIAL RATE (M x 10<EX.5)-6<EXHX)
```



```

s<EX.5)-1<EXHX))". y axis type log. y min is 0.00001. x axis label is "METAL
COMPLEXES PRESENT". independent division labels
are-"CONTROL"- "V-Des"- "Cu-ADP"-- "Fe-EDTA"- "Mn-Des"- "Al-EDTA". sequence data.
"pH=4.0" 1.26E-4 9.07E-3 4.12E-3 6.78E-4 4.63E-4 2.44E-4 "pH=3.2" 0.117 1.32
0.183 0.224 0.114 0.095 "pH=2.6" 0.723 4.59 0.620 0.738 0.528 0.54 " "
0.0000116 0.0002 0.000072 0.000046 0.000023 0.000046 " " 0.0137 0.03 0.0241
0.0200 0.012 0.012 " " 0.15 0.06 0.13 0.1 0.10 0.13 end data. legend text="
". frame the plot. Title is "FIG. 3 EFFECTS OF pH ON " "AEROBIC OXIDATION OF
FERROCYTOCHROME C". every dist documentation content = user-text. dist 1
shade pattern is 0. DIST 4 WIDTH 2 . DIST 4 LEGEND ENTRY EXISTENCE OFF . DIST
4 STACKED . dist 2 shade pattern is 45150 . DIST 5 WIDTH 2 . DIST 5 LEGEND
ENTRY EXISTENCE OFF . DIST 5 STACKED . dist 3 shade pattern is 90110. DIST 6
WIDTH 2 . DIST 6 LEGEND ENTRY EXISTENCE OFF . DIST 6 STACKED . dist 1 doc
text " " "*" "*" "*" "*" "*" ". dist 2 doc text " " "*" "*" "*" " " " ". dist 3
doc text " " "*" " " " " " " " ". clustering is 3. SEND.

```

Figure 2-4

```

CASE IS ASCII. generate a bar. window destination 1 10 1 8. layout=lrh. TITLE
HEIGHT IS 0.25 . X PAGE 11, Y PAGE 11 . LEGEND FRAME OFF . LENGTH X=6.5,
Y=7.5. AXIS FRAME . LEGEND UNITS PLOT% . LEGEND FRAME=OFF. LEGEND BOX 2.5 35
75 97.5. LEGEND ' ' . y axis label is "INITIAL RATE (M x 10<EX.5)-6<EXHX)
s<EX.5)-1<EXHX))". y axis type log. y min is 0.001. x axis label is
"TRANSITION METAL ION PRESENT". independent division labels are-"No
metal"- "V"- "Cu"- "Fe"-- "Al"- "Mn". sequence data. "Aq" .1110 .4896 .1830 .1004
.1120 .0923 "ADP" .0871 .4608 .1660 .1050 .1190 .0826 "EDTA" .0896 1.2060
.1030 .2240 .1200 .0936 "Des" .0991 1.3200 .1440 .1240 .1130 .1010 "His"
.1030 1.1590 .1310 .1240 .1120 .0913 " " .0080 .0100 .0150 .0108 .0122 .0108
" " .0041 .0120 .0180 .0094 .0130 .0079 " " .0038 .0200 .0110 .0130 .0115
.0108 " " .0081 .0250 .0130 .0120 .0108 .0108 " " .0081 .0100 .0140 .0130
.0108 .0094 end data. legend text is " ". frame the plot. Title is "Fig. 4
EFFECTS OF METALS AND METAL COMPLEXES" -"ON AEROBIC OXIDATION OF
FERROCYTOCHROME c". every dist documentation content = user-text. dist 1
shade pattern is 0. DIST 6 WIDTH 2 . DIST 6 LEGEND ENTRY EXISTENCE OFF . DIST
6 STACKED . DIST 2 shade pattern is 45150. DIST 7 WIDTH 2 . DIST 7 LEGEND
ENTRY EXISTENCE OFF . DIST 7 STACKED . dist 3 shade pattern is 45351. DIST 8
WIDTH 2 . DIST 8 LEGEND ENTRY EXISTENCE OFF . DIST 8 STACKED . dist 4 shade
pattern is 45141. DIST 9 WIDTH 2 . DIST 9 LEGEND ENTRY EXISTENCE OFF . DIST 9
STACKED . dist 5 shade pattern is 90100. DIST 10 WIDTH 2 . DIST 10 LEGEND
ENTRY EXISTENCE OFF . DIST 10 STACKED . dist 1 doc text " " "*" "*" " " " " "
". dist 2 doc text " " "*" "*" " " " " "*" ". dist 3 doc text " " "*" " " "*" "
" " ". dist 4 doc text " " "*" "*" " " " " " ". dist 5 doc text " " "*" " " "
" " " " ". clustering is 5. send.

```

### Chapter 3

Figure 3-1

