KINETIC STUDIES OF THE OXIDATION OF HORSE HEART CYTOCHROME *c*(II), *PSEUDOMONAS AERUGINOSA* CYTOCHROME c_{551}(II), AND *PSEUDOMONAS AERUGINOSA* AZURIN(I) BY TRIS COMPLEXES OF 1,10-PHENANTHROLINE AND MODIFIED 1,10-PHENANTHROLINE WITH COBALT(III)

Thesis by

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ABSTRACT

Anaerobic stopped flow kinetic studies of the oxidation of horse heart cytochrome c(II), Pseudomonas aeruginosa cytochrome c_{551}(II), and Pseudomonas aeruginosa azurin(I) have been performed. The oxidants were the tris complexes of 1,10-phenanthroline; 5-chloro-1,10-phenanthroline; 5,6-dimethyl-1,10-phenanthroline; 4,7-dimethyl-1,10-phenanthroline; and 4,7-di(phenyl-4'-sulfonate)-1,10-phenanthroline with cobalt(III).

Second-order rate constants found for the oxidation of the cytochromes by $Co(phen)_3^{3+}$ give good correlation with rate constants predicted by Marcus theory. Outer-sphere electron transfer at a site common to both proteins is implied, and this site is proposed to be the exposed heme edge.

Rate and activation parameters for the oxidation of the proteins by all of the above mentioned oxidants were acquired. Reactivities of the proteins toward the various oxidants were found to be similar except for the oxidant $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$. Known structural differences between the proteins in the vicinity of the heme edge are proposed to account for the reactivity difference found for oxidation by $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$. No dependence of the activation parameters upon charge was observed. Accumulated activation parameters were interpreted to mean that efficient electron transfer from the cytochromes involves considerable activation energy.

Ionic strength dependence studies showed that horse heart cytochrome c(II) has a small positive active site charge, and that

P. aeruginosa cytochrome c_{551}(II) has a small negative active site charge.

A similar series of oxidation experiments was performed using P. aeruginosa azurin(I). Once again excellent correlation between Marcus theory and experiment was found. The occurrence of simple outer-sphere electron transfer between the three proteins of this study was thus indicated. The oxidant $Co(phen)_3^{3+}$ was also thereby shown to be a suitable reagent for the study of these electron transfer proteins.

The oxidation of azurin(I) was shown to proceed only with considerable protein activation. All modified-phenanthroline oxidants cause significant perturbation of the activation parameters compared to those of the unmodified-phenanthroline oxidant. A very sterically inaccessible electron transfer site for azurin was therefore suggested.

To define the effects of the substitutions on the properties of the oxidants, spectral data and reduction potential data were collected. In addition, electron transfer studies involving each of the oxidants with the reductants $Co(terpy)_2^{2+}$ and $Ru(NH_3)_5py^{2+}$ were completed. Excluding charge effects, the oxidants were shown to have only slightly varying redox properties.

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CHAPTER I

OXIDATION STUDIES OF CYTOCHROME *c*

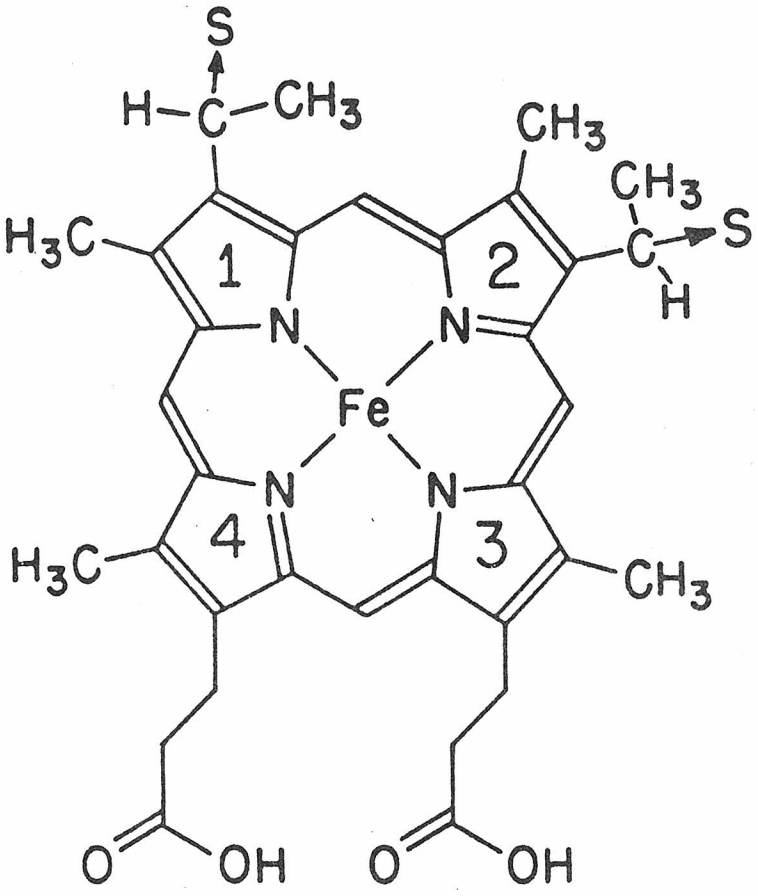
INTRODUCTION

The cytochromes are a series of heme proteins which function in the respiratory chain of all aerobic organisms. In each of the cytochromes the heme iron may exist in either the ferrous or ferric form, thus enabling reducing equivalents to pass down the chain. Through the terminal enzyme cytochrome oxidase, the system of cytochromes is able to reduce dioxygen directly.[1]

The distribution of the cytochromes among every animal species was recognized at the time of the discovery of the protein by MacMunn in 1884.[2] However, MacMunn's findings were not accepted among his contemporaries because of the criticism of the much respected Hoppe-Seyler[3] and because of difficulties in his experiments that MacMunn himself recognized. Not until their rediscovery by Keilin[4] in 1925 did experimentation begin to discover the various properties of the cytochromes. Much of the work has centered upon cytochrome c because of its unusual stability to temperature and pH, and because of its ease of extraction. That cytochrome c may be extracted with nearly full retention of its physiological properties has been demonstrated by the ability of bovine cytochrome c to maintain both electron transfer and oxidative phosphorylation in rat liver mitochondria.[5] Cytochrome c may now routinely be extracted from muscle tissue in highly purified form.[6]

The "c" type cytochromes are so named because all have a "c" type heme (Figure 1) covalently attached to the polypeptide chain. Linkage of the heme to the chain usually occurs through two thioether bridges to cysteine residues. The cytochromes c have polypeptide

Figure 1. The structure of heme c (Figures 1 and 2 are taken from reference 7.)

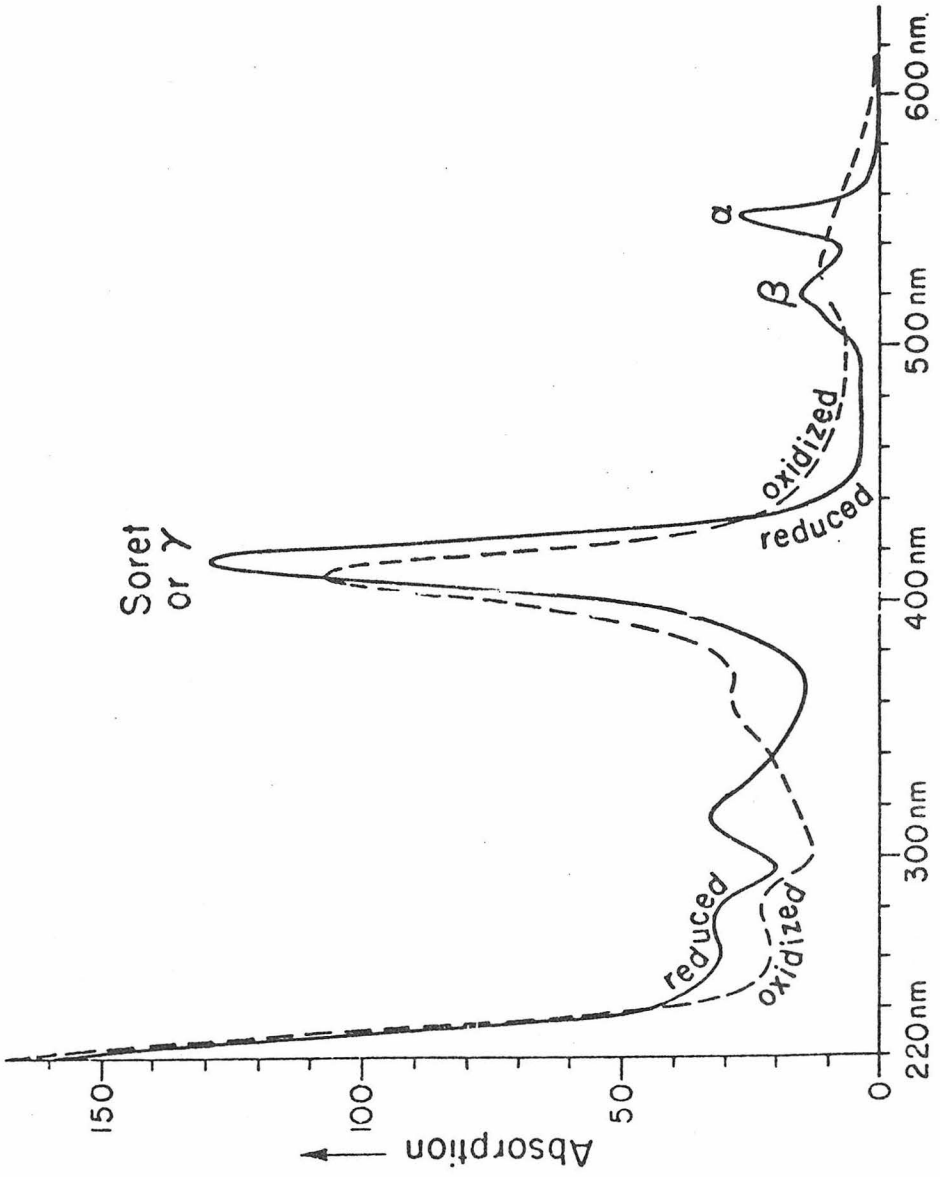

S
H
C
CH3
CH3
CH3
S
H3C
1
2
C
H
N
N
Fe
N
N
4
3
H3C
CH3
O
OH
O
OH

chains of 103 to 113 amino acids, depending upon the organism from which the cytochrome is extracted. Both d^5 Fe(III) and d^6 Fe(II) are low spin; thus, the Fe(III)-heme complex has a single unpaired electron and a formal charge of +1.

The heme also gives rise to the visible absorption spectrum of cytochrome c. Figure 2 compares the reduced and oxidized spectra of a typical cytochrome c.[7] An intense absorption at about 400 nm characteristic of heme groups was first discovered in 1883 by J. L. Soret.[8] The Soret or γ band is the most intense of the porphyrin absorptions, with a molar extinction coefficient of the order of 10^5. Two other visible absorption peaks, the α and β bands, lie at longer wavelength. The molar extinction coefficients for the α and β bands are about one-twentieth that of the Soret band, which is in fact a general property of extended two-dimensional π-electron systems. All three absorptions arise from the $A_{2u} \rightarrow E_g$ transition of the neutral porphyrin. However, the lower lying states have higher spin multiplicity and transitions to them are strongly forbidden.[9,10]

Thus all cytochromes c display some common structural parameters. However, variances in the amino acid sequences of the different cytochromes can provide a very detailed picture of the evolution of this particular protein. The subject has been discussed in many recent reviews, including the excellent review by Dickerson and Timkovich.[7] From an evolutionary standpoint, the important objectives to be attained from the study of cytochromes c are the construction of phylogenetic trees, and the comparison of these

Figure 2. The reduced and oxidized cytochrome c spectra.


Absorption
150
100
50
0
220nm
300nm
400nm
500nm
600nm
Soret
or γ
reduced
oxidized
α
β
reduced
oxidized

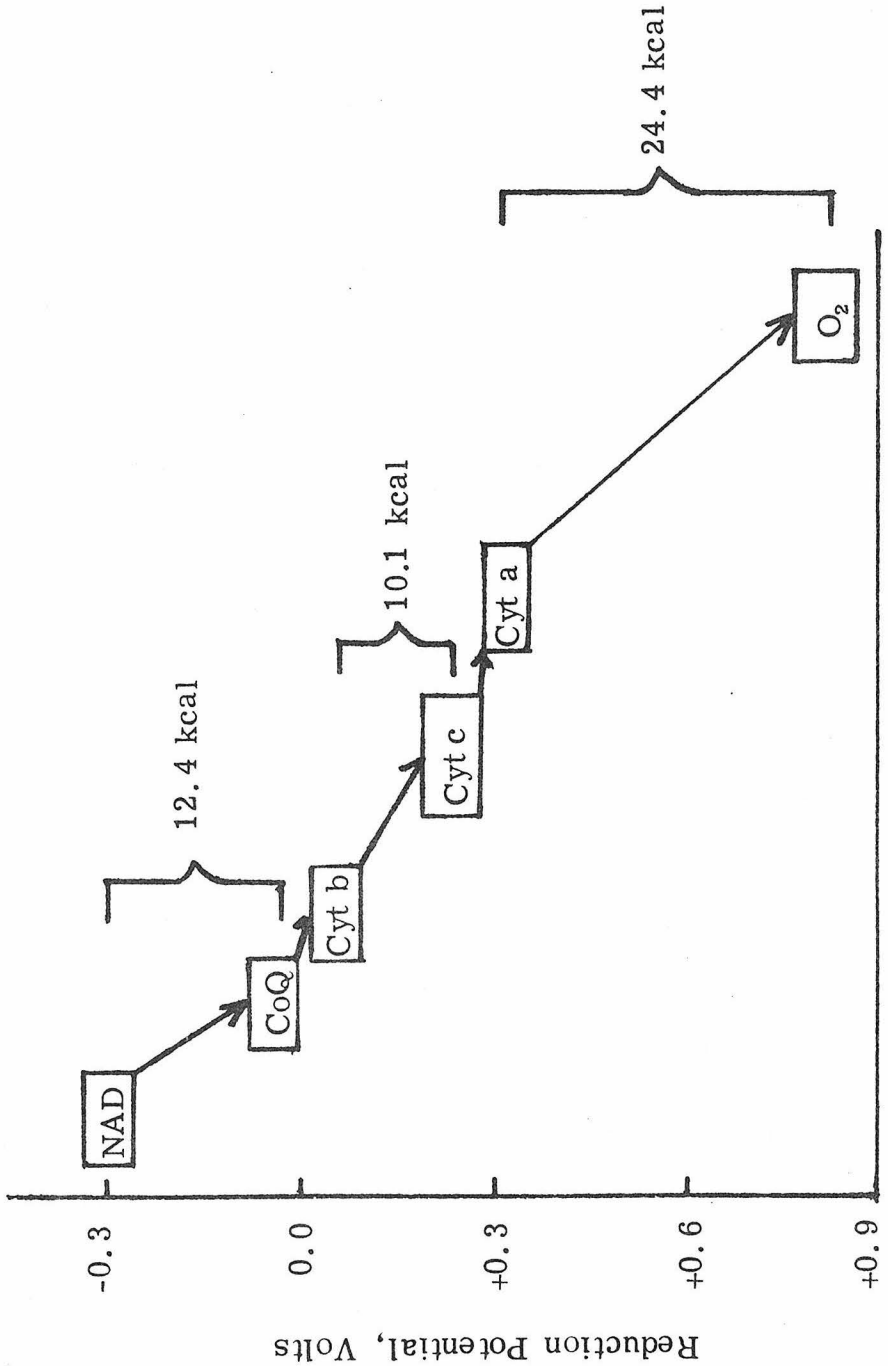
trees with phylogenetic trees constructed on other bases.

The position of cytochrome c in the respiratory chain is depicted schematically in Figure 3. At three positions along the chain, the drop in reduction potential is of such significance that the energy released may be conserved by the process of oxidative phosphorylation. ATP is the energy carrier that sustains life; thus, the study of any facet of ATP synthesis is potentially very rewarding.

The exact nature of the interaction of cytochrome c with either its oxidase or reductase has not been determined. It is known that cytochrome c is located at the outer surface of the inner membrane of mitochondria and is not membrane bound. In contrast, both the oxidase and reductase of cytochrome c are bound to the inner membrane and cannot be extracted from the mitochondria without significant loss in activity.[11,12,13] Thus cytochrome c in some manner acts as an electron shuttle between the immobile oxidase and reductase.

The tertiary structure of cytochrome c may contain valuable clues concerning the *in vivo* mechanism of these proteins. Therefore, the X-ray structure analysis of oxidized horse[14,15,16,17] and tuna[18] cytochrome c and of reduced tuna[19,20,21] and bonito[22,23,24,25] cytochrome c have been undertaken. The mechanistically most intriguing aspects of the tertiary structure of cytochrome c are a left hydrophobic channel and a right hydrophobic channel (as defined in reference 15) leading from the heme to the surface of the protein; a heme crevice between the left and right halves of the polypeptide chain with a heme pocket leading from it to the surface of the protein;

Figure 3. Components of the respiratory redox chain.



Flow of Electrons Along Respiratory Chain

an exposed edge of the porphyrin ring; and segregated areas of positive and negative charge at the surface of the protein. In addition, aromatic side chains tend to occur in pairs within the interior of the molecule. The heme is held in position by bonds through the sulfurs of cysteines 14 and 17, and the fifth and sixth ligand positions of iron are occupied by nitrogen of histidine 18 and sulfur of methionine 80. Each of these features is highly conserved through all species whose cytochrome c primary structure is known.[17]

The oxidized and reduced proteins vary only slightly.[20] Upon reduction, the heme group shifts slightly within the polypeptide framework, although it must be noted that an edge of the porphyrin ring remains exposed at the surface of the protein. The reduced protein also appears to be more compact.[26]

Elucidation of the tertiary structure of a number of cytochromes is the most important recent advance toward a thorough understanding of the structure-function relationship, which must be considered the primary goal in the study of cytochrome c. Yet the *in vivo* mechanism of electron transfer remains undetermined. Based on the results of experiments with antibodies produced in rabbits against human cytochrome c,[27,28,29] and with the chemically modified protein,[30,31,32,33,34] it has been proposed that the oxidase and reductase binding sites differ.[35] Further, the upper heme crevice region of cytochrome c has been associated with binding to the oxidase,[34] whereas the left side has been suggested as the reductase binding site.[36] Separate binding sites have been taken as evidence that two distinct electron transfer pathways do indeed exist.[33] Later

interpretations, however, indicate that the data from chemically modified cytochrome c do not support the idea of separate electron pathways for oxidation and reduction of the protein any more strongly than they support the idea of separate binding sites for the oxidase and the reductase and a common electron pathway for oxidation and reduction.[37]

Another method of arriving at the redox mechanism of cytochrome c is the kinetic study of reactions of inorganic electron transfer reagents with the protein. An important question regarding this approach has been raised by Dickerson[20]: "Can the in vivo electron transfer mechanism of cytochrome c be 'short circuited' during in vitro reactions involving metalloorganic complexes?" It seems that this can happen. But it also seems probable that an inorganic reagent will react with the protein by the simplest mechanism available to it. Thus far, no reason has been advanced why nature should not also. Therefore, if the inorganic reagent is subject to the same kinds of restrictions as the in vivo reagents, extrapolation of the results of experiments involving inorganic reagents to the in vivo mechanism may be rather straightforward. In fact, rate and activation parameters for the oxidation of ferrocytochrome c suspended in phospholipid bilayers[38] were found to be indistinguishable from rate and activation parameters for the oxidation of the protein in water solution.[39]

Mechanistic studies of ferricytochrome c reduction reactions have implicated attack at the heme and suggested that electron

transfer may proceed either at the exposed edge of the heme or via the heme crevice, depending on the properties of the reducing agent. Evidence for the latter reaction mode has come from recent kinetic and mechanistic investigations of the chromous ion reduction of ferricytochrome c.[40,41,42,43] Chromium(II) becomes bound to the cytochrome molecule[42] in the area of the heme crevice, and electron transfer involving tyrosine 67 has been suggested.[43] Chromium(II) attack at the crevice is consistent with the results of ligand binding studies which have emphasized the lability of the iron-methionine-80 bond.[44] Outer-sphere reduction of ferricytochrome c(III) has been accomplished using ferrocyanide[45] and the kinetics of the ferrocyanide-ferricytochrome c reaction have been interpreted as support for a heme edge electron transfer mechanism.[46] A more recent study has shown that iron hexacyanide may electrostatically associate with cytochrome c in the area of the heme edge, thus facilitating electron transfer.[47] Extremely low values of $\Delta H^{\ddagger}$ have been found for this system, suggesting complex formation and intramolecular electron transfer.[48] In the dithionite reduction, parallel pathways are operative.[49,50] One pathway involves direct "outer-sphere" or "remote" attack by $S_2O_4^{2-}$; the other pathway (which is less than first order in dithionite) is consistent with either "inner-sphere" or "adjacent" attack by dithionite[49] or with reduction by the SO_2^- radical.[49,50] Ferrous ethylenediaminetetraacetate[51,52] and hexammineruthenium(II)[53] have also been employed as outer-sphere reductants for cytochrome c(III). In both cases, support was again found for heme edge electron transfer, although the possibility of

attack at some site removed from this edge could not be excluded. Pulse radiolysis techniques have been used to generate small, highly reactive reducing species which mechanistically appear to be very non-specific.[54,55]

For the reduction of ferricytochrome c, attack from the heme crevice is rendered possible by the relative lability of the iron-sulfur bond and the accessibility of the crevice to the solution; in sharp contrast is the inertness of the Fe(II)-S bond in native ferrocytochrome c.[56] As a consequence, rapid oxidation of the reduced heme protein via direct adjacent attack on the iron(II) is unlikely and only heme-edge attack or electron transfer at another site removed from the iron remain as pathways for rapid oxidation. To date these predictions have not been verified to any great extent, owing in part to the dearth of oxidants which are tractable in the neutral pH range. Thus the need for kinetic studies of the oxidation of ferrocytochrome c is apparent.