```

CASE IS ASCII. EVERY COLOR IS BLACK . LAYOUT LRH . GENERATE A PLOT. x
numbered. x page 11, y page 11. X AXIS LENGTH=6.5 . Y AXIS LENGTH=7.5 . curve
1 symbol type=17 . curve 2 symbol type=17 . curve 3 symbol type=18 . curve 4
symbol type=19 . curve 5 symbol type=2 . curve 6 symbol type=6 EVERY CURVE
COLOR=BLACK . EVERY CURVE SYMBOL BLANKING ON . every curve legend line

```

existence off . every curve solid GRACE 0 . frame. LEGEND HEIGHT .2 LEGEND  
 FRAME=OFF . LEGEND TEXT ' ' . every curve symbol count=1. LEGEND UNITS PLOT%  
 . LEGEND BOX 2.5 35 2.5 35. LEGEND ' ' . WINDOW DESTINATION 1 10 1 8. AXIS  
 FRAME . TITLE IS 'FIGURE 1. ANAEROBIC OXIDATION OF FERROCYTOCHROME c' 'BY  
 VANADATE OR VANADATE/ATP COMPLEX AT pH 6.0'. X AXIS LABEL TEXT IS 'TIME  
 (minutes)' . Y AXIS LABEL TEXT IS 'ABSORBANCE AT 550nm' . y min is 0.150. Y  
 MAX IS 0.380. INPUT DATA . 'CONTROL' 0, 0.365 1, 0.365 2, 0.365 3, 0.365 4,  
 0.365 5, 0.365 'CONTROL' 0, 0.365 1, 0.365 2, 0.365 3, 0.365 4, 0.365 5,  
 0.365 'VANADATE' 0, 0.36 1, 0.3561 2, 0.3529 3, 0.3499 4, 0.3469 5, 0.3445  
 'VANADATE' 0, 0.36 1, 0.3561 2, 0.3529 3, 0.3499 4, 0.3469 5, 0.3445  
 'VANADATE/ATP' 0, 0.3560 1, 0.293 2, 0.2510 3, 0.223 4, 0.204 5, 0.1915  
 'VANADATE/ATP' 0, 0.3560 1, 0.293 2, 0.2510 3, 0.223 4, 0.204 5, 0.1915 END  
 OF DATA. CURVE 1 SCATTERED. CURVE 2 legend entry existence off. CURVE 2  
 SMOOTH. CURVE 3 SCATTERED. CURVE 4 legend entry existence off. CURVE 4  
 SMOOTH. CURVE 5 SCATTERED. CURVE 6 legend entry existence off. CURVE 6  
 SMOOTH. SEND. Figure 3-2

CASE is ASCII. EVERY COLOR IS BLACK . FANCY IS SWISS MEDIUM . LAYOUT LRH .  
 GENERATE A FANCY BAR . window destination 1 10 1 8. TITLE HEIGHT IS 0.25 . X  
 PAGE 11, Y PAGE 11 . LEGEND FRAME OFF . LENGTH X=6.5, Y=7.5. AXIS FRAME .  
 LEGEND UNITS PLOT% . LEGEND FRAME=OFF. LEGEND BOX 2.5 35 75 97.5. LEGEND ' '  
 . EVERY DISTRIBUTION COLOR IS BLACK . AXIS FRAME . TITLE IS 'FIGURE 2.  
 ANAEROBIC OXIDATION OF FERROCYTOCHROME c'-'BY VANADATE COMPLEXES: EFFECTS OF  
 LIGANDS'. y axis label is "INITIAL RATE (M x 10<EX.5)-6<EXHX)  
 s<EH.5)-1<EXHX))". Y AXIS TYPE LOG. y min is 0.00001. X AXIS LABEL IS  
 "LIGANDS PRESENT". INDEPENDENT DIVISION LABELS -- 'CONTROL'-- 'ATEE'--  
 'BSA'-- 'EDTA'--'ATP'-- 'Histidine'--'Desferrioxamine'. SEQUENCE DATA. 'pH=6.0'  
 1.67E-3 9.47E-4 2.58E-3 3.40E-3 0.028 0.031 5.63 'pH=7.0' 1.57E-5 1.57E-5  
 1.57E-5 4.45E-4 1.18E-4 1.57E-5 0.0117 ' ' 0.000513 0.00013 0.00013 0.00096  
 0.0024 0.00132 0.2843 ' ' 0.00000012 0.00000012 0.00000012 0.00013 0.0000363  
 0.00000012 0.00132 END OF DATA . EVERY DIST SHADE PATTERN IS 90110 . DIST 1  
 SHADE PATTERN IS 0. dist documentation content = user-text. dist 1 doc text "  
 " " " "\*" "\*" "\*" "\*" "\*" . DIST 3 WIDTH 2 . DIST 3 LEGEND ENTRY EXISTENCE OFF.  