To be presented in this work are the experimental results of a study of the kinetics of the oxidation of ferrocytochrome c by tris complexes of 1,10-phenanthroline and its derivatives[57] with cobalt(III). By modifying the outer edges of the tris-(1,10-phenanthroline)cobalt(III) ion, we hope to be able to learn through kinetic studies something about the steric requirements and the mechanism of the oxidation of ferrocytochrome c. In addition, information may be gained concerning the detailed description of the structure-function relationship for cytochrome c.

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EXPERIMENTAL

I. Preparation of Reagents

Reagent grade chemicals were used throughout. Deionized distilled water was used in the preparation of all solutions used for synthetic or kinetic experiments. Nitrogen gas was passed through two chromous scrubbing towers to remove oxidizing impurities. Horse heart cytochrome c (Type VI) obtained from Sigma Chemical Co. was used without further purification. Cytochrome c_{551} from Pseudomonas aeruginosa was prepared according to the method of Ambler and Wynn.[1] Dr. R. C. Rosenberg was responsible for growing the bacteria and performing the initial steps of the extraction.

Solutions of reduced protein were prepared by adding a twenty-fold excess of $Fe(EDTA)^{2-}$ to nitrogen-saturated, buffered solutions of the oxidized protein. Excess $Fe(EDTA)^{2-}$ and $Fe(EDTA)^{1-}$ were removed from the reduced protein solutions using the hollow fiber Dow Beaker Dialyzer obtained from Bio-Rad Laboratories. Protein solutions of 75 to 100 mls were dialyzed under a constant flow of nitrogen against approximately 1.75 liters of nitrogen-saturated buffer solution of ionic strength 0.1 M. Alternatively, reduced protein solutions were purified using Sephadex G-25 Gel Filtration Beads obtained from Sigma Chemical Co. The reduced protein was loaded onto the column, eluted with the appropriate buffer, and diluted to the desired volume.

The $Fe(EDTA)^{2-}$ solutions used to prepare ferrocytochrome c were made by combining aliquots of ferrous chloride solution with buffered solutions containing a 20% excess over the stoichiometric amount of Na_2H_2EDTA. The ferrous chloride solutions were prepared by dissolving high purity iron wire (Allied Chemical) in excess HCl under a stream of nitrogen. The Fe(II) concentration of the ferrous chloride solutions was determined by transferring aliquots into excess Ce(IV) and back titrating to a ferroin end point with As(III).[2]

Tris complexes of 1,10-phenanthroline and its derivatives with Co(III) were prepared by a method analogous to that of Pfeiffer and Werdelmann,[3] and characterized spectrally in the region 380-210 nm.[4] The $Co(terpy)_2Cl_2 \cdot H_2O$ was prepared according to the method of Baker, et al.[5] The $Ru(NH_3)_5py(PF_6)_2$ was prepared according to the method of Ford, et al.[6] as modified by Diane Cummins. The $Ru(NH_3)_5py(PF_6)_2$ was a generous gift from Dr. Cummins.

II. Analyses

The absorbance ratio A_{521}/A_{551} for cytochrome c_{551} at the conclusion of the extraction procedure[1] was 0.58, compared to the published value of 0.58 to 0.62 for the purified protein.[7] Polyacrylamide gel electrophoresis of the purified protein resulted in a single band with a calculated molecular weight of 9500 ± 10%, which agrees well with literature values.[8] Barry Rothman of the Division of Biology very kindly performed the gel electrophoresis experiment.

The substituted 1,10-phenanthroline complexes with cobalt were analyzed by Schwarzkopf Microanalytical Laboratory. The analytical data are summarized in Table I. In addition, spectral data were used to characterize each complex. Table II contains molar extinction coefficients for local maxima and minima in the ultraviolet absorption spectrum of each complex.

The $Co(terpy)_2Cl_2 \cdot H_2O$ and $Ru(NH_3)_5py(PF_6)_2$ complexes were characterized according to the spectra published in reference 5 and reference 6, respectively.

III. Preparation of Solutions

Buffered solutions in the range pH 6-9 were used for the kinetic measurements. Type VI cytochrome c solutions were stored in nitrogen-purged, serum-capped bottles. Nitrogen was passed only slowly through the cytochrome c solutions or above them to prevent protein denaturation. Buffers were prepared to contribute 0.05 M to the ionic strength of the solutions. Both reactant solutions contained identical buffering systems, except any reaction that involved $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$. It was found that, after a period of time, buffered solutions of $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ became cloudy. It was therefore necessary to store $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ in deionized distilled water and to perform buffer jump experiments when $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ was involved. In such cases, the buffer of the reductant was prepared to give the proper pH and ionic strength after mixing of the reactant solutions.

Table I

	Co	C	N	H
$Co(5\text{-}Cl\text{-}1,10\text{-}phen)_3Cl_3 \cdot 5H_2O$ (MW = 899.28)				
% calculated	6.55	48.08	9.35	3.48
% found	6.11	48.60	9.50	3.86
$Co(5,6\text{-}Me_2\text{-}1,10\text{-}phen)_3Cl_3 \cdot 7H_2O$ (MW = 916.12)				
% calculated	6.43	55.01	9.18	5.50
% found	6.46	55.12,54.82	9.21	5.36
$Co(4,7\text{-}Me_2\text{-}1,10\text{-}phen)_3Cl_3 \cdot 14H_2O$ (MW = 1042.1)				
% calculated	5.65	48.40	8.06	6.20
% found	5.05	48.08	8.50	5.98
$Co(4,7\text{-}[\phi\text{-}SO_3Na]_2\text{-}1,10\text{-}phen)_3Cl_3 \cdot 8H_2O$ (MW = 1918.9)				
% calculated	3.07	45.06	4.38	3.05
% found	3.07	43.83	4.40	3.96

Table II. Extinction Coefficients of Substituted 1,10-Phenanthroline Complexes of Cobalt(III)

Complex	λ nm	$10^{-4}\epsilon$ $M^{-1}cm^{-1}$
$Co(5\text{-}Cl\text{-}phen)_3^{3+}$	270.5	8.87
	247.5	2.54
$Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$	282.0	8.33
	274.5	8.96
	251.0	3.81
$Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$	288.5	6.50
	263.0	2.91
$Co(4,7\text{-}[\phi\text{-}SO_3]_2\text{-}phen)_3^{3+}$	293.0	12.0
	257.5	3.84

The solutions of complexes of cobalt with the various phenanthrolines were stored in serum-capped, round bottom flasks fitted with a nitrogen inlet tube and a glass luer-lock fitting, thus allowing introduction of the solutions into the stopped-flow apparatus through an all glass and Kel-F Teflon system. Earlier work showed that stainless steel needles had to be avoided when handling $Co(phen)_3^{3+}$. Both reactant solutions were nitrogen-saturated for at least fifteen minutes prior to introduction into the stopped-flow machine. Solutions of the tris complexes of substituted 1,10-phenanthroline and cobalt were made by weight, although in some earlier work $Co(phen)_3^{3+}$ solutions were prepared by diluting various amounts of stock solution of $Co(phen)_3^{3+}$.

The $Co(terpy)_2^{2+}$ and $Ru(NH_3)_5py^{2+}$ solutions were prepared by weight to give suitable changes in absorbance upon oxidation.

IV. Kinetic Measurement and Data Analysis

The kinetics of oxidation of cytochrome c were followed at 550 nm ($\Delta\epsilon = 18.5 \times 10^3\ M^{-1}cm^{-1}$ [9]). The kinetics of oxidation of $Co(terpy)_2^{2+}$ were followed at 510 nm ($\Delta\epsilon = 1.4 \times 10^3\ M^{-1}cm^{-1}$ [10]), and the kinetics of oxidation of $Ru(NH_3)_5py^{2+}$ were followed at 428 nm ($\Delta\epsilon = 3.75 \times 10^3\ M^{-1}cm^{-1}$ [6]). The concentration of the oxidant was varied between 9.0×10^{-5} and 4.0×10^{-3} M and always kept in large excess over the reductant. At least four kinetic runs were performed with each oxidant solution.

A Durrum Model 110 stopped-flow spectrophotometer was used in all kinetic experiments. The flow subsystem of the

instrument was all glass or Kel-F Teflon, permitting safe handling of the phenanthroline complexes. The light source was a tungsten lamp with a grating monochromator. The power supply for the tungsten lamp was a Power/Mate, while the photomultiplier tube was powered by a Kepco Model ABC 1500 (M) power supply.

The reagent solutions before mixing sat in glass syringes immersed in water. A Forma Scientific temperature control unit circulated the water at a selected temperature. At least fifteen minutes were allowed for temperature equilibration for runs done near room temperature (20-25°), and at least thirty minutes were allowed for runs done at other temperatures.

Electron transfer reactions which are first order in each reactant follow the rate law

$$\frac{-d[\text{red}]}{dt} = k[\text{red}][\text{ox}] \qquad (1)$$

where [red] represents the concentration of the reducing agent, and [ox] is the concentration of the oxidizing agent. By maintaining $[\text{ox}] \gg [\text{red}]$, it is assured that [ox] does not change significantly during the course of the reaction. Thus equation (1) may be treated as follows:

$$\frac{-d[\text{red}]}{dt} = k[\text{red}][\text{ox}] = k_{obsd}[\text{red}] \qquad (2)$$

$$\frac{-d[red]}{[red]} = k_{obsd}dt \tag{3}$$

$$-\int_{[red]_o}^{[red]} \frac{d[red]}{[red]} = k_{obsd} \int_o^t dt \tag{4}$$

In equation (4), the limits of integration are $[red] = [red]_o$ at $t = 0$ and $[red] = [red]$ at $t = t$.

$$\ln \left(\frac{[red]_o}{[red]}\right) = k_{obsd}\, t \tag{5}$$

Any physical parameter, such as absorbance, which is linearly proportional to concentration may be used to follow a reaction kinetically. Actually, output voltage from the photomultiplier was used. However, output voltage is proportional to absorbance and is thus proportional to concentration. Therefore,

$$v_t - v_{t=\infty} = p[red] \tag{6}$$

where v_t = photomultiplier output voltage at time t

p = proportionality constant

$$\ln(v_t - v_{t=\infty}) = \ln(v_{t=o} - v_{t=\infty}) - k_{obsd}t \tag{9}$$

A plot of $\ln(v_t - v_{t=\infty})$ vs t thus gives a straight line with a slope of $-k_{obsd}$.

Photomultiplier output voltages were sent to a Tektronix 564B storage oscilloscope to be displayed against time, and were also sent to an analog to digital converter. The A/D converter, constructed by James Elliot of the Caltech Computing Center, transmitted accumulated

data to the Caltech PDP-10 computer. A computer program entitled Interactive Data Collector (IDC), written by Tom Dailey of the Caltech Computing Center, made possible reduction of the data by the computer. In addition to storing the data, IDC was used to label data files with reaction condition descriptions, type the numerical data in tabular form, plot the data in the form $\ln(v_t - v_{t=\infty})$ vs t, and to calculate the least squares slope of a data plot. IDC was not used to calculate k_{obsd} for reactions involving $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$. Because of the problem of clouding mentioned above, reliable absorbance values for the completed reaction could not be obtained. In such cases, the Guggenheim method[11] was used to calculate k_{obsd}.

V. Other Equipment

All spectral data were measured on a Cary 17 spectrophotometer. A Brinkman pH 101 instrument was used in all pH determinations. The composition of precipitated metal complexes was checked with a DuPont 950 thermogravimetric analyzer.

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RESULTS AND DISCUSSION

I. Oxidation of Horse Heart Cytochrome c by $Co(phen)_3^{3+}$

First-order plots of the absorbance-time data observed at 550 nm are linear for greater than 90% of the reaction. The first-order dependence of observed rate constants on the $Co(phen)_3^{3+}$ concentration is illustrated in Figure 1. The least-squares slope of the data of Figure 1 gives a second-order rate constant $k = (1.50 \pm 0.05) \times 10^3$ M^{-1} sec^{-1} for the oxidation of Type VI horse heart ferrocytochrome c in 0.05 M pH 7.0 phosphate buffer of ionic strength 0.1 M at 25°. Under all conditions reported in this work, the rate law obtained for the oxidation of ferrocytochrome c is

$$\frac{-d[\text{cyt c(II)}]}{dt} = k[Co(phen)_3^{3+}][\text{cyt c(II)}]. \qquad (1)$$

Table I gives the second-order rate constants found at various pH values in phosphate and tris buffers for horse heart cytochrome c. The activation parameters at pH 7.0 in phosphate buffer obtained from the plot of log(k/T) vs. 1/T shown in Figure 2 are $\Delta H^{\ddagger} = 11.3$ kcal mol^{-1} and $\Delta S^{\ddagger} = -6$ cal deg^{-1} mol^{-1}.

The second-order rate constants obtained for the oxidation of horse heart ferrocytochrome c by $Co(phen)_3^{3+}$ are seen to be nearly independent of pH. No evidence was found for biphasic kinetics at pH 9 (550 nm), as has been observed in the reduction of ferricytochrome c.[1,2,3,4] The kinetic behavior of the reduction reaction

Figure 1. Plot of k_{obsd} vs $[Co(phen)_3^{3+}]$ for the oxidation of horse heart Type VI ferrocytochrome c [25°, pH = 7.0 (phosphate), μ = 0.1 M (NaCl)]. The reaction was followed at 550 nm. Points plotted in all figures represent averages of at least four data points.

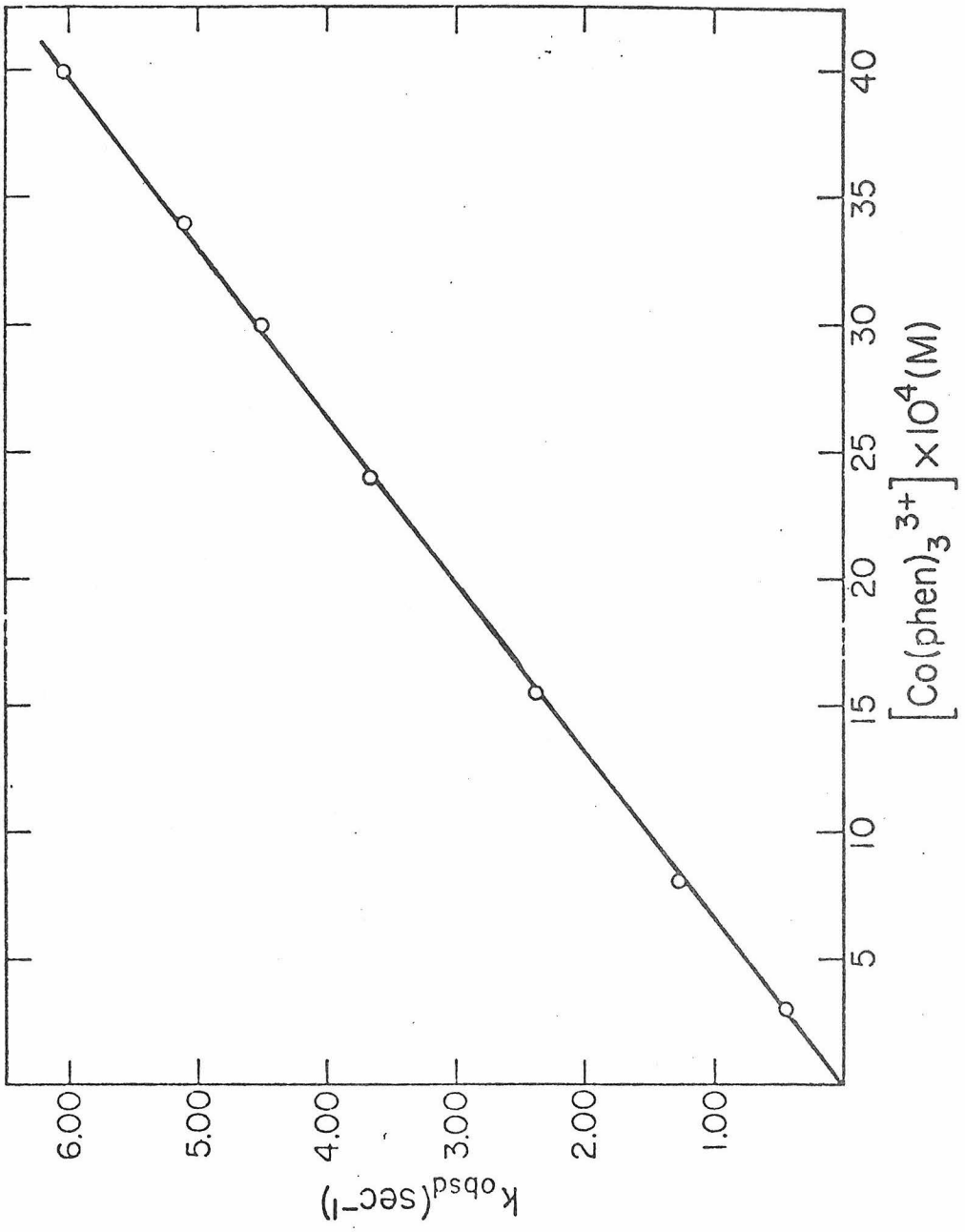
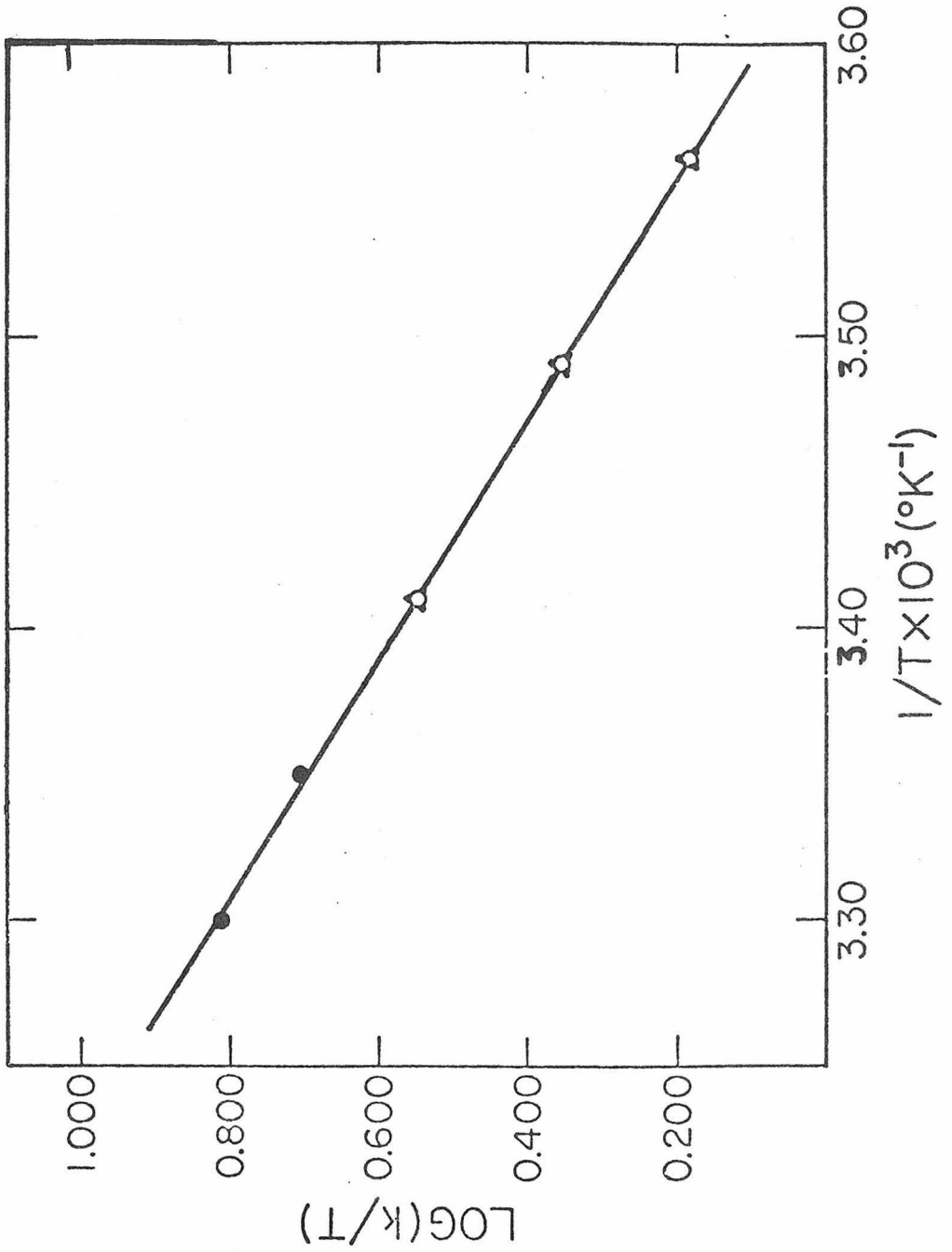