 . DIST 3 STACKED . DIST 2 SHADE PATTERN IS 90110. dist documentation content  
 = user-text. dist 2 doc text " " " " " " "\*" "\*" " " "\*" . DIST 4 WIDTH 2 .  
 DIST 4 LEGEND ENTRY EXISTENCE OFF . DIST 4 STACKED . clustering is 2. send.

Figure 3-3

CASE IS ASCII. EVERY COLOR IS BLACK . LAYOUT LRH . GENERATE A PLOT. x  
 numbered. x page 11, y page 11. X AXIS LENGTH=6.5 . Y AXIS LENGTH=7.5 . curve  
 1 symbol type=17 . curve 2 symbol type=17 . curve 3 symbol type=18 . curve 4  
 symbol type=19 . curve 5 symbol type=2 . curve 6 symbol type=6 EVERY CURVE  
 COLOR=BLACK . EVERY CURVE SYMBOL BLANKING ON . every curve legend line  
 existence off . every curve solid GRACE 0 . frame. LEGEND HEIGHT .2 LEGEND  
 FRAME=OFF . LEGEND TEXT ' ' . every curve symbol count=1. LEGEND UNITS PLOT%  
 . LEGEND BOX 2.5 35 75 97.5 LEGEND ' ' . WINDOW DESTINATION 1 10 1 8. AXIS  
 FRAME . TITLE IS 'FIGURE 3 ANAEROBIC REDUCTION OF FERRICYTOCHROME c BY'  
 'DEFERRIOXAMINE OR VANADATE/DEFERRIOXAMINE COMPLEX'. X AXIS LABEL TEXT IS  
 'TIME (minutes)' . Y AXIS LABEL TEXT IS 'ABSORBANCE AT 550nm'. Y MIN IS 0.13.  
 Y MAX IS 0.27. INPUT DATA . 'CONTROL' 0, 0.15 3, 0.15 6, 0.15 9, 0.15 12,  
 0.15 'CONTROL' 0, 0.15 3, 0.15 6, 0.15 9, 0.15 12, 0.15 'DES' 0, 0.1530 3,  
 0.1643 6, 0.1723 9, 0.1785 12, 0.1854 'DES' 0, 0.1530 3, 0.1643 6, 0.1723 9,

0.1785 12, 0.1854 'vanadate/DES' 0, 0.1549 3, 0.1972 6, 0.2237 9, 0.2441 12, 0.2596 'vanadate/DES' 0, 0.1549 3, 0.1972 6, 0.2237 9, 0.2441 12, 0.2596 END OF DATA. CURVE 1 SCATTERED. CURVE 2 legend entry existence off. CURVE 2 SMOOTH. CURVE 3 SCATTERED. CURVE 4 legend entry existence off. CURVE 4 SMOOTH. CURVE 5 SCATTERED. CURVE 6 legend entry existence off. CURVE 6 SMOOTH. SEND. Figure 3-4

CASE IS ASCII. LAYOUT LRH . GENERATE A BAR . window destination 1 10 1 8. TITLE HEIGHT IS 0.25 . X PAGE 11, Y PAGE 11 . LEGEND FRAME OFF . LENGTH X=6.5, Y=7.5. AXIS FRAME . LEGEND UNITS PLOT% . LEGEND FRAME=OFF. LEGEND BOX 2.5 35 75 97.5. LEGEND ' ' . EVERY DISTRIBUTION COLOR IS BLACK . AXIS FRAME . TITLE IS 'FIGURE 4. ANAEROBIC REDUCTION OF FERRICYTOCHROME c BY'-'VANADIUM(III) OR VANADYL(IV) COMPLEXES AT pH 6.0: EFFECTS OF LIGAND'. y axis label is "INITIAL RATE (M x 10<EX.5)-6<EXHX) s<EH.5)-1<EXHX))". X AXIS LABEL TEXT IS 'LIGANDS PRESENT'. INDEPENDENT DIVISION LABELS ARE-- 'CONTROL'-- 'EDTA'-'ATP'-- 'ATEE'-'HIS'-- 'BSA'-'DES'. SEQUENCE DATA. 'VANADIUM(III)' 0.064 1.29E-3 5.73E-03 0.0505 0.0384 0.075 0.072 'VANADYL(IV)' 0.0366 9.24E-3 1.88E-03 0.0465 0.0496 0.0693 0.0716 ' ' 6.90E-03 9.29E-4 0.51E-03 4.78E-3 4.50E-03 4.22E-03 2.58E-04 ' ' 4.11E-03 2.2E-3 4.40E-04 2.5E-3 4.30E-03 4.50E-03 4.40E-03 END OF DATA. DIST 1 SHADE PATTERN IS 0 . every dist documentation content = user-text. dist 1 doc text " " "\*" "\*" " " "\*" " " " ". DIST 3 WIDTH 2 . DIST 3 LEGEND ENTRY EXISTENCE OFF . DIST 3 STACKED . DIST 2 SHADE PATTERN IS 90110. dist 2 doc text " " "\*" "\*" " " " " "\*" "\*" . DIST 4 WIDTH 2 . DIST 4 LEGEND ENTRY EXISTENCE OFF . DIST 4 STACKED. CLUSTERING IS 2. SEND.