6.00
5.00
4.00
3.00
2.00
1.00
$k_{obsd}(sec^{-1})$
5
10
15
20
25
30
35
40
$[Co(phen)_3^{3+}] \times 10^4 (M)$

Figure 2. Eyring plot of rate data for the oxidation of horse heart Type VI ferrocytochrome c by $Co(phen)_3^{3+}$ [pH = 7.0 (phosphate), μ = 0.1 M (NaCl)]. ○, $[Co(phen)_3^{3+}] = 2.4 \times 10^{-3}$ M; Δ, $[Co(phen)_3^{3+}] = 3.0 \times 10^{-3}$ M; ●, $[Co(phen)_3^{3+}] = 3.4 \times 10^{-3}$ M.


LOG(k/T)
1.000
0.800
0.600
0.400
0.200
3.30
3.40
3.50
3.60
1/T X 10³ (°K⁻¹)

at high pH has been interpreted[1-5] in terms of the presence of a high pH isomer,[6] possibly containing a lysine 79 as sixth ligand,[7,8] in addition to the native, methionine 80-ligated protein. The failure to observe biphasic kinetics at 550 nm is not inconsistent with previous observations, as at this wavelength the absorbance difference between the lysine- and methionine-bound forms of ferricytochrome c is minimal.[5] In fact, kinetic runs utilizing 695 nm as monitoring wavelength clearly show both the oxidation and isomerization steps. After an initial increase in absorbance at 695 nm owing to oxidation of ferrocytochrome c to native ferricytochrome c, spectral changes corresponding to isomerization of the protein were observed. The initial phase of the reaction was analyzed by the Guggenheim method,[9] yielding values for k_{obsd} at 695 nm. A plot of k_{obsd} (695 nm) vs. $[Co(phen)_3^{3+}]$ gave a second-order rate constant of 1.77×10^3 M^{-1} sec^{-1}, in excellent agreement with the value found at 550 nm (Table I). The isomerization reaction is independent of the concentration of $Co(phen)_3^{3+}$ (0.75 to 4.0×10^{-3} M) and is characterized by a rate constant k(native → high pH) of 0.071 sec^{-1}. Our value of 0.071 sec^{-1} accords well with the first-order rate constant for isomerization obtained by Wilson and Greenwood at pH 9 (20°).[4]

The rate of oxidation of horse heart ferrocytochrome c by $Co(phen)_3^{3+}$ increases with increasing ionic strength at pH 7, as would be expected for a reaction between positively charged species. Theory predicts a linear relationship between log k and $\sqrt{\mu}$ for ionic strengths below 0.01 M.[9] Although the interval $0.06 \leq \mu \leq 0.20$ M we have examined is well above the upper limit demanded by theory, a plot of

Table I. Rate Constants (k) for the Oxidation of Horse Heart Ferrocytochrome c by Tris(1,10-phenanthroline)cobalt(III) at 25° and μ = 0.1 M

pH	Buffer	10^{-3} k, M^{-1} sec^{-1} (550 nm)
6.0	41 mM phosphate	1.01
7.0	24 mM phosphate	1.50
7.0	54 mM tris	1.28
7.8	18 mM phosphate	1.65
9.0	414 mM tris	1.82
9.0	414 mM tris	1.77[a]

[a]Reaction followed at 695 nm.

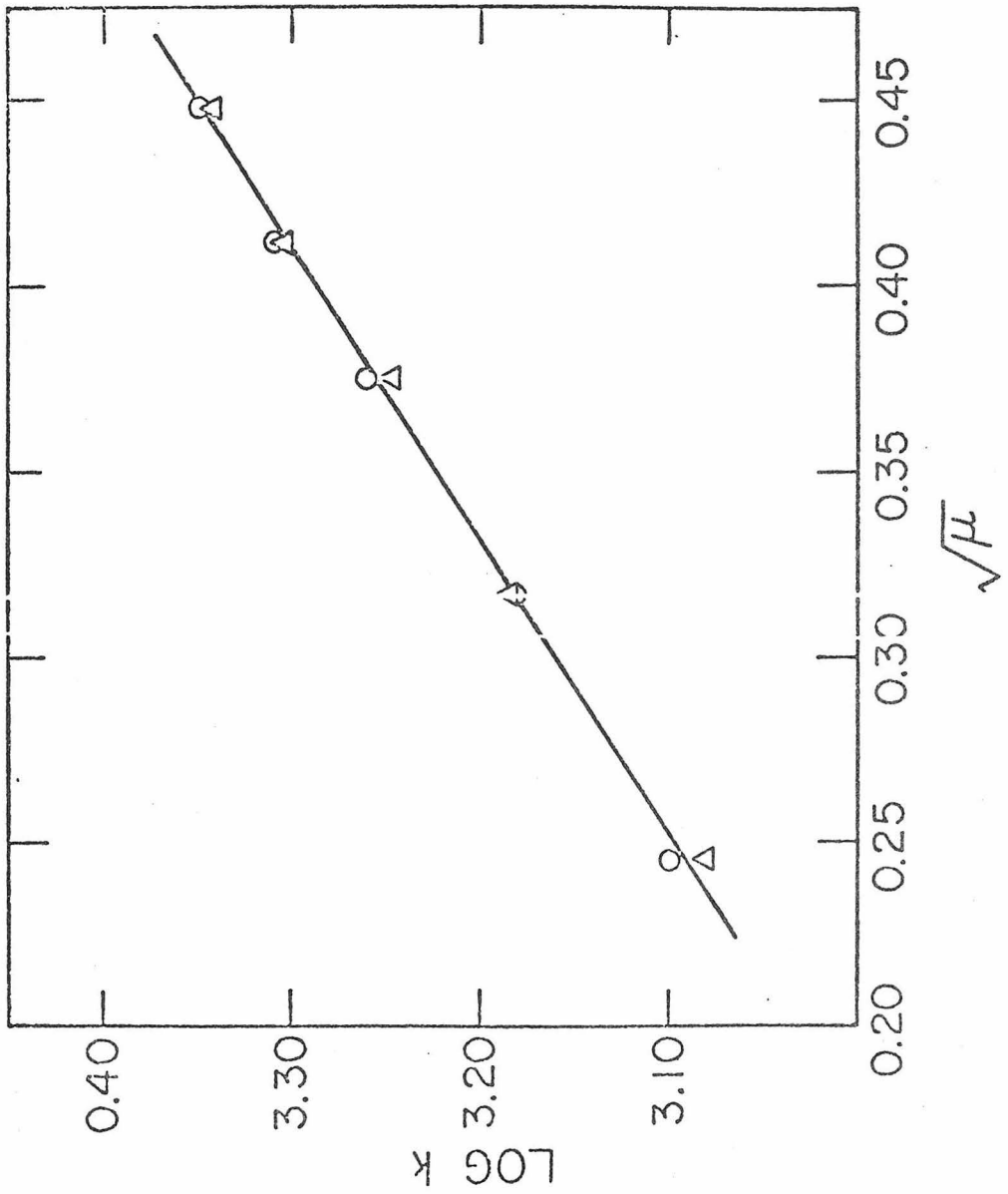
log k vs $\sqrt{\mu}$ nevertheless yields an excellent straight line (Figure 3). In earlier work, Hodges and coworkers obtained a linear log k vs $\sqrt{\mu}$ plot for the reduction of ferricytochrome c by $Fe(EDTA)^{2-}$ and estimated an "active site charge" of +1.7 for the oxidized protein. An analogous calculation based on the data presented in Figure 3 gives an "active site charge" of +0.4 for ferrocytochrome c. The exact values cannot of course be taken seriously, but it is clear that they are much smaller than the overall charge of about +7 [2] on the protein in neutral solution. It is also interesting that the "active site charge" is approximately one unit less positive for ferrocytochrome c.

The oxidant $Co(phen)_3^{3+}$ is known to behave according to the Marcus theory for outer-sphere electron transfer in some,[10,11] but not all,[12-17] of its redox reactions. Typical examples of good behavior include the oxidation of $Co(terpy)_2^{2+}$ [11] and V_{aq}^{2+},[10] where theory and experiment are in reasonably close agreement. In addition, the calculated rate constant for the reaction between Fe_{aq}^{3+} and $Co(phen)_3^{2+}$ is within a factor of 3 of the experimental value.[10] The theoretical expression for the rate constant (k_{12}) of the ferrocytochrome c-$Co(phen)_3^{3+}$ reaction is [18]

$$\log k_{12} = 0.5[\log k_{11} + \log k_{22} + 16.9\,\Delta E_{12}^{\circ}] \qquad (2)$$

where k_{11} and k_{22} are the appropriate self-exchange rate constants. The horse heart cytochrome c self-exchange rate is in the range $(0.2\text{-}1.0) \times 10^3\ \underline{M}^{-1}\ sec^{-1}$ at pH 7.0, $\mu = 0.1\ \underline{M}$, and 25°,[19-21] and the $Co(phen)_3^{2+/3+}$ self-exchange rate, extrapolated to 25° from the data of Baker, et al.,[22] is $2.1 \times 10^1\ \underline{M}^{-1}\ sec^{-1}$. Taking reduction

Figure 3. Plot of log k vs $\sqrt{\mu}$ for the oxidation of horse heart Type VI ferrocytochrome c by $Co(phen)_3^{3+}$ [25°, pH = 7.0 (phosphate).] ○, $[Co(phen)_3^{3+}] = 1.5 \times 10^{-3}$ M; Δ, $[Co(phen)_3^{3+}] = 3.0 \times 10^{-3}$ M.


LOG k
0.40
3.30
3.20
3.10
0.20
0.25
0.30
0.35
0.40
0.45
$\sqrt{\mu}$

potentials for the $Co(phen)_3^{2+/3+}$ and cytochrome c(II)/(III) couples as +0.42[23] and +0.261 V,[24] respectively, we calculate k_{12} to fall in the range $(1.2\text{-}2.7) \times 10^3$ $\underline{M}^{-1}$ sec^{-1} for the horse heart reaction, which is in rather good agreement with experiment. Using the same data for the cytochrome c(II)/(III) self-exchange rate and redox couple, Ewall and Bennett[25] have reported an equally successful Marcus calculation of the rate of reduction of ferricytochrome c by $Ru(NH_3)_6^{2+}$ [calculated: $(3\text{-}6) \times 10^4$ $\underline{M}^{-1}$ sec^{-1}; found: 3.78×10^4 $\underline{M}^{-1}$ sec^{-1}]. As a further check on the interpretation, it should be noted that the rate of oxidation of $Ru(NH_3)_6^{2+}$ by $Co(phen)_3^{3+}$ is also in fair agreement with the Marcus relation [calculated: 1×10^5 $\underline{M}^{-1}$ sec^{-1}; found: $1\text{-}2 \times 10^4$ $\underline{M}^{-1}$ sec^{-1}].[26]

It is important to note that neither reduction nor oxidation of cytochrome c by outer-sphere reagents would be expected to yield rate constants which are predictable by the Marcus theory if the electron transfer mechanism in question were radically different from that employed in the self-exchange reaction. It should also be noted that since $\log k_{12} - \log k_{21} = 16.9\ \Delta E_{12}^0$, reversibility of the cross-reaction is implicit in eq. 2. Thus, if the forward rate satisfies eq 2, then the reverse rate must also, and vice versa. An apparent breakdown of reversibility can occur if certain equilibria or conformation changes take place that are not sufficiently rapid, in which case conformity to eq 2 must to some extent have been accidental in the first place. The fact that excellent agreement with eq 2 has been obtained for both a reduction and an oxidation reaction lead to the

conclusion that, in these cytochrome c systems, electron transfer to and from the heme iron of the protein follows the same pathway as in the self-exchange reaction.

The simplest and most straightforward interpretation of the kinetic studies both of the protein self-exchange and the reaction of ferricytochrome c with model outer-sphere reductants is that electron transfer occurs via the exposed heme edge.[2,25,27] The present experiments on the oxidation of ferrocytochrome c from horse heart by tris-(1,10-phenanthroline)cobalt(III) are also most easily reconciled with a mechanistic model featuring outer-sphere electron transfer utilizing contact between the heme edge and one of the phenanthroline rings. Indeed, given an edge-edge mechanism for the self-exchange reaction, edge transfer to $Co(phen)_3^{3+}$ must be the path of choice in order to understand the excellent Marcus-theory correlation. In view of all the results on model systems, then, it is reasonable to propose that the remote-attack reactions utilize a common heme-edge site for both oxidation and reduction of cytochrome c. This model is in contrast to the proposal that electron transfer takes place in vivo at separate oxidase and reductase binding sites.[12,28-31] However, it is difficult to understand why a common heme-edge pathway should not also be employed in vivo, unless access to the edge is blocked as a consequence of binding to the membrane or to the oxidase or reductase.

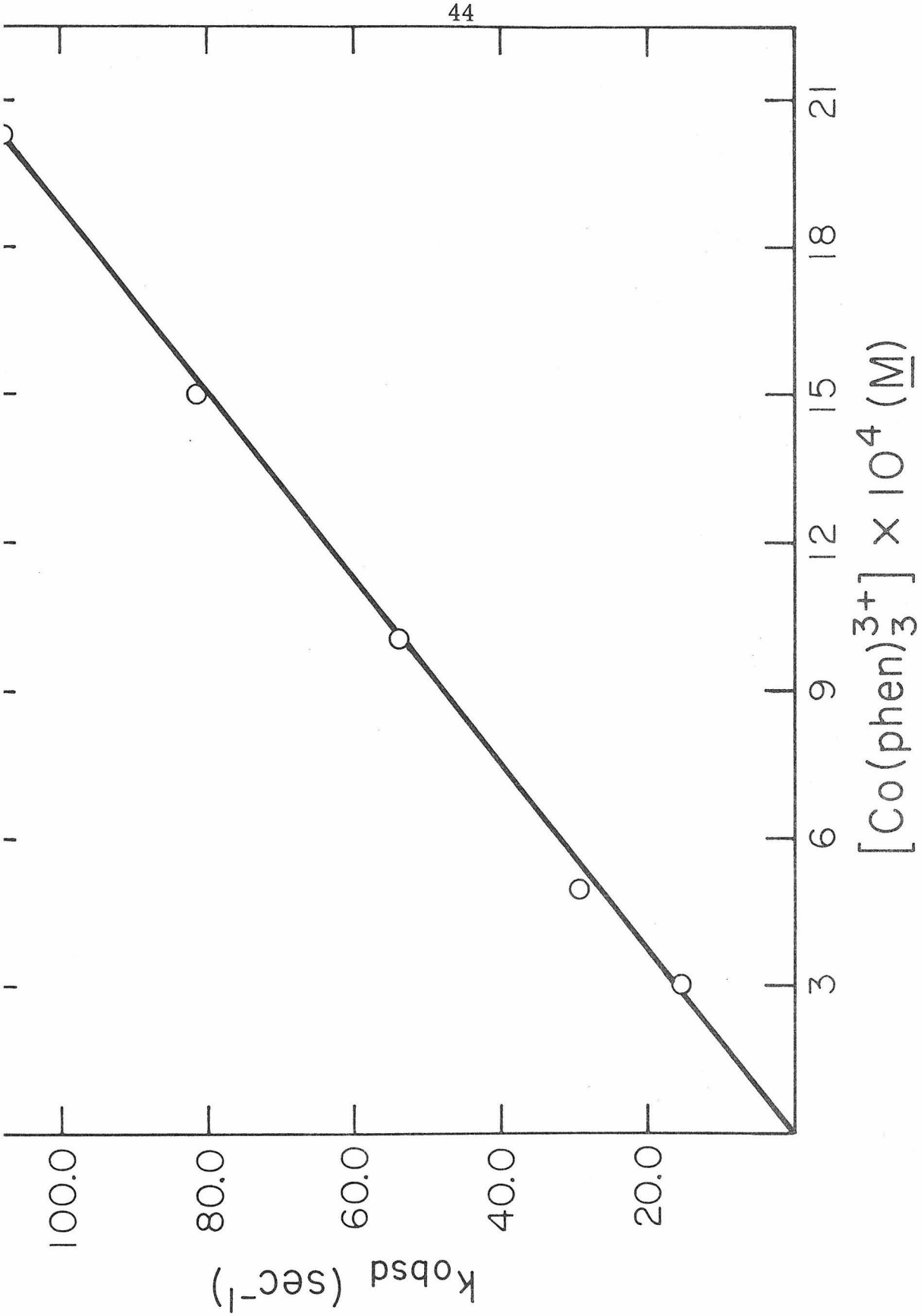
II Oxidation of Cytochrome c_{551} by $Co(phen)_3^{3+}$

Following the successful study of the oxidation of horse heart cytochrome c, it was decided that oxidation studies should be performed on a second c-type cytochrome. It was hoped that the path of electron transfer from the protein could be further defined by correlating any reactivity differences that might be found with the known differences in the structures of the proteins. The idea of treating cytochrome from one species as a modification of cytochrome from another was first proposed by Kamen.

Cytochrome c_{551} from the bacteria Pseudomonas aeruginosa has the same reduction potential[32] as cytochrome c from horse heart, but has a polypeptide chain of only 82 amino acids[33] compared to 104 for horse heart cytochrome c. In addition, cytochrome c_{551} has a net negative charge.[33] Because the two proteins are equipotential, electron transfer reactivity differences between them must be related to structural differences. Thus, cytochrome c_{551} was chosen to be investigated through the kinetics of oxidation by $Co(phen)_3^{3+}$.