Figure 3-5

CASE IS ASCII. LAYOUT LRH . GENERATE A BAR . window destination 1 10 1 8. TITLE HEIGHT IS 0.25 . X PAGE 11, Y PAGE 11 . LEGEND FRAME OFF . LENGTH X=6.5, Y=7.5. AXIS FRAME . LEGEND UNITS PLOT% . LEGEND FRAME=OFF. LEGEND BOX 2.5 35 75 97.5. LEGEND ' ' . EVERY DISTRIBUTION COLOR IS BLACK . AXIS FRAME . TITLE IS 'FIGURE 5. ANAEROBIC REDUCTION OF FERRICYTOCHROME c. BY'-'VANADIUM(III) OR VANADYL(IV) COMPLEXES AT pH 7.0: EFFECTS OF LIGAND'. y axis label is "INITIAL RATE (M x 10<EX.5)-6<EXHX) s<EH.5)-1<EXHX))". X AXIS LABEL TEXT IS 'LIGANDS PRESENT'. INDEPENDENT DIVISION LABELS ARE-- 'CONTROL'-- 'EDTA'-'ATP'-- 'ATEE'-'BSA'-'DES'-- 'HIS'. SEQUENCE DATA. 'VANADIUM(III)' 0.132 0.0164 0.0156 0.107 0.106 0.144 0.119 'VANADYL(IV)' 0.0852 0.0591 6.20E-03 0.0591 0.067 0.0975 0.119 ' ' 0.0071 0.0038 0.00089 0.0072 0.00515 0.0121 0.00992 ' ' 5.70E-03 1.5E-5 6.59E-04 5.7E-3 2.10E-03 4.61E-03 4.51E-03 END OF DATA. DIST 1 SHADE PATTERN IS 0 . every dist documentation content = user-text. dist 1 doc text " " "\*" "\*" " " " " " " " ". DIST 3 WIDTH 2 . DIST 3 LEGEND ENTRY EXISTENCE OFF . DIST 3 STACKED . DIST 2 SHADE PATTERN IS 90110. DIST 4 WIDTH 2 . DIST 4 LEGEND ENTRY EXISTENCE OFF . DIST 4 STACKED . dist 2 doc text " " "\*" "\*" " " " " " " "\*" . CLUSTERING IS 2. SEND.

Figure 3-6

CASE IS ASCII. LAYOUT LRH . GENERATE A BAR . window destination 1 10 1 8. TITLE HEIGHT IS 0.25 . X PAGE 11, Y PAGE 11 . LEGEND FRAME OFF . LENGTH X=6.5, Y=7.5. AXIS FRAME . LEGEND UNITS PLOT% . LEGEND FRAME=OFF. LEGEND BOX 2.5 35 75 97.5. LEGEND ' ' . EVERY DISTRIBUTION COLOR IS BLACK . AXIS FRAME .

TITLE IS 'FIGURE 6. ANAEROBIC REDUCTION OF FERRICYTOCHROME c BY'-'VANADIUM(III) OR VANADYL(IV) COMPLEXES AT pH 7.4: EFFECTS OF LIGAND'. y axis label is "INITIAL RATE (M x 10<EX.5)-6<EXHX) s<EH.5)-1<EXHX))". X AXIS LABEL TEXT IS 'LIGANDS PRESENT'. INDEPENDENT DIVISION LABELS ARE-- 'CONTROL'-- 'EDTA'-'ATP'-- 'ATEE'-'BSA'-'DES'-- 'HIS'. SEQUENCE DATA. 'VANADIUM(III)' 0.133 0.04 0.0153 0.1005 0.111 0.144 0.130 'VANADYL(IV)' 0.102 1.5E-3 0.025 0.0802 0.0825 0.1162 0.18 ' ' 1.25E-02 5.71E-4 4.44E-03 5.59E-3 7.58E-03 1.11E-02 8.70E-03 ' ' 0.0045 1.5E-4 0.0027 0.0018 0.00195 0.00557 0.00118 END OF DATA. every dist documentation content = user-text. dist 1 doc text " " "\*" "\*" " " " " " " ". DIST 1 SHADE PATTERN IS 0 . DIST 3 WIDTH 2 . DIST 3 LEGEND ENTRY EXISTENCE OFF . DIST 3 STACKED . DIST 2 SHADE PATTERN IS 90110. DIST 4 WIDTH 2 . DIST 4 LEGEND ENTRY EXISTENCE OFF . DIST 4 STACKED . dist 2 doc text " " "\*" "\*" " " " " " " "\*". CLUSTERING IS 2. send.

Figure 3-7