First-order plots of the absorbance-time data observed at 551 nm are linear for greater than 90% of the reaction. The first-order dependence of observed rate constants on the $Co(phen)_3^{3+}$ concentration is illustrated in Figure 4. The least squares slope of the data of Figure 4 gives a second order rate constant $k = (5.30 \pm 0.05) \times 10^4$ M^{-1} sec^{-1} for the oxidation of ferrocytochrome c_{551} in 0.05 M pH 7.0 phosphate buffer of ionic strength 0.1 M at 25°. Under all conditions reported in this work, the rate law obtained for the oxidation of

Figure 4. Plot of k_{obsd} vs $[Co(phen)_3^{3+}]$ for the oxidation of *P. aeruginosa* ferrocytochrome *c* [25°, pH = 7.0 (phosphate), μ = 0.1 M (NaCl)].


k_{obsd} (sec^{-1})
100.0
80.0
60.0
40.0
20.0
3
6
9
12
15
18
21
[Co(phen)$_3^{3+}$] X 10^4 (M)

ferrocytochrome $\underline{c}_{551}$ is

$$\frac{-d[\text{cyt } \underline{c}_{551}(II)]}{dt} = k[\text{Co(phen)}_3^{3+}][\text{cyt } \underline{c}_{551}(II)]. \qquad (3)$$

Table II gives the second-order rate constants found at various pH values in phosphate and tris buffers for the oxidation of ferrocytochrome $\underline{c}_{551}$. The second-order rate constants obtained for the oxidation of ferrocytochrome $\underline{c}_{551}$ by $Co(phen)_3^{3+}$ are seen to be nearly independent of pH. The activation parameters at pH 7.0 in phosphate buffer obtained from the plot of log (k/T) vs 1/T shown in Figure 5 are $\Delta H^{\ddagger} = 12.3 \pm 0.5$ kcal mole^{-1} and $\Delta S^{\ddagger} = +4 \pm 1$ cal degree^{-1} mole^{-1}.

The rate of oxidation of ferrocytochrome $\underline{c}_{551}$ by $Co(phen)_3^{3+}$ decreases with increasing ionic strength at pH 7.0, as would be expected for oppositely charged species.[9] The data are presented in Figure 6 and give an "active site charge" of -0.4 for ferrocytochrome $\underline{c}_{551}$. As was found for horse heart ferrocytochrome $\underline{c}$, the estimated charge at the active site is near zero and of much smaller magnitude than the overall charge of the protein in neutral solution.

Although the self-exchange rate of cytochrome $\underline{c}_{551}$ has not been determined, it may be calculated from the rate of oxidation of ferrocytochrome $\underline{c}_{551}$ by ferricytochrome $\underline{c}$ from horse heart. The theoretical expression relating the self-exchange rates k_{11} and k_{22} and the reduction potentials of the reagents with the cross reaction rate k_{12} according to the Marcus theory for outer-sphere electron transfer is[18]

Table II. Rate Constants (k) for the Oxidation of P. aeruginosa Ferrocytochrome c_{551} by Tris(1,10-phenanthroline)cobalt(III) at 25° and $\mu = 0.1$ M.

pH	Buffer	10^{-4} k, M^{-1} sec^{-1} (551 nm)
6.0	41 mM phosphate	3.67
7.0	24 mM phosphate	5.30
7.0	54 mM tris	5.36
7.8	18 mM phosphate	9.44
9.0	414 mM tris	8.52

Figure 5. Eyring plot of rate data for the oxidation of P. aeruginosa ferrocytochrome c_{551} by $Co(phen)_3^{3+}$ [pH = 7.0 (phosphate), μ = 0.1 M (NaCl)]. ●, $[Co(phen)_3^{3+}] = 2.0 \times 10^{-3}$ M; ○, $[Co(phen)_3^{3+}] = 1.0 \times 10^{-3}$ M; ×, $[Co(phen)_3^{3+}] = 3 \times 10^{-4}$ M; Δ, $[Co(phen)_3^{3+}] = 5 \times 10^{-4}$ M.

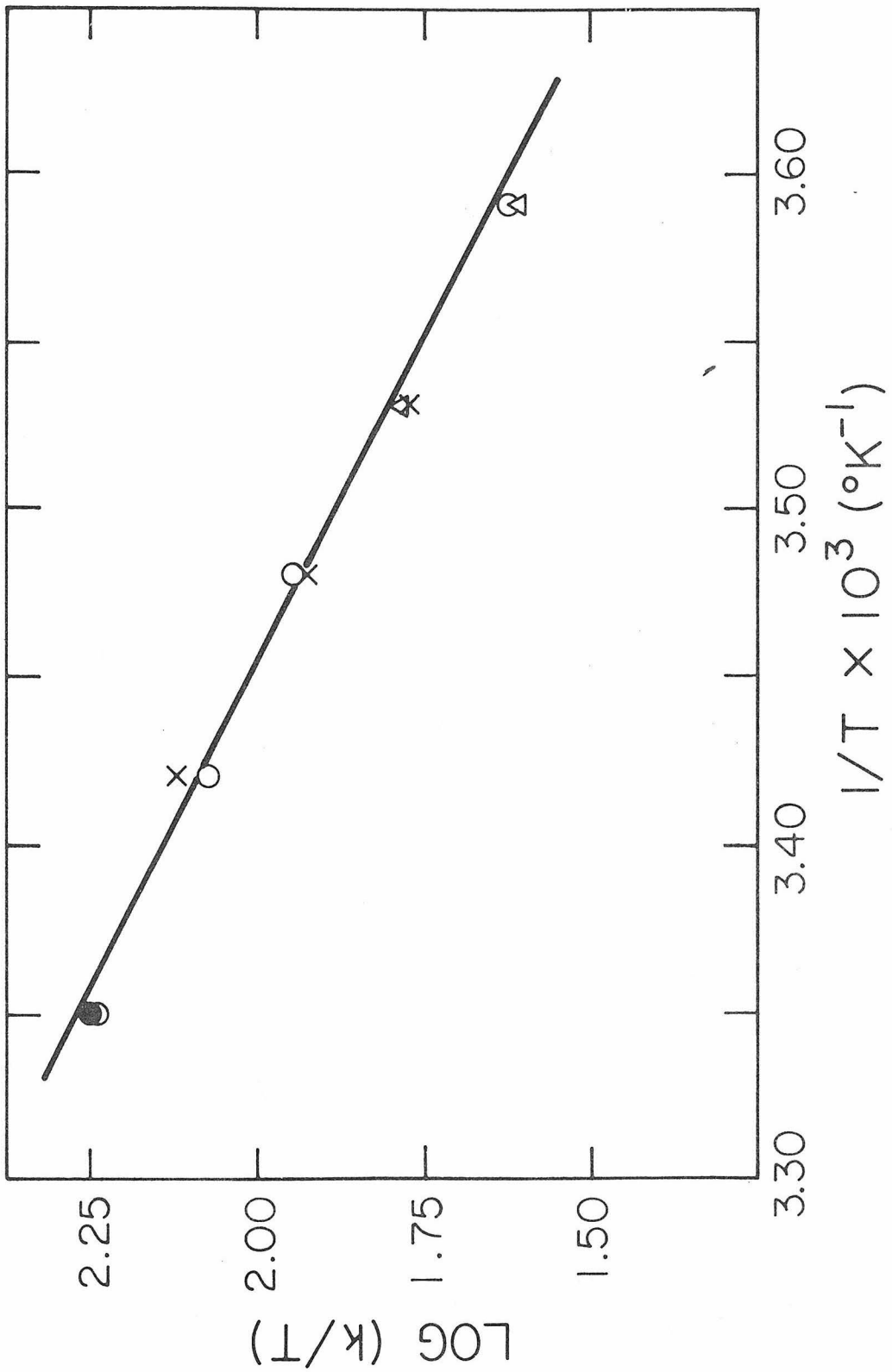
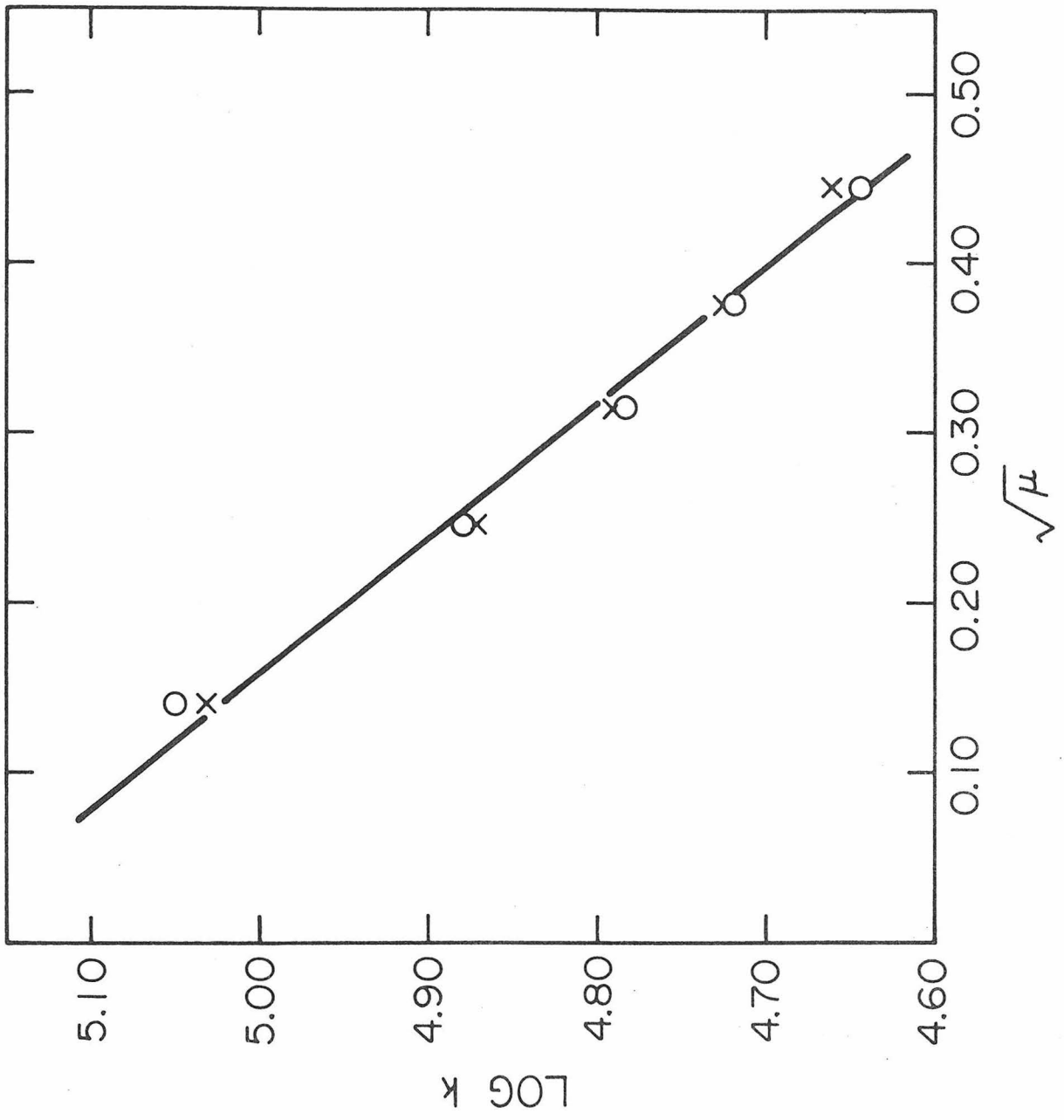

LOG (k/T)
2.25
2.00
1.75
1.50
3.30
3.40
3.50
3.60
1/T X 10^3 (°K^-1)

Figure 6. Plot of log k vs $\sqrt{\mu}$ for the oxidation of P. aeruginosa ferrocytochrome c_{551} by $Co(phen)_3^{3+}$ [25°, pH = 7.0 (phosphate).] ○, $[Co(phen)_3^{3+}] = 3 \times 10^{-4}$ M; ×, $[Co(phen)_3^{3+}] = 6 \times 10^{-4}$ M.


LOG k
5.10
5.00
4.90
4.80
4.70
4.60
0.10
0.20
0.30
0.40
0.50
$\sqrt{\mu}$

$$\log k_{12} = 0.5[\log k_{11} + \log k_{22} + 16.9\,\Delta E^{\circ}]. \qquad (2)$$

Taking $k_{12} = 7.1 \times 10^{4}\ \underline{M}^{-1}\ sec^{-1}$ (reference 34), $\Delta E^{\circ} = 0$, and k_{11} for horse heart cytochrome c to be $2.1 \times 10^{2}\ \underline{M}^{-1}\ sec^{-1}$ (references 19-21, 35), k_{22} is calculated to be $2.4 \times 10^{7}\ \underline{M}^{-1}\ sec^{-1}$. The rate constant for the cross reaction of ferrocytochrome c_{551} with $Co(phen)_3^{3+}$ may now be calculated. The $Co(phen)_3^{2+/3+}$ self-exchange rate, extrapolated to 25° from the data of Baker, et al.,[22] is $2.1 \times 10^{1}\ \underline{M}^{-1}\ sec^{-1}$. Taking reduction potentials for the $Co(phen)_3^{2+/3+}$ and cytochrome c_{551}(II)/(III) couples as +0.42[23] and +0.26V,[32] respectively, k_{12} is calculated to be $5.0 \times 10^{5}\ \underline{M}^{-1}\ sec^{-1}$, which is in fair agreement with experiment.

Recently, Haim and Sutin[36] have proposed a refinement of electron transfer theory to account for electrostatic effects if the reactant ions are of opposite charge. The difficulty arises from the fact that self-exchange reactions usually involve ions of the same charge; therefore, the assembly of the activated complex of the cross reaction involves significantly different work terms. A more complete expression for the free energy change of the cross reaction is

$$\Delta G^{*}_{12} = \Delta G^{**}_{12} + w_{12} \qquad (4)$$

ΔG^{*}_{12} is the free energy change which is measured for the cross reaction; ΔG^{**}_{12} is the sum of the free energy changes of the self-exchange reactions corrected for electrostatic work terms; w_{12} is the work term for the assembly of the reagents of the cross reaction. The expression for ΔG^{**}_{12} is

$$\Delta G^{**}_{12} = \frac{\Delta G^{*}_{11} - w_{11} + \Delta G^{*}_{22} - w_{22} + \Delta G^{\circ}_{12} - w_{12} + w_{21}}{2} \qquad (5)$$

where w_{21} is the work term for the separation of the charged species after electron transfer. The work terms are given by the expression[37]

$$w_{ab} = \frac{Z_a Z_b e^2}{Da} \exp(-\kappa a) \qquad (6)$$

where Z_a, Z_b = charge of reacting species

e^2 = square of unit charge = 3.31×10^{-6} kcal cm mole^{-1}

D = dielectric constant of water = 78.5

a = distance between centers of reactants = 14×10^{-8} cm[38]

The expression for κ is

$$\kappa = \left(\frac{8\pi \mu e^2}{1000\, D\, k_B T}\right)^{\frac{1}{2}} \qquad (7)$$

where μ = ionic strength (mole cm^{-3})

e^2 = square of unit charge = 8.31×10^{28} g cm^3 mole^{-2} sec^{-2}

D = dielectric constant of water = 78.5

k_B = Boltzmann's constant = 8.31×10^7 g cm^2 sec^{-2} mole^{-1} °K^{-1}

T = temperature (°K)

The work term must be averaged between the values found at the ionic strength of interest (which over-corrects for electrostatic effects[39]) and the value found at ionic strength of zero (which under-corrects).

The values of ΔG^*_{ab} may be gotten from the known rates of reaction and the expression

$$k_{ab} = \frac{k_B T}{h} \exp\left(\frac{-\Delta G^*_{ab}}{RT}\right) \quad (8)$$

where k_B = Boltzmann's constant = 8.31 joules $mole^{-1}\,°K^{-1}$

T = temperature (°K)

h = Planck's constant = 3.99×10^{-10} joule sec $mole^{-1}$

The value of $\Delta G_{12}°$ is given by the expression

$$\Delta G_{12}° = -\eta F \Delta E° \quad (9)$$

where F = Faraday's constant = 23.06 kcal $equiv^{-1}\ V^{-1}$

and $\Delta E°$ = difference in reduction potentials of reactant complexes

Using equations (4) through (9) and the parameters presented above, a self-exchange rate of 2.39×10^7 $\underline{M}^{-1}$ sec^{-1} may be calculated for P. aeruginosa cytochrome $\underline{c}_{551}$ from its reaction with horse heart cytochrome c. The self-exchange rates calculated from Marcus theory with and without charge corrections must agree because $\Delta G° = 0$ so that the work terms involved must also total zero. A cross reaction rate of 8.36×10^5 $\underline{M}^{-1}$ sec^{-1} may then be calculated for the oxidation of ferrocytochrome $\underline{c}_{551}$ by $Co(phen)_3^{3+}$. The near agreement of cross reaction rates predicted with and without charge corrections shows that the work term corrections involved in the protein redox reactions are small, as expected. But the data also seem to indicate that perhaps active site charges are not very well known, since the rate

predicted including charge effects agrees not as well as the rate predicted from simple Marcus theory. Indeed, the active site charge data are not given without caution as to their literal interpretation (see above). Even so, the fair agreement of Marcus theory and experimental determination for the rate of oxidation of ferrocytochrome $\underline{c}_{551}$ by $Co(phen)_3^{3+}$ may be informative.

Considering the derivation of the cytochrome $\underline{c}_{551}$ self-exchange rate, the agreement of experiment and Marcus calculation found for the oxidation of the protein leads to the conclusion that the removal of an electron from the reduced bacterial protein by $Co(phen)_3^{3+}$ follows the same mechanism as the removal of an electron by horse heart ferricytochrome $\underline{c}$. That this mechanism involves edge-edge transfer is supported by several studies. The case for outer-sphere redox processes involving horse heart cytochrome $\underline{c}$ has been summarized in Part I of the Results and Discussion section of this thesis. In addition, the absence of a dependence on ionic strength of the horse heart cytochrome $\underline{c}$-*P. aeruginosa* cytochrome $\underline{c}_{551}$ electron transfer reaction[34] suggests transfer occurs through the heme rings.

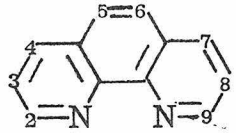
The rates of electron transfer from *P. aeruginosa* ferrocytochrome $\underline{c}_{551}$ to itself and to $Co(phen)_3^{3+}$ are considerably faster than the corresponding rates of electron transfer from horse heart ferrocytochrome $\underline{c}$. An obvious explanation of these rate differences is that the area of the heme edge is more exposed in the bacterial

protein due to its 20% shorter polypeptide chain. It is also possible that the bacterial polypeptide chain is less densely wrapped about the area of the heme edge, thus permitting more efficient encounters between the reactants.

In any case, the correlation of theory and experiment for the $Co(phen)_3^{3+}$ oxidation of both horse heart ferrocytochrome c and P. aeruginosa ferrocytochrome c_{551} nearly confirms that these two widely different proteins share a common pathway for the removal of an electron.

III Oxidation of Horse Heart Cytochrome *c*(II) and *Pseudomonas aeruginosa* Cytochrome c_{551}(II) by Tris Complexes of Modified 1,10-Phenanthroline with Cobalt(III)

If an edge-edge mechanism is employed by cytochrome *c* in its *in vivo* outer-sphere electron transfer processes and in the *in vitro* process of oxidation by $Co(phen)_3^{3+}$, then hindrance of edge-edge contact should markedly decrease the rate of electron transfer. Therefore, kinetic studies were performed on the oxidation of both horse heart cytochrome *c*(II) and *Pseudomonas aeruginosa* cytochrome c_{551}(II) by tris complexes of modified 1,10-phenanthroline with cobalt(III). The planar structure of 1,10-phenanthroline and the numbering scheme used to identify its derivatives is shown below.



The modified 1,10-phenanthrolines that were chosen are 5-chloro-1,10-phenanthroline (5-Cl-phen); 5,6-dimethyl-1,10-phenanthroline (5,6-Me_2-phen); 4,7-dimethyl-1,10-phenanthroline (4,7-Me_2-phen); and 4.7-di(phenyl-4'-sulfonate)-1,10-phenanthroline ($[4,7\text{-}(\phi\text{-}SO_3)_2\text{-phen}]^{2-}$).

The general types of effects modifications of the ring system have upon the 1,10-phenanthroline ligand itself and upon the metal complex will now be considered. The following discussion is derived from reference 40.

Steric effects are of two kinds. Substituent groups may discourage coplanarity of the heterocyclic rings. Loss of resonance

energy results and metal chelates are lowered in stability. Substituent groups may also act to prevent successive coordination of two or more ligands to the same metal center. Thus, tris complexes of 1,10-phenanthrolines substituted in the 2,9 positions are not known.

Substituents in the molecule will also cause a change in the relative electron distribution in the ring system. The relative net charges of the π-electrons at the various positions of the unsubstituted 1,10-phenanthroline have been calculated as the following[41]:

Position	Relative Net Charge
1,10	-0.377
2,9	0.138
3,8	0.019
4,7	0.120
5,6	0.015

Electrophilic substitutions would be expected at the 5,6 positions, while nucleophilic substitutions would be expected at the 2,9,4, and 7 positions. Similarly, the electron releasing effect of substituted methyl groups follows the order 2,9 > 4,7 > 3,8,5,6. Thus the pK_a of 3,5,6,8-tetramethyl-1,10-phenanthroline is 5.54, while that of 2,9-dimethyl-1,10-phenanthroline is 6.17.[42] Stability constants of tris chelates are related to basicities and have been found to be linearly dependent upon pK_a for iron(II) complexes.[43] Linear relationships have also been found to exist between ligand pK_a and redox potential for iron[43-45] and ruthenium[46] substituted phenanthroline complexes.

Unfortunately, most of the specific investigations that have been carried out to determine quantitatively the effects of substitutions in the ring system of 1,10-phenanthroline upon properties of the metal tris chelates have concentrated upon ferrous and ferric ions. Nevertheless, it will be useful to consider the cobaltous and cobaltic tris chelates, keeping in mind the general substitution effects discussed above.

It has already been mentioned that methyl groups raise the pK_a's of substituted 1,10-phenanthrolines, and that the effect quantitatively varies according to the position of substitution. Stability constants also are expected to rise for metal tris-ligand complexes (except where steric hindrance becomes important). The few stability constants that have been measured for Co(II) complexes confirm this expectation. Using the equations

$$Co^{2+} + L \rightleftharpoons CoL^{2+} \qquad k_1 = \frac{[CoL^{2+}]}{[Co^{+2}][L]} \tag{10}$$

$$CoL^{2+} + L \rightleftharpoons CoL_2^{2+} \qquad k_2 = \frac{[CoL_2^{2+}]}{[CoL^{2+}][L]} \tag{11}$$

$$CoL_2^{2+} + L \rightleftharpoons CoL_3^{2+} \qquad k_3 = \frac{[CoL_3^{2+}]}{[CoL_2^{2+}][L]} \tag{12}$$

$$\beta_3 = k_1k_2k_3 = \frac{[CoL_3^{2+}]}{[Co^{2+}][L]} \tag{13}$$

where L is 1,10-phenanthroline and its methyl derivatives, the following values have been determined:

L	log k_1	log k_2	log k_3	log β_3	Ref
1,10-phen	6.96	6.73	6.08	19.77	47
5-Me-1,10-phen	7.14	6.86	6.60	20.60	47
5,6-Me_2-1,10-phen	7.47	8.00	8.14	23.63	48
4,7-Me_2-1,10-phen	8.08	8.00	8.43	24.51	48

The only phenyl derivative that will be of interest in this study is 4,7-diphenyl-1,10-phenanthroline. The pK_a of this derivative is 4.8,[42] compared to 4.9[48] for the unsubstituted 1,10-phenanthroline. The stability constants of this derivative with metal ions would be expected to be nearly the same as those for the unsubstituted 1,10-phenanthroline. Indeed, for Fe(II) complexes, the values of log β_3 are 21.3 for the unsubstituted ligand,[49] and 21.8 for the 4,7-diphenyl derivative.[50] Thus the substituted 1,10-phenanthroline complexes are even more labile inert than the unsubstituted 1,10-phenanthroline complex, and their stability qualifies them as good reagents for kinetic study.

Another kinetically critical factor in the use of modified 1,10-phenanthroline cobalt complexes in redox reactions is the electron self-exchange rate between the +2 and +3 ionic species. Quantitative work has been done on self-exchange rates of Fe(II)/Fe(III) tris complexes of modified 1,10-phenanthroline.[51] The data are summarized below:

Ligand	$k\ (10^7\ M^{-1}\ sec^{-1})$
1,10-phen	0.62
4,7-diphenyl-1,10-phen	0.90
5,6-dimethyl-1,10-phen	1.03
4,7-dimethyl-1,10-phen	1.67
3,4,7,8-tetramethyl-1,10-phen	1.77

It is seen that in all complexes the self-exchange rate differs by a factor much less than one order of magnitude. Once again it is obvious that the position of the substitution is very important in terms of the quantitative effect. The electron releasing power of the methyl group best explains the observations.

The reduction potentials are also obviously important in considering the usefulness of the cobalt tris- (substituted-1,10-phenanthroline) complexes. The reduction potentials measured by cyclic voltammetry are presented in Table III. The measurements were kindly performed by Dr. A. Randall Bowen. The data are consistent with that from earlier investigations of such systems. For example, it was found[42] that a linear relationship exists for the redox potential among a series of iron and ruthenium tris-(5-substituted-1,10-phenanthroline) complexes. It was similarly found[44] that for a series of methyl substitutions in the 1,10-phenanthroline ligand, substitutions in the 4 or 7 position lowered the reduction potential of the $Fe^{2+/3+}$ tris complexes by 0.11 volts; substitutions in the 5 or 6 position lowered the reduction potential by 0.04 volts; and substitutions in the 3 or 8 position lowered the reduction potential by 0.03 volts. These effects were found to be additive. The reduction potentials

Table III. Reduction Potentials (E°) for Tris Complexes of 1,10-Phenanthroline and Modified 1,10- Phenanthroline with Cobalt(III).

Complex	E°, volts
$Co(phen)_3^{3+}$	+0.42[a]
$Co(5\text{-}Cl\text{-}phen)_3^{3+}$	+0.43[b]
$Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$	+0.42
$Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$	+0.34
$Co(4,7\text{-}[\phi\text{-}SO_3]\text{-}phen)_3^{3-}$	+0.33

[a] Data from reference 23.

[b] Potential determinations were made in 0.05 M NaCl solution.

are less affected in the $Co^{2+/3+}$ system. The activation energy for electron exchange between the $Co(phen)_3^{2+}$ and $Co(phen)_3^{3+}$ ions has been attributed largely to the rearrangement of the ligands in the solvated ions of the reacting species, concurrent with the change in electronic ground state from $(t_{2g})^5(e_g)^2$ to $(t_{2g})^6$. Apparently, this factor is comparatively insensitive to substitutions in the ligand.

Now that evidence has been presented that modified phenanthroline ligands will make tris complexes with cobalt which are satisfactory redox reagents in terms of stability, electron exchange, and reduction potential, the kinetic results will be given.

To further define the effects that the ring substitutions have upon the properties of the oxidizing agents, electron transfer studies were performed using as oxidants the same set of 1,10-phenanthroline and modified 1,10-phenanthroline tris complexes with cobalt(III). One of the reductants studied was $Co(terpy)_2^{2+}$, chosen because it is a strictly outer-sphere reagent with a conjugated ligand system surrounding the buried metal center; the other was $Ru(NH_3)_5py^{2+}$, chosen because it is a d^6/d^5 metal center analogous to that found in the cytochromes. In addition, each of these reductants is sufficiently stable at the conditions used in the study of the cytochromes to render them convenient reagents. The plot of observed first-order rate constants vs. cobalt concentration for the oxidation of $Co(terpy)_2^{2+}$ by $Co(phen)_3^{3+}$ is shown in Figure 7, and the corresponding plot for the oxidation of $Ru(NH_3)_5py^{2+}$ is shown in Figure 8. The rate law obtained for the oxidation of $Co(terpy)_2^{2+}$ by any of the cobalt(III) oxidants is

Figure 7. Plot of k_{obsd} vs $[Co(phen)_3^{3+}]$ for the oxidation of $Co(terpy)_2^{2+}$ [25°, pH = 7.0 (phosphate), μ = 0.5 (NaCl)].

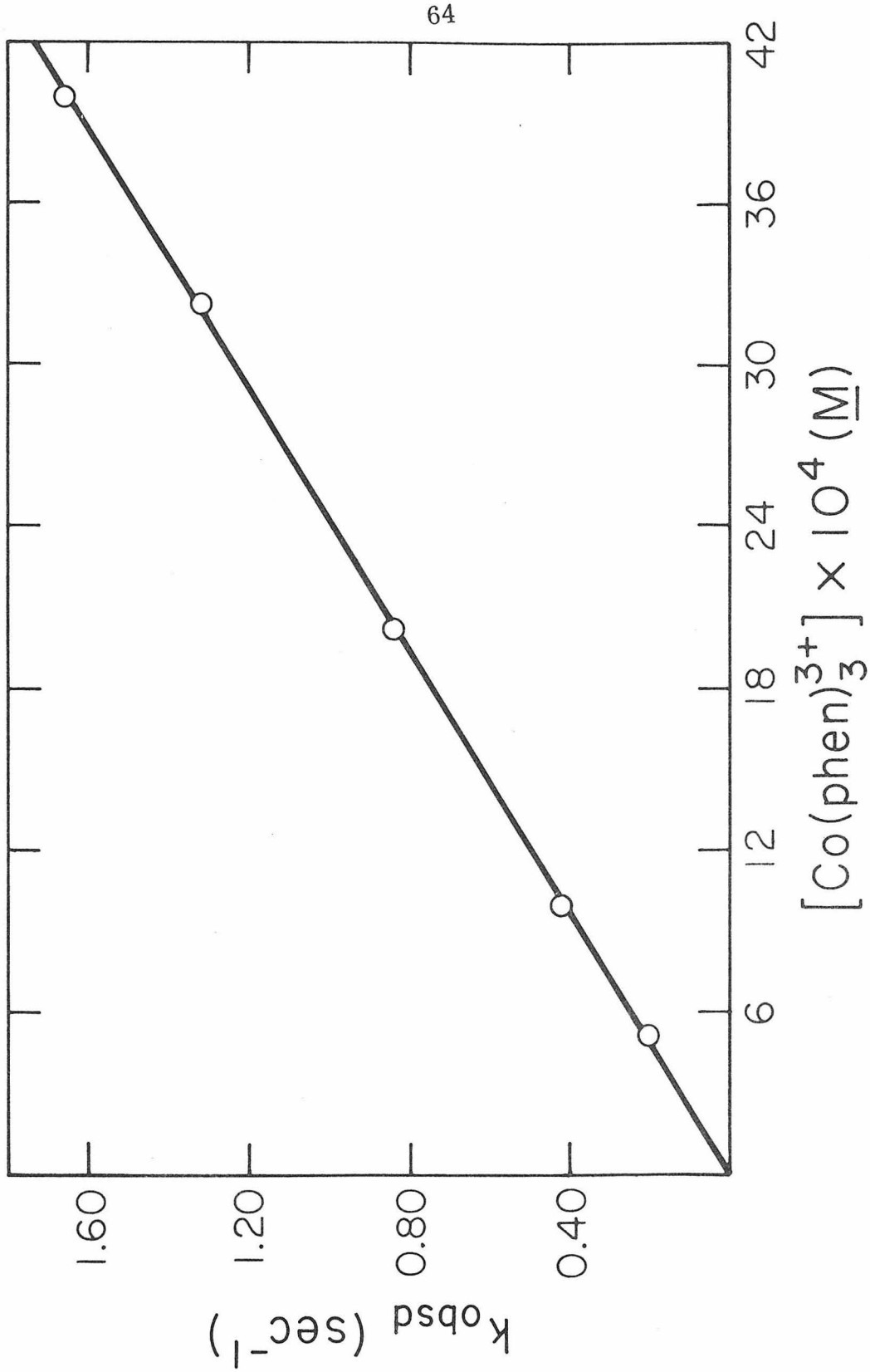
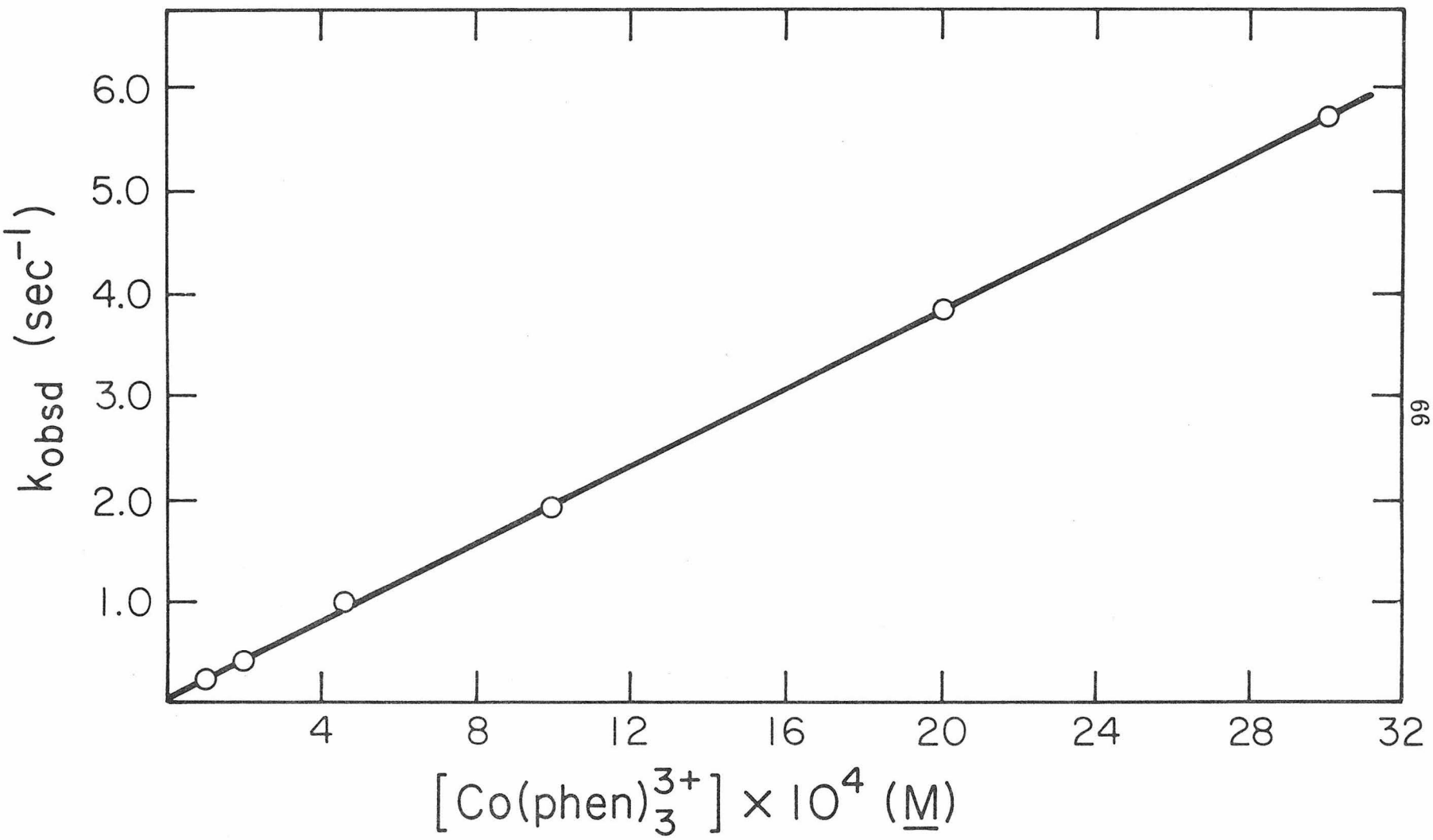

1.60
1.20
0.80
0.40
k_{obsd} (sec^{-1})
6
12
18
24
30
36
42
$[Co(phen)_3^{3+}] \times 10^4$ (M)

Figure 8. Plot of k_{obsd} vs $[Co(phen)_3^{3+}]$ for the oxidation of $Ru(NH_3)_5py^{2+}$ [25 °, pH = 7.0 (phosphate), μ = 0.5 NaCl].


6.0
5.0
4.0
3.0
2.0
1.0
k_{obsd} (sec^{-1})
4
8
12
16
20
24
28
32
$[Co(phen)_3^{3+}] \times 10^4$ (M)

$$\frac{-d[Co(terpy)_2^{2+}]}{dt} = k[Co(terpy)_2^{2+}][\,ox(III)] \qquad (14)$$

and that for the oxidation of the $Ru(NH_3)_5py^{2+}$ is

$$\frac{-d[Ru(NH_3)_5py^{2+}]}{dt} = k_1[Ru(NH_3)_5py^{2+}][ox(III)] + k_2[Ru(NH_3)_5py^{2+}] \qquad (15)$$

The fact that $Ru(NH_3)_5py^{2+}$ undergoes slow autoxidation is evident from the non-zero intercept of Figure 8. Autoxidation was slowed by storing all solutions of $Ru(NH_3)_5py^{2+}$ in the absence of light until introduction of the reagnets into the observation cell of the stopped flow machine. The kinetic parameters are presented in Table IV. Representative Eyring plots are given in Figures 9 and 10. It is evident that the properties of the oxidants are accountable for only small rate reductions. Also evident from Table IV is the expected dependence upon charge of the activation parameters for electron transfer between small molecules. The rate of reaction between $Co(terpy)_2^{2+}$ and $Co(phen)_3^{3+}$ agrees well with that found by Farina and Wilkins[11] under slightly different conditions.

Calculation of the diffusion controlled rates of electron transfer between an oxidant of charge +3 or -3 and a reductant of charge +2 gives $k = 6.4 \times 10^8$ $\underline{M}^{-1}$ sec^{-1} and $k = 4.6 \times 10^{10}$ $\underline{M}^{-1}$ sec^{-1}, respectively. The Debye equation[52] and values of the parameters used are as follows:

$$k = \frac{4\pi q_A a_B DN^2}{1000\ \epsilon RT[\,e^{q_A q_B N/\epsilon RT\sigma} - 1]} \qquad (16)$$

Table IVa. Kinetic Parameters for the Oxidation of $Co(terpy)_2^{2+}$ [25°, pH = 7.0 (phosphate), μ = 0.5 (NaCl)].

Oxidant	$k \times 10^{-2}$, $M^{-1}sec^{-1}$	$\Delta H^{\ddagger}$, kcal mol^{-1}	$\Delta S^{\ddagger}$ cal deg^{-1} mol^{-1}
$Co(phen)_3^{3+}$	4.16 (8)[a]	6.6(5)	-24(1)
$Co(5\text{-}Cl\text{-}phen)_3^{3+}$	0.77	10.9	-13
$Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$	1.22	6.7	-26
$Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$	1.04	11.6	-11
$Co(4,7\text{-}[\phi\text{-}SO_3]_2\text{-}phen)_3^{3-}$	265	2.6	-29

[a] Figure in parentheses marks the error of the last digit given.