CASE IS ASCII. LAYOUT LRH . GENERATE A BAR . window destination 1 10 1 8. TITLE HEIGHT IS 0.25 . X PAGE 11, Y PAGE 11 . LEGEND FRAME OFF . LENGTH X=6.5, Y=7.5. AXIS FRAME . LEGEND UNITS PLOT% . LEGEND FRAME=OFF. LEGEND BOX 2.5 35 75 97.5. LEGEND ' ' . EVERY DISTRIBUTION COLOR IS BLACK . AXIS FRAME . TITLE IS 'FIGURE 7. COMPARISON OF INITIAL RATE OF OXIDATION OF' 'FERROCYTOCHROME c BETWEEN AEROBIC AND ANAEROBIC CONDITIONS'. y axis label is "INITIAL RATE (M x 10<EX.5)-6<EXHX) s<EH.5)-1<EXHX))". Y AXIS TYPE LOG. Y MIN IS 0.001. X AXIS LABEL TEXT IS 'LIGANDS PRESENT'. INDEPENDENT DIVISION LABELS -- 'CONTROL'-'ATEE'-- 'BSA'-'EDTA'-'ATP'-'HISTIDINE'-'DEFERRIOXAMINE'. sequence data. 'AEROBIC' 1.39E-03 1.48E-03 2.0E-3 0.0113 0.038 0.0324 0.2184 'ANAEROBIC' 1.67E-3 1.32E-3 2.58E-3 3.4E-3 0.028 0.031 5.63 ' ' 2.34E-04 2.31E-04 2.46E-4 9.25E-4 3.48E-3 1.69E-3 0.0218 ' ' 0.000513 0.00014 0.00013 0.00096 0.0024 0.00132 0.0443 end of data. DIST 1 SHADE PATTERN IS 0. every dist documentation content = user-text. dist 1 doc text " " " " " " "\*" "\*" "\*" "\*". DIST 3 WIDTH 2 . DIST 3 LEGEND ENTRY EXISTENCE OFF . DIST 3 STACKED . DIST 2 SHADE PATTERN IS 90110. dist 2 doc text " " " " "\*" "\*" "\*" "\*" "\*". DIST 4 WIDTH 2 . DIST 4 LEGEND ENTRY EXISTENCE OFF . DIST 4 STACKED . clustering is 2. SEND.

Figure 3-8

CASE IS ASCII. EVERY COLOR IS BLACK . LAYOUT LRH . GENERATE A PLOT. x page 11, y page 11. X AXIS LENGTH=6.5 . Y AXIS LENGTH=7.5 . EVERY CURVE COLOR=BLACK . every curve legend line existence off . every curve solid frame. LEGEND HEIGHT .2 LEGEND FRAME=OFF . WINDOW DESTINATION 1 10 1 8. AXIS FRAME . TITLE IS 'FIGURE 8 SPECTRUM OF VANADATE DEFERRIOXAMINE COMPLEX'. X AXIS LABEL TEXT IS 'nm' . Y AXIS LABEL TEXT IS 'TRANSMITTANCE %'. Y MAX IS 100. Y MIN IS 0. INPUT DATA . ' ' 460, 90 440, 70 420, 49 400, 31 390, 23 380, 17 370, 11.55 360, 7.8 354, 6.2 350, 5.4 344, 4.1 340, 3.8 332, 3.5 324, 3.8 320, 4.2 318, 4.8 316, 5.45 314, 6.5 312, 7.7 310, 10 300, 29 294, 55 290, 63 288, 66 286, 70 284, 73 282, 76 280, 78 278, 78.2 276, 77.8 274, 75 272, 70 270, 64 264, 40 260, 30 258, 27.5 256, 26.5 254, 26 250, 26 240, 26 230, 26 END OF DATA. CURVE 1 legend entry existence off. send. **Chapter-4**

Figure 4-1

CASE IS ASCII. EVERY COLOR IS BLACK . LAYOUT LRH . GENERATE A PLOT. x

numbered. x page 11, y page 11. X AXIS LENGTH=6.5 . Y AXIS LENGTH=7.5 . curve 1 symbol type=16 . curve 2 symbol type=17 . curve 3 symbol type=18 . curve 4 symbol type=19 . curve 5 symbol type=2 . curve 6 symbol type=6 EVERY CURVE COLOR=BLACK . EVERY CURVE SYMBOL BLANKING ON . every curve legend line existence off . every curve solid GRACE 0 . frame. LEGEND HEIGHT .2 LEGEND FRAME=OFF . LEGEND TEXT ' ' . every curve symbol count=1. LEGEND UNITS PLOT% . LEGEND BOX 2.5 35 2.5 35. LEGEND ' ' . WINDOW DESTINATION 1 10 1 8. AXIS FRAME . TITLE IS 'FIGURE 1 REDUCTION OF OXYGEN BY VANADYL 'AND VANADYL COMPLEXES AT pH 7.4'. X AXIS LABEL TEXT IS 'TIME (minute)' . Y AXIS LABEL TEXT IS '% OF ORIGINAL OXYGEN REMAINING'. Y MAX IS 100. Y MIN IS 50. INPUT DATA . 