Table IVb. Kinetic Parameters for the Oxidation of $Ru(NH_3)_5py^{2+}$ [25°, pH = 7.0 (phosphate), μ = 0.5 (NaCl)]

Oxidant	$k_1 \times 10^{-2}$, $M^{-1}sec^{-1}$	k_2, sec^{-1}	$\Delta H^{\ddagger}$, kacl mol^{-1}	$\Delta S^{\ddagger}$ cal deg^{-1} mol^{-1}
$Co(phen)_3^{3+}$	19.1(7)[a]	0.04(5)	5(1)	-24(2)
$Co(5\text{-Cl-phen})_3^{3+}$	9.0	0.15	3	-35
$Co(5,6\text{-Me}_2\text{-phen})_3^{3+}$	3.3	0.05	7	-24
$Co(4,7\text{-Me}_2\text{-phen})_3^{3+}$	3.0	0.02	8	-17
$Co(4,7\text{-}[\phi\text{-SO}_3]_2\text{-phen})_3^{3-}$	200	--	2	-32

[a] Figure in parentheses marks the error of the last digit given.

Figure 9. Eyring plot of rate data for the oxidation of $Co(terpy)_2^{2+}$ by $Co(phen)_3^{3+}$ [pH = 7.0 (phosphate), μ = 0.5 M (NaCl)]. □, $[Co(phen)_3^{3+}] = 5 \times 10^{-4}$ M; Δ, $[Co(phen)_3^{3+}] = 3.2 \times 10^{-3}$ M; ×, $[Co(phen)_3^{3+}] = 4 \times 10^{-3}$ M; ○, $[Co(phen)_3^{3+}] = 1 \times 10^{-3}$ M; ○, $[Co(phen)_3^{3+}] = 2 \times 10^{-3}$ M.

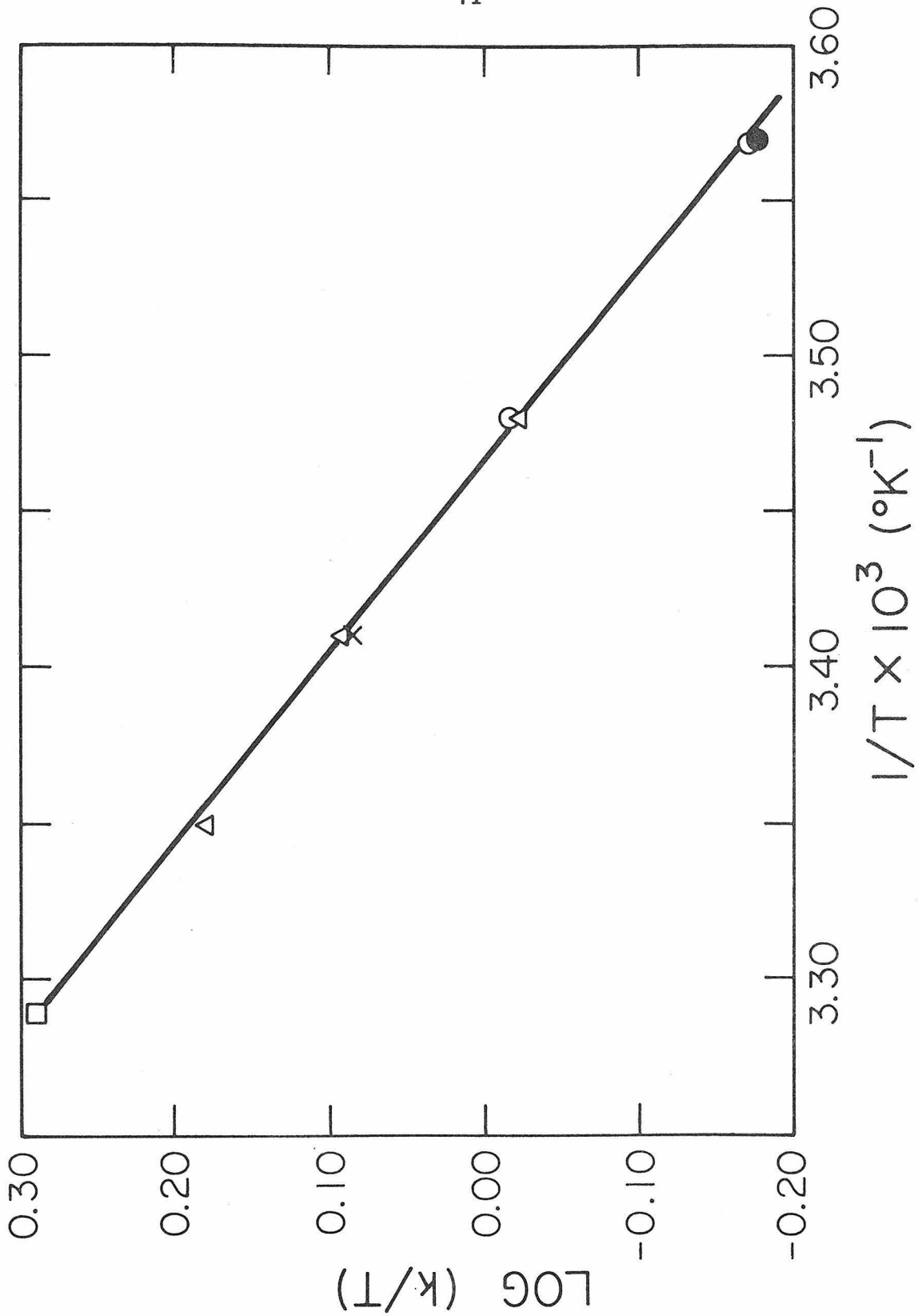
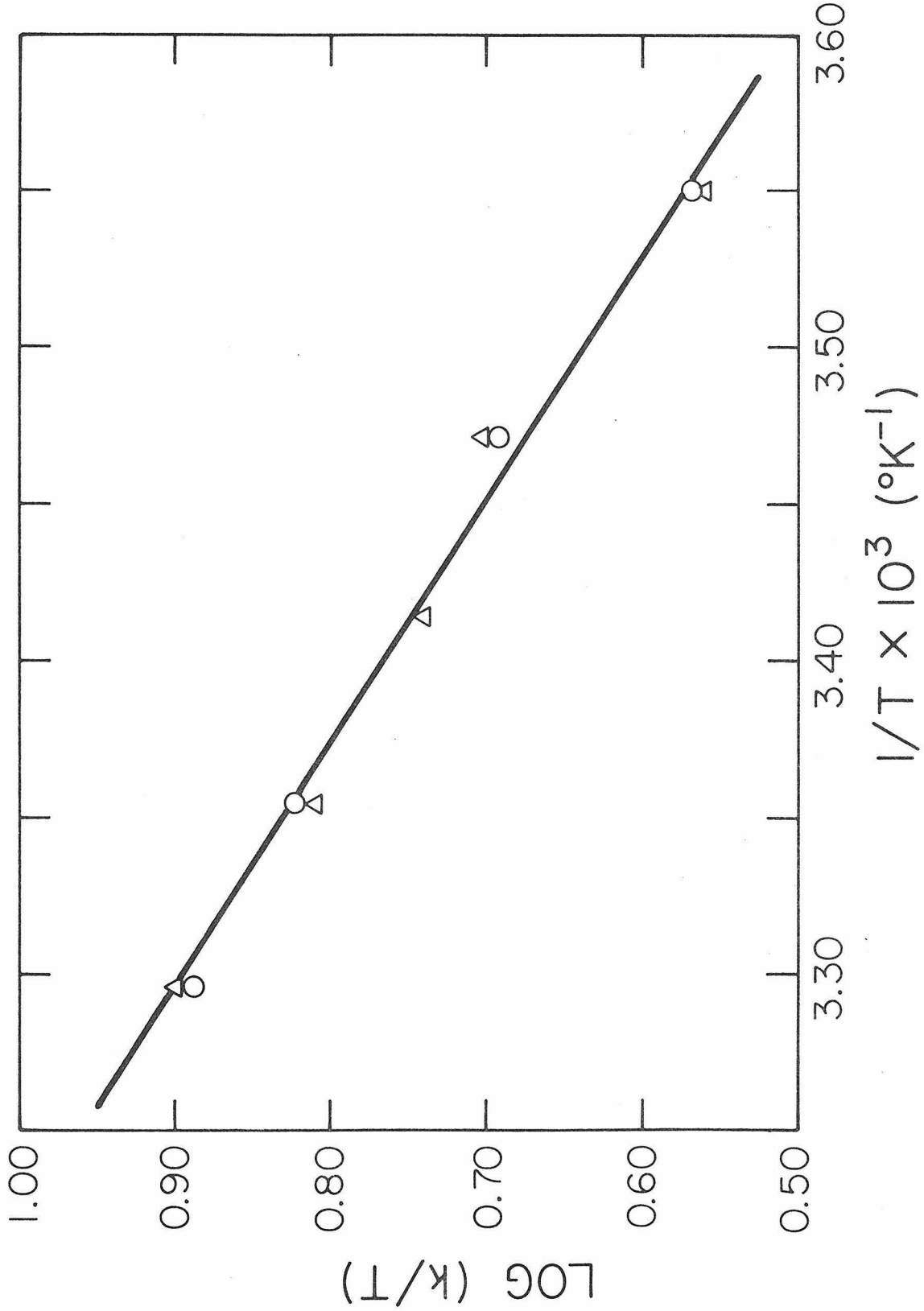

0.30
0.20
0.10
0.00
-0.10
-0.20
LOG (k/T)
3.30
3.40
3.50
3.60
1/T X 10^3 (°K^-1)

Figure 10. Eyring plot of rate data for the oxidation of $Ru(NH_3)_5py^{2+}$ by $Co(phen)_3^{3+}$ [pH = 7.0 (phosphate), μ = 0.5 M (NaCl)]. ○, $[Co(phen)_3^{3+}] = 1 \times 10^{-3}$ M; Δ, $[Co(phen)_3^{3+}] = 2 \times 10^{-3}$ M.


LOG (k/T)
1.00
0.90
0.80
0.70
0.60
0.50
3.30
3.40
3.50
3.60
1/T X 10^3 (°K^-1)

q_A, q_B = charges of reactive species

D = sum of diffusion coefficients of reactive particles = 1.4×10^{-5} cm^2 sec^{-1}

N = Avogadro's number = 6.02×10^{23} $mole^{-1}$

ϵ = dielectric constant of water = 78.5

R = gas constant = 8.31 joules $mole^{-1}$ $°K^{-1}$

T = temperature = 298.2 °K

σ = collision diameter = 10 A

The factor of approximately 70 between the two calculated rates accounts nicely for the observed difference in rates of oxidation of the small complexes by $Co(phen)_3^{3+}$ and $Co(4,7\text{-}[\phi\text{-}SO_3]_2\text{-}phen)_3^{3-}$.

First-order plots of the absorbance-time data observed at 550 nm for horse heart ferrocytochrome c and at 551 nm for P. aeruginosa ferrocytochrome c_{551} are linear for greater than 90% of the reaction.

The plots of observed first-order rate constants vs the concentration of the cobalt(III) complexes all feature zero intercepts and straight lines over the entire range of cobalt concentrations. The rate law followed for the oxidation of either reduced protein (pro (II)) by any of the cobalt oxidants (ox(III)) is

$$\frac{-d[pro(II)]}{dt} = k[ox(III)]\,[pro(II)] \qquad (17)$$

Table V summarizes the kinetic parameters found for the oxidation of each of the two proteins in 0.05 M pH 7.0 phosphate buffer of ionic strength 0.1 M at 25°.

Interpretation of the considerable number of accumulated activation parameters will now be undertaken. The free energy of activation of an outer-sphere electron transfer reaction[53] is given as a sum of contributions:

$$\Delta G^{\ddagger} = RT \ln \frac{kT}{hZ} + \Delta G_c^{\ddagger} + \Delta G_i^{\ddagger} + \Delta G_o^{\ddagger} \qquad (18)$$

The first term of equation (18) represents the loss of rotational and translational free energy upon forming the activated complex. The second term represents the free energy change due to coulombic interactions. The third term represents the free energy change necessary to rearrange the inner-coordination-sphere ligands, and the last term represents the free energy change needed to rearrange the solvation spheres of the complexes. It is apparent that changes in the activation parameters among the various oxidants with a single reductant will contain little useful information. However, comparison of the differences in the way the activation parameters change for a group of oxidants and a group of reductants may be revealing.

Before a discussion of the rate and activation parameters is given, it must be emphasized that the steric properties of the substitutions on the phenanthroline ligand will be a critical factor in that discussion. Only by viewing the space-filling models of the tris complexes can the steric factors be correctly interpreted. Each of the

Table Va. Kinetic Parameters for the Oxidation of Horse Heart Type VI Cytochrome c(II)

[25°, pH = 7.0 (phosphate), μ = 0.1 (NaCl)]

Oxidant	$k \times 10^{-2}$, $M^{-1} sec^{-1}$	$\Delta H^{\ddagger}$, kcal mol^{-1}	$\Delta S^{\ddagger}$, cal deg^{-1} mol^{-1}
$Co(phen)_3^{3+}$	15.0(5)[a]	11.3(4)	-6(1)
$Co(5\text{-}Cl\text{-}phen)_3^{3+}$	1.26	9.6	-16
$Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$	2.66	12.4	-6
$Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$	0.276	14.6	-3
$Co(4,7\text{-}[\phi\text{-}SO_3]_2\text{-}phen)_3^{3-}$	28.7	12.8	0

[a] Figure in parentheses marks the error of the last digit given.

Table Vb. Kinetic Parameters for the Oxidation of P. aeruginosa cytochrome c_{551}(II)

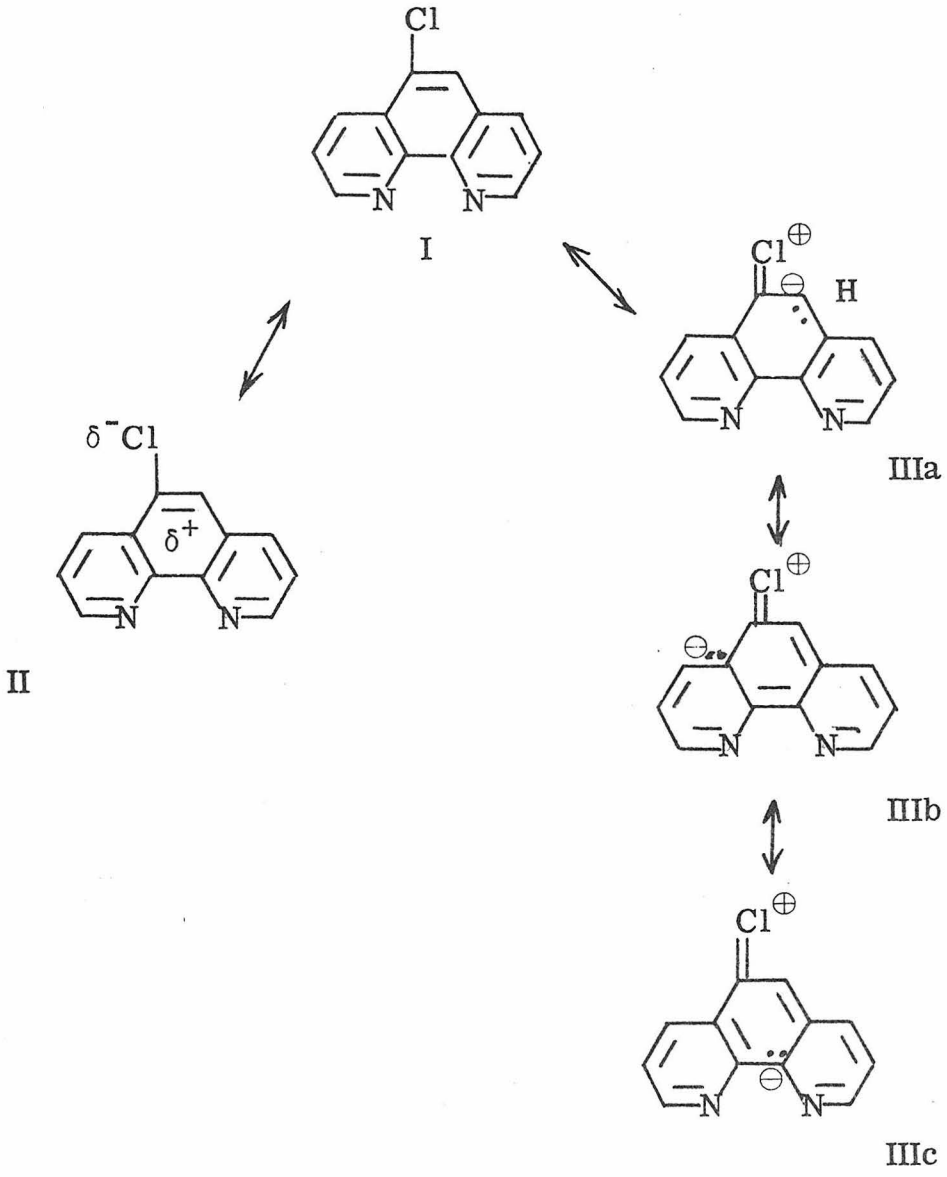
[25°, pH = 7.0 (phosphate), μ = 0.1 (NaCl)]

Oxidant	$k \times 10^{-3}$, $M^{-1}sec^{-1}$	$\Delta H^{\ddagger}$, kcal mol^{-1}	$\Delta S^{\ddagger}$, cal deg^{-1} mol^{-1}
$Co(phen)_3^{3+}$	53.0(5)[a]	12.3(4)	+4(1)
$Co(5\text{-Cl-phen})_3^{3+}$	4.42	9.4	-10
$Co(5,6\text{-Me}_2\text{-phen})_3^{3+}$	27.0	13.6	+7
$Co(4,7\text{-Me}_2\text{-phen})_3^{3+}$	3.17	10.9	-6
$Co(4,7\text{-}[\phi\text{-SO}_3]_2\text{-phen})_3^{3-}$	27.5	13.8	+8

[a] Figure in parentheses marks the error of the last digit given.

oxidants shall now be considered in turn.

The oxidant $Co(5\text{-}Cl\text{-}phen)_3^{3+}$ places a more electronegative element at the edge of the 1,10-phenanthroline rings, and is seen to have remarkably similar effects on the kinetic parameters of oxidation of the proteins. Compared to the "reference" parameters of oxidation of the proteins by $Co(phen)_3^{3+}$, the rate constants are reduced by a factor of twelve, and both sets of activation parameters become more negative. The bacterial protein is able to minimize its energy of activation by lowering $\Delta H^{\ddagger}$ more than the horse heart protein (2.9 kcal $mole^{-1}$ compared to 1.7 kcal $mole^{-1}$). However, the corresponding decreases in $\Delta S^{\ddagger}$ are also larger for the bacterial protein (14 e.u. compared to 10 e.u.). Oxidation by $Co(5\text{-}Cl\text{-}phen)_3^{3+}$ of $Co(terpy)_2^{2+}$ but not $Ru(NH_3)_5py^{2+}$ shows an increase in the activation parameters compared to the activation parameters for the oxidation of the respective reagents by $Co(phen)_3^{3+}$. The anomalous behavior of the $Co(5\text{-}Cl\text{-}phen)_3^{3+}$ ligand may in part be explained by contributions from the following ligand resonance structures:



Contributions from the charged chlorosubstituent as in structures II and III a-c may lead to greater steric requirements for successful electron acceptance from the proteins. There also will be a negative contribution to $\Delta S^{\ddagger}$ from the necessity of reconjugating the ligand after complex formation but prior to electron transfer. Similar considerations may apply to the oxidation of $Ru(NH_3)_5py^{2+}$ by $Co(5\text{-Cl-phen})_3^{3+}$. For the oxidation of $Co(terpy)_2^{2+}$, the steric

requirements appear to be removed, but a considerably larger "push" in the form of 4 kcal $mole^{-1}$ is required to complete the reaction.

Oxidation of the proteins by $Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$ shows only small differences in kinetic parameters as compared to the parameters of oxidation by $Co(phen)_3^{3+}$. The rate decreases by less than a factor of six for the horse heart protein, and less than a factor of two for the bacterial protein. The molecular model shows that although the 5,6-dimethyl substitution in the phenanthroline ligand blocks completely the 5,6 edge, other edges of the conjugated system remain at nearly as great a distance from the cobalt as the 5,6 edge. The effect $Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$ has on the oxidation of either protein or on either small molecule reductant is very slight compared to oxidation by $Co(phen)_3^{3+}$. A small increase in $\Delta H^{\ddagger}$ is needed to yield oxidation of the proteins, which may mean slightly more energetic collisions are required. It is interesting that although approach to the conjugated heme now requires about one extra kcal per mole, the enthalpy of activation of oxidation of $Co(terpy)_2^{2+}$ does not change. This is the first bit of evidence that the "exposed" heme edge may not really be very exposed.