'CONTROL' 0, 100 3, 90.5 6, 86.8 9, 84.5 12, 83 'CONTROL' 0, 100 3, 90.5 6, 86.8 9, 84.5 12, 83 'V(IV)/ATP' 0, 100 3, 97.5 6, 96.2 9, 95.5 12, 94.8 'V(IV)/ATP' 0, 100 3, 97.5 6, 96.2 9, 95.5 12, 94.8 'V(IV)/des' 0, 100 3, 84 6, 74.4 9, 67 12, 63 'V(IV)/des' 0, 100 3, 84 6, 74.4 9, 67 12, 63 END OF DATA. CURVE 1 SCATTERED. CURVE 2 legend entry existence off. CURVE 2 SMOOTH. CURVE 3 SCATTERED. CURVE 4 legend entry existence off. CURVE 4 SMOOTH. CURVE 5 SCATTERED. CURVE 6 legend entry existence off. CURVE 6 SMOOTH. SEND.

Figure 4-2

CASE IS ASCII. FANCY IS SWISS MEDIUM . LAYOUT LRH . GENERATE A FANCY BAR . window destination 1 10 1 8. TITLE HEIGHT IS 0.25 . X PAGE 11, Y PAGE 11 . LEGEND FRAME OFF . LENGTH X=6.5, Y=7.5. AXIS FRAME . LEGEND UNITS PLOT% . LEGEND FRAME=OFF. LEGEND BOX 2.5 35 75 97.5. LEGEND ' ' . EVERY DISTRIBUTION COLOR IS BLACK . AXIS FRAME . TITLE IS 'FIGURE 2. REDUCTION OF OXYGEN BY VANADIUM(III) COMPLEXES'. y axis label is "INITIAL RATE (M x 10<EX.5)-6<EXHX) s<EX.5)-1<EXHX))". X AXIS LABEL TEXT IS 'LIGANDS PRESENT'. INDEPENDENT DIVISION LABELS ARE-- 'CONTROL'-- 'ATEE'--'ATP'-- 'BSA'--'DESFERRIOXAMINE'-- 'EDTA'--'HISTIDINE'. SEQUENCE DATA. 'pH=6.0' 0.0202 0.018 9.72E-03 8.86E-03 0.05 0.014 0.0216 'pH=7.0' 0.06 0.058 0.0174 0.051 0.072 0.044 0.07 'pH7.4' 0.101 0.094 0.019 0.1104 0.0792 0.085 0.101 ' ' 1.66E-03 2.47E-03 2.10E-03 1.10E-03 6.14E-03 7.80E-04 4.20E-04 ' ' 5.70E-03 5.40E-03 4.20E-03 4.61E-03 9.81E-03 1.50E-03 8.30E-03 ' ' 0.013 2.40E-03 6.60E-03 2.73E-03 9.12E-03 8.02E-03 2.20E-03 END OF DATA. DIST 1 SHADE PATTERN IS 0. DIST 4 WIDTH 2. DIST 4 LEGEND ENTRY EXISTENCE OFF . DIST 4 STACKED . DIST 2 SHADE PATTERN IS 45150. DIST 5 WIDTH 2 . DIST 5 LEGEND ENTRY EXISTENCE OFF . DIST 5 STACKED . DIST 3 SHADE PATTERN IS 90110. DIST 6 WIDTH 2 . DIST 6 LEGEND ENTRY EXISTENCE OFF . DIST 6 STACKED . every dist documentation content = user-text. dist 1 doc text " " " " "\*" "\*" "\*" "\*" " ". dist 2 doc text " " " " "\*" " " " " "\*" " ". dist 3 doc text " " " " "\*" " " " " " " " ". clustering is 3. SEND.

Figure 4-3

CASE IS ASCII. FANCY IS SWISS MEDIUM . GENERATE A FANCY BAR . window destination 1 10 1 8. TITLE HEIGHT IS 0.25 . X PAGE 11, Y PAGE 11 . LEGEND FRAME OFF . LENGTH X=6.5, Y=7.5. AXIS FRAME . LEGEND UNITS PLOT% . LEGEND FRAME=OFF. LEGEND BOX 2.5 35 75 97.5. LEGEND ' ' . LAYOUT LRH . EVERY DISTRIBUTION COLOR IS BLACK . AXIS FRAME . TITLE IS 'FIGURE 3. REDUCTION OF OXYGEN BY VANADYL COMPLEXES'. y axis label is "INITIAL RATE (M x 10<EX.5)-6<EXHX) s<EX.5)-1<EXHX))". X AXIS LABEL TEXT IS 'LIGANDS PRESENT'. INDEPENDENT DIVISION LABELS ARE-- 'CONTROL'-- 'ATEE'--'ATP'-- 'BSA'--'DESFERRIOXAMINE'-- 'EDTA'--'HISTIDINE'. SEQUENCE DATA. 'pH=6.0' 0.079

```

0.061 0.029 0.061 0.53 0.016 0.049 'pH=7.0' 0.135 0.134 0.0326 0.14 0.329
0.011 0.307 'pH=7.4' 0.162 0.156 0.0314 0.146 0.29 0.0195 0.274 ' ' 9.73E-03
4.10E-03 1.50E-03 0.013 0.036 1.01E-03 2.51E-03 ' ' 0.013 0.014 2.80E-03
5.41E-03 0.039 5.44E-04 0.028 ' ' 3.41E-03 3.41E-04 6.41E-03 0.018 5.12E-03
8.12E-03 0.013 end of data. DIST 1 SHADE PATTERN IS 0 . DIST 4 WIDTH 2 . DIST
4 LEGEND ENTRY EXISTENCE OFF . DIST 4 STACKED . DIST 2 SHADE PATTERN IS
45150. DIST 5 WIDTH 2 . DIST 5 LEGEND ENTRY EXISTENCE OFF . DIST 5 STACKED .
DIST 3 SHADE PATTERN IS 90110. DIST 6 WIDTH 2 . DIST 6 LEGEND ENTRY EXISTENCE
OFF . DIST 6 STACKED . every dist documentation content = user-text. dist 1
doc text " " " " "*" " " "*" "*" "*" ". dist 2 doc text " " " " "*" " " "*" "*"
*". dist 3 doc text " " " " "*" " " "*" "*" "*" . CLUSTERING IS 3. SEND.

```

## Chapter 5

Figure 5-1

```

CASE IS ASCII. EVERY COLOR IS BLACK . LAYOUT LRH . GENERATE A PLOT. x
numbered. x page 11, y page 11. X AXIS LENGTH=6.5 . Y AXIS LENGTH=7.5 . curve
1 symbol type=17. curve 3 symbol type=18. curve 5 symbol type=2. curve 7
symbol type=6. curve 9 symbol type=16. EVERY CURVE COLOR=BLACK . EVERY CURVE
SYMBOL BLANKING ON . every curve legend line existence off . every curve
solid GRACE 0 . frame. LEGEND HEIGHT .2 LEGEND FRAME=OFF . LEGEND TEXT ' ' .
every curve symbol count=1. LEGEND UNITS PLOT% . LEGEND BOX 2.5 35 2.5 35.
LEGEND ' ' . WINDOW DESTINATION 1 10 1 8. AXIS FRAME . TITLE IS 'FIGURE 1.
OXIDATION OF FERROCYTOCHROME c' X AXIS LABEL TEXT IS 'TIME (minutes)' . Y
AXIS LABEL TEXT IS 'ABSORBANCE AT 550nm'. y min is 0.330. Y MAX IS 0.350.
INPUT DATA . 'CONTROL' 0, 0.349 5, 0.349 10, 0.349 15, 0.349 20, 0.349 25,
0.349 'CONTROL' 0, 0.349 5, 0.349 10, 0.349 15, 0.349 20, 0.349 25, 0.349
'V(IV)' 0, 0.3495 5, 0.3495 10, 0.3496 15, 0.3496 20, 0.3495 25, 0.3495
'V(IV)' 0, 0.3495 5, 0.3495 10, 0.3496 15, 0.3496 20, 0.3495 25, 0.3495
'V(V)-DES' 0, 0.348 5, 0.3479 10, 0.3478 15, 0.34773 20, 0.3477 25, 0.34768
'V(V)-DES' 0, 0.348 5, 0.3479 10, 0.3478 15, 0.34773 20, 0.3477 25, 0.34768
'N2-V(V)/DES' 0, 0.3478 5, 0.3477 10, 0.3476 15, 0.3476 20, 0.34765 25,
0.34765 'N2-V(V)/DES' 0, 0.3478 5, 0.3477 10, 0.3476 15, 0.3476 20, 0.34765
25, 0.34765 'V(IV)/DES' 0, 0.3487 5, 0.3429 10, 0.3391 15, 0.3360 20, 0.3334
25, 0.3312 'V(IV)/DES' 0, 0.3487 5, 0.3429 10, 0.3391 15, 0.3360 20, 0.3334
25, 0.3312 END OF DATA. CURVE 1 SCATTERED. CURVE 2 legend entry existence
off. CURVE 2 SMOOTH. CURVE 3 SCATTERED. CURVE 4 legend entry existence off.
CURVE 4 SMOOTH. CURVE 5 SCATTERED. CURVE 6 legend entry existence off. CURVE
6 SMOOTH. CURVE 7 SCATTERED. CURVE 8 legend entry existence off. CURVE 8
SMOOTH. CURVE 9 SCATTERED. CURVE 10 legend entry existence off. CURVE 10
SMOOTH. send.

```

Figure 5-2