Oxidation of the proteins by $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ does, however, produce a large variation in kinetic parameters (Figures 11-14). Inspection of the molecular model of $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ shows that the methyl groups have the ability to block access to the phenanthroline not only at the 4 or 7 position, but also at the 3, 5, 6, and 8 positions to an only slightly lesser extent. The rate of oxidation of horse heart ferrocytochrome c by $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ is almost

Figure 11. Plot of k_{obsd} vs $[Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}]$ for the oxidation of horse heart ferrocytochrome c [25°, pH = 7.0 (phosphate), μ = 0.1 M (NaCl)].

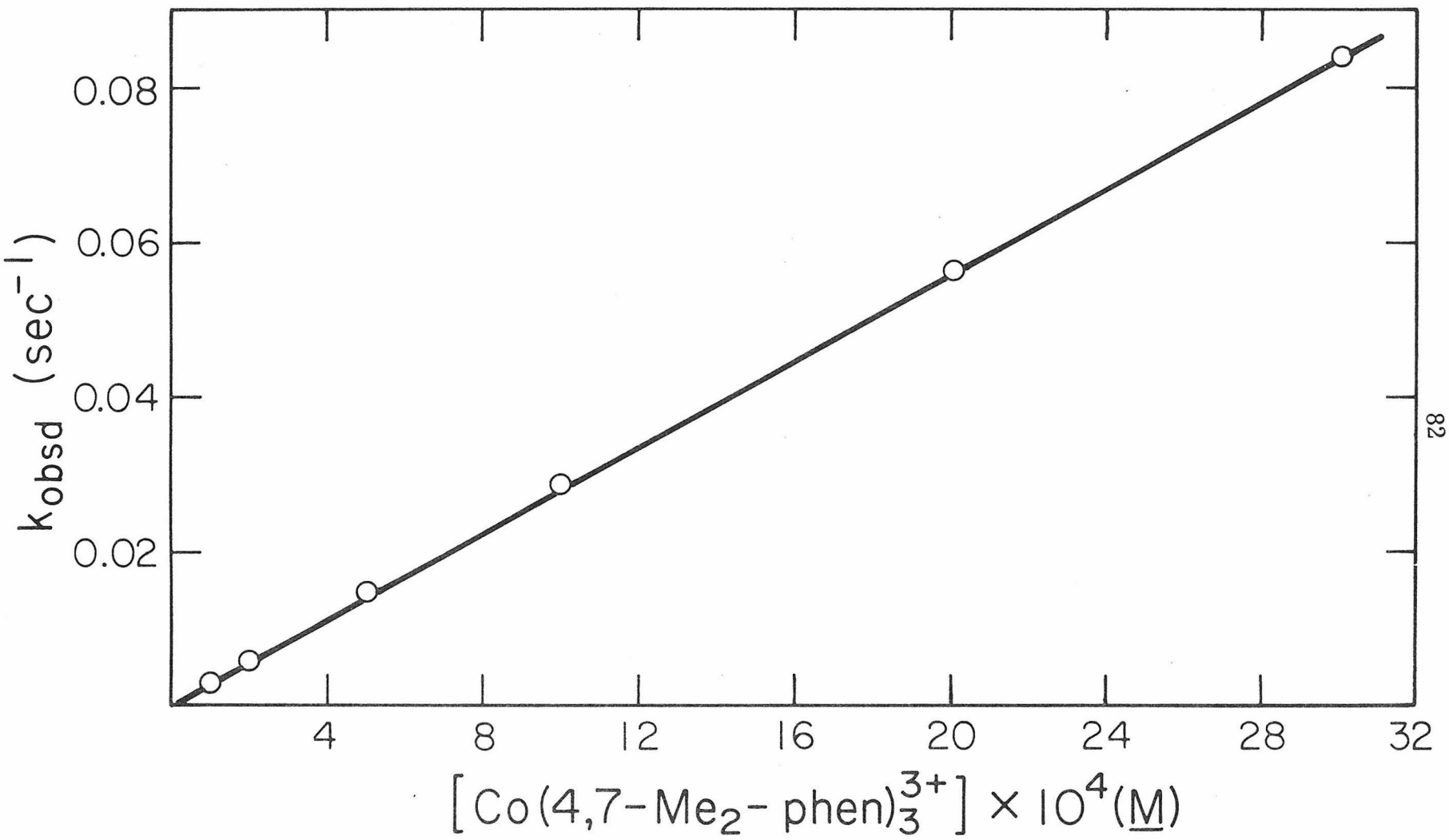

0.08
0.06
0.04
0.02
k_{obsd} (sec^{-1})
4
8
12
16
20
24
28
32
$[Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}] \times 10^4$ (M)

Figure 12. Eyring plot of rate data for the oxidation of horse heart ferrocytochrome c by $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ [pH = 7.0 (phosphate), μ = 0.1 M (NaCl)]. Δ, $[Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}] = 1 \times 10^{-3}$ M; $\circ$, $[Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}] = 2 \times 10^{-3}$ M; $\times$, $[Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}] = 3 \times 10^{-3}$ M.

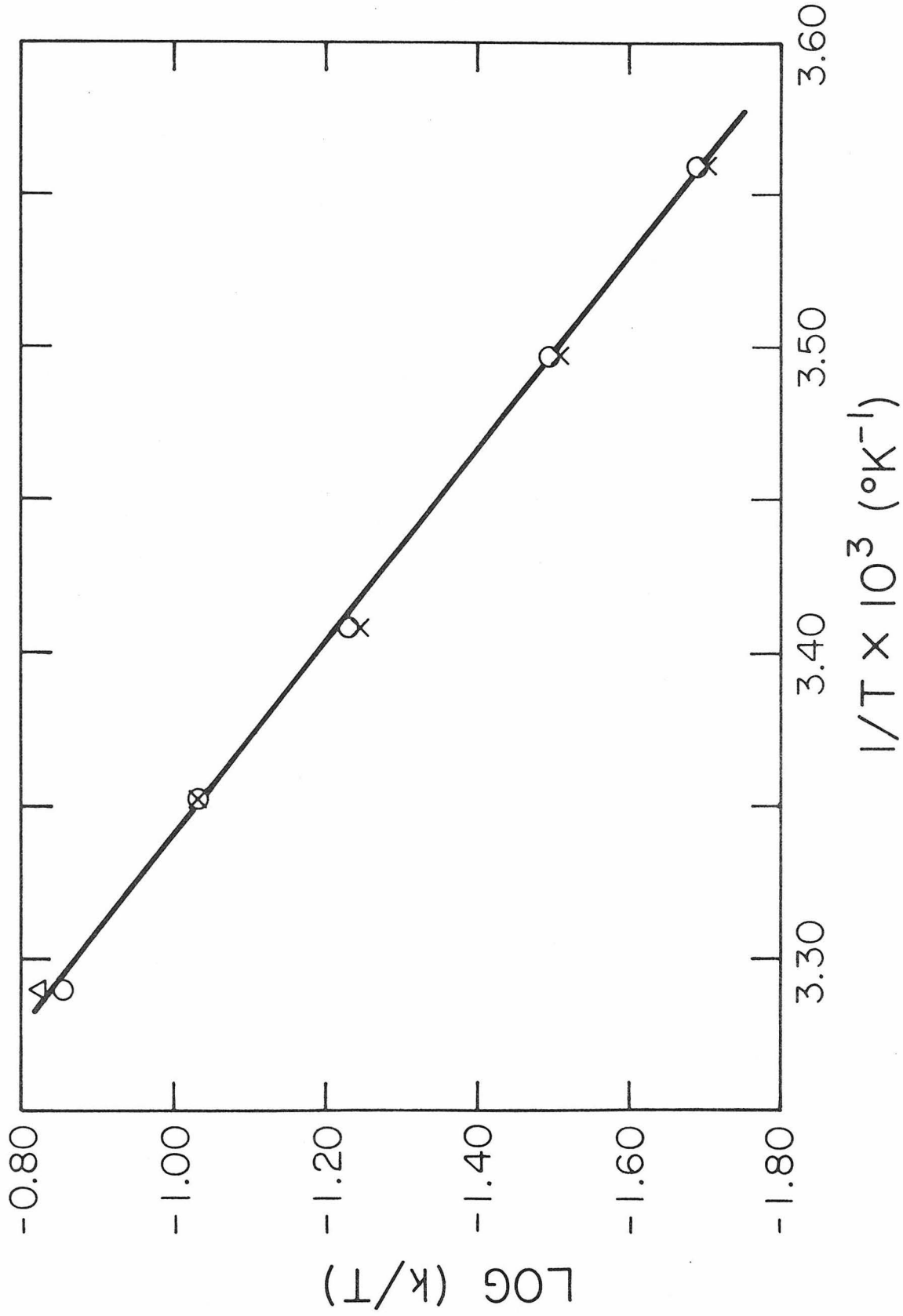

-0.80
-1.00
-1.20
-1.40
-1.60
-1.80
LOG (k/T)
3.30
3.40
3.50
3.60
1/T X 10^3 (°K^-1)

Figure 13. Plot of k_{obsd} vs $[Co(4,7\text{-}Me_2\text{-}phen)_3]^{3+}$ for the oxidation of P. aeruginosa ferrocytochrome c [25°, pH = 7.0 (phosphate), μ = 0.1 M (NaCl)].

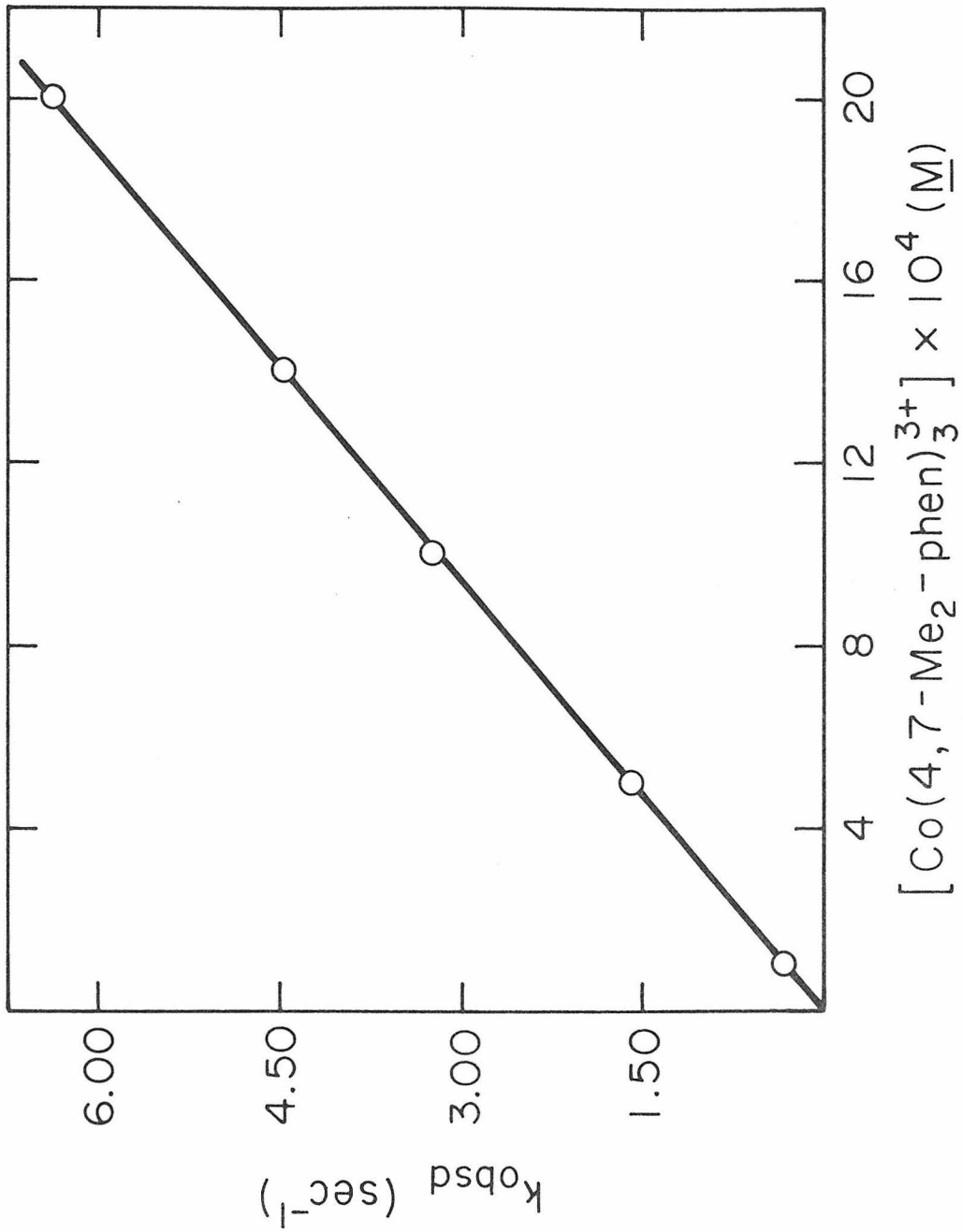
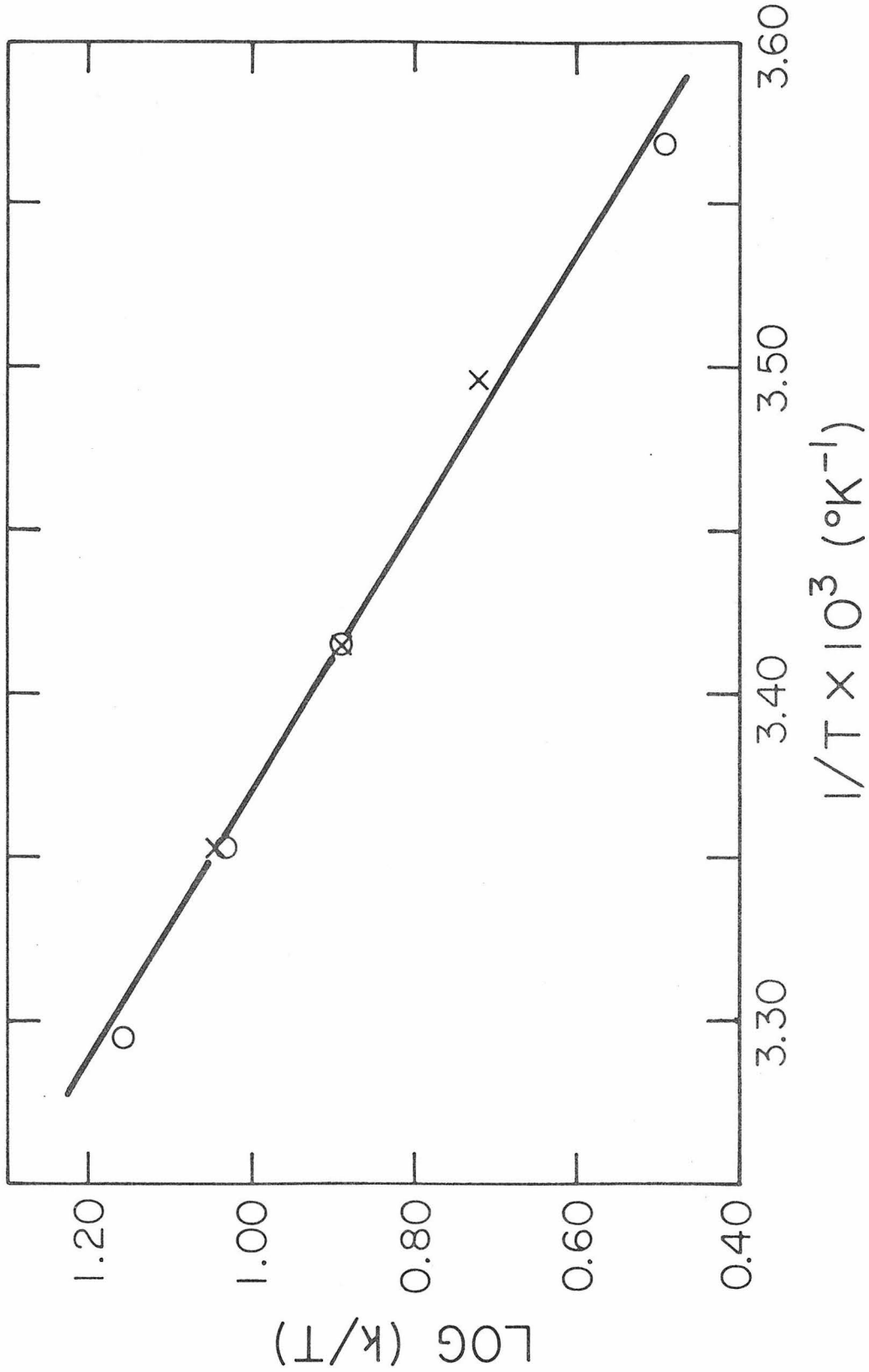

6.00
4.50
3.00
1.50
k_{obsd} (sec^{-1})
4
8
12
16
20
$[Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}] \times 10^4$ (M)

Figure 14. Eyring plot of rate data for the oxidation of P. aeruginosa ferrocytochrome c_{551} by $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ [pH = 7.0 (phosphate), μ = 0.1 M (NaCl)]. ○, $[Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}]$ = 5×10^{-4} M; ×, $[Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}]$ = 1×10^{-3} M.


LOG (k/T)
1.20
1.00
0.80
0.60
0.40
3.30
3.40
3.50
3.60
1/T X 10³ (°K⁻¹)

one sixtieth that of oxidation by $Co(phen)_3^{3+}$, while the rate of oxidation of P. aeruginosa ferrocytochrome c_{551} by $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ falls only to one-sixteenth that of oxidation by $Co(phen)_3^{3+}$. In addition, the proteins act in opposite manners to achieve the minimum activation energy. The enthalpy of activation for the oxidation of horse heart ferrocytochrome c by $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ increases by more than 3 kcal $mole^{-1}$, and the entropy of activation increases by about 3 e.u. compared to the activation parameters obtained for the oxidation of the protein by $Co(phen)_3^{3+}$. In contrast, the enthalpy of activation for the oxidation of P. aeruginosa ferrocytochrome c_{551} by $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ decreases by almost 1.5 kcal $mole^{-1}$, and the entropy of activation decreases by about 10 e.u. compared to the activation parameters of oxidation by $Co(phen)_3^{3+}$. Oxidation of the small molecules shows an increase in the activation parameters for oxidation by $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ compared to oxidation by $Co(phen)_3^{3+}$.

The very different reactivities of the proteins toward $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ merits further discussion. The now very large $\Delta H^{\ddagger}$ for the oxidation of horse heart cytochrome c(II) supports the earlier suggestion that the exposed heme edge may be only very slightly exposed in solution. The $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ oxidant requires a much more energetic reactive collision to result in oxidation of the protein. The rate of oxidation of the bacterial protein by $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ is slowed by a decrease in $\Delta S^{\ddagger}$. In contrast to the horse heart protein and the rigid small molecules, it appears that the bacterial protein is structured such that accommodation of the $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ oxidant occurs with significantly

less activation enthalpy. Considering the 20% shorter polypeptide chain of P. aeruginosa cytochrome c_{551}, structural alteration near the active site seems reasonable. That this structural alteration should occur in the area of the heme edge is strongly supported by the preliminary 4A P. aeruginosa cytochrome c_{551} X-ray structure determination now completed.[54] It appears residues 39 to 56 of horse heart protein have been cut away and the polypeptide chain reconnected by joining residue 39 to 48 in the horse heart protein run from the back to joining residue 38 to 57. This information is significant because residues 39 to 48 in the horse heart protein run from the back to the front at the bottom of the molecule just below the heme. Also, residues 28, 29, and 30, which in horse heart protein sit very near the right face of the heme, are moved downward to help close the gap from the missing chain length.