```

CASE IS ASCII. EVERY COLOR IS BLACK . LAYOUT LRH . GENERATE A PLOT. x
numbered. x page 11, y page 11. X AXIS LENGTH=6.5 . Y AXIS LENGTH=7.5 . curve
1 symbol type=17 . curve 3 symbol type=18. curve 5 symbol type=2 . curve 7
symbol type=6. curve 9 symbol type=16. curve 11 symbol type=16. curve 13
symbol type=2. EVERY CURVE COLOR=BLACK . EVERY CURVE SYMBOL BLANKING ON .
every curve legend line existence off . every curve solid GRACE 0 . frame.
LEGEND HEIGHT .2 LEGEND FRAME=OFF . LEGEND TEXT ' ' . every curve symbol
count=1. LEGEND UNITS PLOT% . LEGEND BOX 75 97.5 50 72.5. LEGEND ' ' . WINDOW

```



```

VANADATE'. INDEPENDENT DIVISION LABELS -- 'CONTROL'-'0.05mM-V(V)'--
'0.20mM-V(V)'-- SEQUENCE DATA. ' ' 6.20E-04 3.50E-03 7.90E-03 ' ' 6.36E-05
1.98E-04 4.24E-04 END OF DATA . EVERY DIST SHADE PATTERN IS 90110. DIST 1
SHADE PATTERN IS 90110. DIST 2 WIDTH 2 . DIST 2 LEGEND ENTRY EXISTENCE OFF .
DIST 2 STACKED . every dist documentation content = user-text. dist 1 doc " "
"*" "*". SEND.

```

Figure 5-5

```

CASE IS ASCII. LAYOUT LRH . GENERATE A BAR . window destination 1 10 1 8.
TITLE HEIGHT IS 0.25 . X PAGE 11, Y PAGE 11 . LEGEND FRAME OFF . LENGTH
X=6.5, Y=7.5. AXIS FRAME . LEGEND UNITS PLOT% . LEGEND FRAME=OFF. LEGEND BOX
2.5 35 75 97.5. LEGEND ' ' . EVERY DISTRIBUTION COLOR IS BLACK . AXIS FRAME .
TITLE IS 'FIGURE 5. INITIAL RATE OF THE AEROBIC OXIDATION OF'
'FERROCYTOCHROME c MEDIATED BY VANADYL/DEFERRIOXAMINE'. Y AXIS LABEL IS
'INITIAL RATE(M x 10<EX.5)-6<EXHX) s<EX.5)-1<EXHX))'. X AXIS LABEL TEXT IS
'CONCENTRATION OF VANADYL'. INDEPENDENT DIVISION LABELS -- 'CONTROL'--
'0.05mM'-'0.10mM'-'0.20mM'. SEQUENCE DATA. 'pH=6.0' 2.23E-04 4.68E-04
7.97E-04 1.13E-03 'pH=7.4' 6.35E-06 1.52E-04 2.33E-04 6.73E-04 ' ' 2.31E-05
4.49E-05 2.92E-05 5.66E-05 ' ' 3.37E-07 1.11E-05 2.13E-05 5.15E-05 END OF
DATA . DIST 1 SHADE PATTERN IS 0. every dist documentation content =
user-text. dist 1 doc " " "*" "*" "*". DIST 3 WIDTH 2 . DIST 3 LEGEND ENTRY
EXISTENCE OFF . DIST 3 STACKED . DIST 2 SHADE PATTERN IS 90110. dist 2 doc "
" "*" "*" "*". DIST 4 WIDTH 2 . DIST 4 LEGEND ENTRY EXISTENCE OFF . DIST 4
STACKED . clustering is 2. SEND.

```