The oxidant $Co(4,7\text{-}[\phi\text{-}SO_3]_2\text{-}phen)_3^{3-}$ places substituents in the same positions as $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ but restores the conjugated system. The phenyl groups must be sulfonated to keep the cobalt(III) complex soluble. The molecular model indicates that the length of exposed conjugated ligand increases, although the phenyls are forced a bit out of the plane of the phenanthroline. Conjugation may therefore not be truly complete. The rates of oxidation of the proteins by $Co(4,7\text{-}[\phi\text{-}SO_3]_2\text{-}phen)_3^{3-}$ are within a factor of two of the rates of oxidation by $Co(phen)_3^{3+}$. For each protein, $\Delta H^{\ddagger}$ increases by about 1.5 kcal $mole^{-1}$, and $\Delta S^{\ddagger}$ increases by about 5 e.u. compared to the activation parameters for oxidation by $Co(phen)_3^{3+}$. It is interesting that the rate of oxidation of the proteins is not very dependent upon charge, but that the negatively charged oxidant gives a slightly higher rate than $Co(phen)_3^{3+}$ when the reductant has a positive active site

charge and a slightly lower rate when the reductant has a negative active site charge.

The $Co(4,7\text{-}[\phi\text{-}SO_3]_2\text{-}phen)_3^{3-}$ also serves to demonstrate the absence of a dependence upon charge of the activation parameters for electron transfer from the proteins. In sharp contrast is the dependence upon charge of the activation parameters for electron transfer from the small molecule reductants. In view of this fact and all of the protein enthalpies of activation given above, an earlier statement[55] that oxidation of cytochrome c involves no unusual features seems inappropriate. A significant activation enthalpy is required for the oxidation of cytochrome c. This conclusion seems more accurate, especially since oxidation by corresponding oxidants of a d^7/d^6 cobalt reductant always involves less activation enthalpy. A not very exposed heme edge is again implicated.

Another important observation should be made in view of all of the protein activation parameters. In no instance do the activation parameters change with respect to the activation parameters of oxidation by $Co(phen)_3^{3+}$ in the opposite direction. Either $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ both become more positive, or both become more negative. In no case is the protein able to achieve a minimum activation energy through a compromise of increased enthalpy of activation and decreased entropy of activation, or vice versa. This is most evident in the oxidation of the proteins by $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$.

In summary, a detailed picture of the structure-function relationship may now be given. Obviously, the iron is the store which allows the protein to accept and donate electrons. The heme group surrounding the iron provides a pathway for the shuttling of the

electrons. The uncommon thioether ligand of iron is necessary to maintain the correct reduction potential[56] for the *in vivo* interactions of cytochrome *c*. The polypeptide chain provides insulation for the electron carrying properties of the protein, as well as providing areas of charge at the surface of the protein to allow solution mobility and recognition sites for oxidase and reductase binding. The highly conservative aromatic groups are also probably involved in oxidase or reductase binding. More specifically, the area of lysine 13 has been implicated in oxidase binding.[30,31] The area of reductase binding has been shown to be different from that of oxidase binding,[30,57] but has not been determined any more specifically.

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CHAPTER II

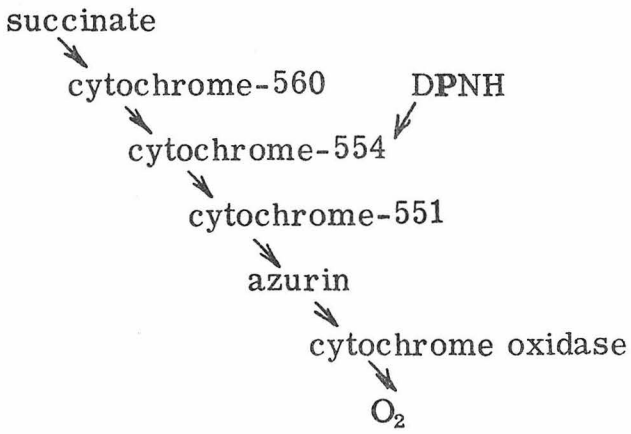
OXIDATION STUDIES OF AZURIN

INTRODUCTION

The discovery of azurin was made by Verhoeven and Takeda who were working with Pseudomonas aeruginosa because of their interest in the cytochromes of denitrifying bacteria. The announcement of their discovery was included at the end of a report of their work on the cytochromes[1]: "A blue protein was also isolated from the bacterial extract...Its physiological function is obscure at present."

Azurin has been found in the bacterial genera Pseudomonas, Bordetella, and Alcaligenes by Sutherland and Wilkinson,[2] who proposed the name "azurin." Azurin was later found in the bacterial genera Achromobacter.[3]

Azurin, like other blue copper proteins, is probably an electron transfer protein. The position of azurin in the respiratory redox chain of P. aeruginosa has been given by Horio, et al.,[4] as the following:



Cytochrome c_{551} may also donate electrons directly tc the oxidase.[5] Specific interaction of the oxidase with cytochrome c_{551} and azurin is evidenced by fast electron transfer compared to electron transfer

from mammalian cytochrome c, yeast cytochrome c, or small molecule electron donors.[5]

Horio performed many of the early investigations of the properties of azurin. Purification methods[6,7] leading to the crystalline protein were first described by him. He also described the reversible oxidation-reduction processes of the protein and demonstrated the presence of copper in the protein.[7] The isoelectric point of the protein was measured by him as 5.70, and the reduction potential at pH 6.4 as +0.328 V.[4]

The presence of copper and the characteristic blue color of azurin and similar proteins led to the obvious suggestion that the absorption spectrum of these proteins is due to d-d electronic transitions of the copper.[8] However, the protein absorptions occur with 10-50 times the intensity of simple model complexes. Thus, the nature of copper binding in these proteins has received much attention.

Electron paramagnetic resonance (EPR) studies of azurin and other blue (Type 1) copper proteins have been extensive. The blue copper proteins present "unusual" (with reference to model complexes) EPR parameters. As found with other blue copper proteins, an extremely small high-field hyperfine splitting constant has been obtained for azurin ($A_{||} = 0.0060$).[9] One explanation offered for the uncommon $A_{||}$ values is that the electron hole of the d^9 Cu^{2+} ground state is highly delocalized from the $d_{x^2-y^2}$ ground state.[10] Recent interpretations indicate that low values of $A_{||}$ reflect distortion of square planar coordination towards octahedral coordination.[11]

Fluorescence studies of blue copper proteins have also been performed. Using excitation light of either 275 or 292 nm, an abnormally low wavelength emission maximum is observed.[12,13] The emission is assigned to the lone tryptophan which must be in a strongly hydrophobic environment in the interior of the protein. The tryptophan is of interest because it is fully conserved through several strains of bacteria whose azurin has been sequenced and therefore may have a functional role.[14] In addition, it was found that the apoprotein had adsorption and emission spectra identical to that of azurin, but that the intensity of emitted light decreases significantly for the native protein. Finazzi-Agro, et al.,[13] found the intensity of emission to decrease by a factor of three, and attributed the effect to binding of the copper in the hydrophobic site near the tryptophan (but not to it[15]) leading to enhanced internal conversion to the ground state. Grinvald, et al.,[16] however, found the intensity of emission to decrease by a factor of six for the native protein compared to the apoprotein. Further, Grinvald and coworkers found that the emission of the apoprotein decayed monoexponentially, whereas the native protein did not. The results were interpreted to mean that all apo-azurin molecules in solution have an identical conformation in the region of the tryptophan, whereas native azurin molecules in solution have structural variability. Their explanation for the reduced emission of the native protein is that approximately 55% of the molecules are fully quenched. They describe the isomerization process as slow and as having an influence on the tryptophan residue if not directly involving the tryptophan.

Another functional group that clearly seems to be located in the hydrophobic area with copper is the sulfhydryl group.[13] Para-mercuribenzoate did not react with native azurin, but titrates with a sharp end-point corresponding to one free sulfhydryl group per molecule for the apoprotein. Stoichiometric amounts of copper added to the p-mercuribenzoate bound protein did not restore the 625 nm absorption of the native protein, although a large excess of copper restored some 625 nm absorption. The p- mercuribenzoate treated protein displayed a fluorescence spectra identical to that of the untreated apoprotein. In contrast, addition of mercuric ion to the apoprotein resulted in a substituted protein which displayed fluorescence quenching and to which copper could not be restored even at high copper concentrations.

If the sulfhydryl group is a ligand of copper, the necessity of maintaining the copper in a highly hydrophobic site becomes clear. Exposure of the sulfhydryl ligand to the water solvent molecules would result in oxidation of the sulfur and reduction of the metal. In fact, autoreduction of the protein under denaturing conditions has been observed in solutions of 6 M guanidine hydrochloride at pH 7.0 or in solutions of pH greater than 8. As expected, denaturation at low pH does not produce autoreduction. The sulfhydryl group may also be important in electron transfer to and from the metal center.[9]

Exposure to the solvent also leads to bleaching of the 625 nm absorption of the protein before reduction of the metal occurs,

suggesting that the peculiar ligand arrangement of blue copper proteins can occur only in very hydrophobic environments.[13]

Silver has also been substituted for copper in azurin.[17] Silver was found to reduce the luminescence of apoazurin by only half as much as either the copper or mercury ligated protein. It was also shown that copper may bind the silver substituted protein and thereby reduce the luminescence to that of the native protein but without restoring the blue color. In the presence of NaCl, copper was observed to replace the silver and restore the visible and EPR spectra of native azurin. It was also reported that NaCl had no effect on the inability of copper to displace mercury in the mercury substituted protein. The results were explained in terms of the favored coordination numbers of two for silver(I), and either two or four for mercury(II). Thus, it was proposed that mercury(II) occupies all the ligands in the coordination site normally held by copper(II), but that silver(I) occupies only two. The sulfhydryl group was thought to be a ligand for all three metal centers.

The apoazurin has also been complexed with cobalt(II), and evidence for the sulfhydryl ligand was again found.[18] Partial quenching of the azurin fluorescence was again observed with the cobalt substituted protein. Also apparent were two ultraviolet absorption bands which are separated by the same energy as the corresponding absorptions in the native protein. The absorption bands were interpreted to arise from a ligand to metal charge transfer which occurs from the same ligand to either metal center in the same

metal binding site. The ligand involved was proposed to be cysteine sulfur, and the transitions involved were proposed to be from the $3p\pi$ (lower energy transition) and $3p\sigma$ (higher energy transition) orbitals of sulfur. The cobalt was thought to occupy a site of low symmetry with a coordination number of four or five. From a consideration of the extinction coefficients obtained for the d-d transitions of the cobalt(II) substituted azurin and analogy to model complexes, coordination of four was favored by the authors. Model systems have admittedly not very well duplicated the geometry of the blue copper site; thus, the favoring of distorted tetrahedral geometry over five coordinate geometry can only be tenuous.

Other authors have favored a five coordinate site for the binding of copper in blue proteins, though their position is no less tenuous at this time. Based on Raman band multiplicity which indicates that there are at least four nitrogen or oxygen ligands in addition to the sulfur ligand of copper, Miskowski, et al.,[19] have reported a trigonal bipyramidal binding site to be more likely. The symmetry is thought to be distorted C_{2v}, with the axial ligands less strongly bound than the equatorial ligands. Support for the coordination of at least one nitrogen has been given by an ENDOR study of the blue copper protein stellacyanin.[20] Miskowski, et al.,[19] have further suggested that trigonal bipyramidal coordination of copper compromises between the normal geometries of copper(I) and copper(II), thus promoting efficient electron transfer. They also contend that variability of the axial ligands accounts for the varying redox potentials found among the blue copper

proteins, while the absorption which is responsible for the intense blue color is due to the constant equatorial sulfhydryl ligand.

Information has also been gathered on blue copper proteins through the technique of proton magnetic resonance relaxation. Boden and coworkers[21] concluded that the copper(II) center is not accessible to water molecules of the solvent, in agreement with the hydrophobicity of the binding site predicted earlier. Another group[22] reached the same conclusion concerning the inaccessibility of the metal center, and also estimated the distance of the copper(II) from the surface of the protein to be 5A. Considering the radius of a spherical protein of the molecular weight of azurin to be about 15A, the metal site is seen to be a significant distance from the center of the molecule. Approach to the metal would thus be limited to only one hemisphere of the protein. It must be mentioned, however, that the validity of interpretations of proton magnetic resonance relaxation techniques applied to blue copper proteins has recently been questioned.[11]

Besides spectral investigations, electron transfer studies of azurin have been undertaken. In vitro electron transfer between the physiologically reacting species azurin and cytochrome c_{551} from P. aeruginosa has been shown to be complicated.[23-27] Earlier workers had postulated complex formation between the two proteins[24] but were contradicted by later experiments.[26,27] Temperature-jump and stopped-flow experiments[27] have been interpreted to display two forms of reduced azurin, only one of which is able to be oxidized by cytochrome c_{551}.[27] Oxidation of reduced cytochrome c_{551} by oxidized azurin yields linear pseudo-first order kinetics over the entire range

of azurin concentrations studied. The second-order rate constant is 6.25×10^6 $\underline{M}^{-1}sec^{-1}$, which is in good agreement with an earlier value.[23] The activation parameters involved are $\Delta H^{\ddagger} = 10.6$ kcal mol^{-1}, and $\Delta S^{\ddagger} = 8.6$ e.u.[28] Oxidation of reduced azurin by cytochrome $\underline{c}_{551}$ was found to also be linear if the azurin concentration was kept in large excess and the reaction performed in phosphate buffer at pH 7.0. However, if oxidized cytochrome $\underline{c}_{551}$ is kept in large excess,the course of the oxidation reaction was found to show two distinct phases: the first corresponds to oxidation of the available reactive form, and the second corresponds to isomerization of the unreactive form to the reactive form. The two forms were reported to have an equilibrium constant of 1 and a rate of interconversion of 40 sec^{-1}. The rate of electron transfer from the azurin was given as $3.4 \times 10^6 \underline{M}^{-1}sec^{-1}$, which again is in agreement with previous results.[23] The very fast rate of electron transfer between the physiologically reacting species has been interpreted[23,27] as a reflection of optimal fit between the reactants.

Electron transfer studies of oxidized P. aeruginosa azurin and reduced cytochromes from other species have also been completed. Thus, Antonini, et al.,[23] found the rate of electron transfer to azurin from mammalian cytochrome c to be 10^3 times slower than transfer from cytochrome $\underline{c}_{551}$. Their observation led to the speculation reported above that the physiological partners may have optimal fit between them. However, electron transfer to azurin from two other cytochromes, cytochrome f from parsley and cytochrome $\underline{c}_{553}$ from Placanium coccineum, were shown to transfer electrons

to azurin at rates just as great as that of P. aeruginosa cytochrome c_{551}.[29] Cytochrome f, cytochrome c_{553} and cytochrome c_{551} are all acidic proteins, whereas mammalian cytochrome c is basic. Wood[29] therefore suggested that charge interactions may be an explanation for the discrimination of the various cytochromes by azurin, even though azurin itself is acidic.

Electron transfer reactions of azurin and small molecules have also been studied. Ferricyanide was found to oxidize reduced azurin with a second order rate constant of 1.2×10^4 $M^{-1}sec^{-1}$.[23] The dithionite reduction of azurin gave a second-order rate constant of 4×10^3 $M^{-1}sec^{-1}$.[23] Again, electron transfer involving small molecules emphasizes the efficiency of the azurin-cytochrome c_{551} exchange.

Reduction of azurin by the outer-sphere reductant $Fe(EDTA)^{2-}$ has very recently been reported.[30] The second order rate constant found is 1.3×10^3 $M^{-1}sec^{-1}$. Activation parameters were given as $\Delta H^{\ddagger} = 2$ kcal mol^{-1} and $\Delta S^{\ddagger} = -37$ e.u. No special protein activation seemed to be involved. The large negative entropy of activation was possibly attributable to electron transfer over a large distance resulting from poor accessibility of $Fe(EDTA)^{2-}$ to the buried metal center of the protein. In addition, a slight dependence of rate upon an ionizable group with a pK_a of about 6.6 has been reported for the $Fe(EDTA)^{2-}$ reduction.[31] It was further reported that the ionizable group probably lies near the site of electron transfer, but not at it. An ionic strength dependence study was also performed, and an

active site charge of -0.1 was obtained for azurin at pH 6.8.[31]

The study of the oxidation of reduced azurin by tris complexes of 1,10-phenanthroline and its derivatives with cobalt(III) seems well suited to probe the electron transfer mechanism of the protein. Considering the large variations in rate and activation parameters for the protein which were included in the above discussion, a more direct comparison of activation parameters is much needed. The utility of oxidation studies using tris complexes of 1,10-phenanthroline and its derivatives with cobalt(III) was adequately demonstrated by the results of the investigation of cytochrome c presented above in Chapter I. Through a similar set of experiments, information may be gained concerning the interpretation of activation parameters, the accessibility of the reactive site, and the mechanism of electron transfer of Pseudomonas aeruginosa azurin(I). The results of these experiments are presented in this work.

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EXPERIMENTAL

I. Preparation of Reagents

Azurin was prepared according to the method of Ambler[1,2] as modified by Rosenberg.[3] The azurin was a gift from Dr. Rosenberg.

Solutions of reduced protein were prepared using the reductant $Fe(EDTA)^{2-}$. Solutions were purified using the hollow fiber Dow beaker dialyzer as described in the Experimental Section of Chapter I of this thesis. The preparative procedures for all reagents were also described above.

II. Analyses

The purified oxidized protein had an A_{625}/A_{280} ratio of 0.44 and showed only one band on starch gel electrophotesis.[3] In very concentrated azurin solutions, a slight band may be present at 415 nm, indicating a trace of cytochrome impurity.

III. Preparation of Solutions

Buffered solutions were prepared as described above, except that the final ionic strength of the reactant solutions was 0.20 M, or 0.50 M for reactions involving $Co(4,7\text{-}[\phi\text{-}SO_3]_2\text{-}phen)_3^{3-}$. Buffers were prepared to contribute 0.05 M to the ionic strength of the solutions.

IV. Kinetic Measurements and Data Analysis

The kinetics of oxidation of azurin were followed at 625 nm ($\Delta\epsilon$ = 3500 [4]). Data collection and reduction followed the same course as outlined in Chapter I.

V. Other Equipment

All spectral data were acquired on a Cary 17 spectrophotometer. A Brinkman pH 101 instrument was used in all pH determinations.

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RESULTS AND DISCUSSION

I. Oxidation of Pseudomonas aeruginosa Azurin(I) by Tris-(1,10-Phenanthroline)cobalt(III)

First-order plots of the absorbance-time data observed at 625 nm are linear for greater than 90% of the reaction of azurin(I) with $Co(phen)_3^{3+}$. Evidence was not found for more than one form of reduced azurin. The first-order dependence of observed rate constants on the $Co(phen)_3^{3+}$ concentration is illustrated in Figure 1. The least-squares slope of the data of Figure 1 gives a second-order rate constant $k = (3.20 \pm 0.05) \times 10^3$ $M^{-1}sec^{-1}$ for the oxidation of P. aeruginosa azurin(I) in 0.05 M pH 7.0 phosphate buffer of ionic strength 0.2 at 25°. The rate law obtained for the oxidation of azurin by $Co(phen)_3^{3+}$ is

$$\frac{-d[Az(I)]}{dt} = k[Az(I)][Co(phen)_3^{3+}] \qquad (1)$$

The activation parameters at pH 7.0 in phosphate buffer obtained from the plot of log (k/T) vs (1/T) shown in Figure 2 are $\Delta H^{\ddagger} = 14.3 \pm 0.5$ kcal mol^{-1}, and $\Delta S^{\ddagger} = +7 \pm 1$ cal $deg^{-1}mol^{-1}$.

The rate of oxidation of azurin(I) by $Co(phen)_3^{3+}$ decreases with increasing ionic strength at pH 7.0, as would be expected for a reaction between oppositely charged particles. Although the ionic strength interval is well above the upper limit demanded by theory, a linear relationship was found between log k and $\sqrt{\mu}$. A plot of log k vs $\sqrt{\mu}$ yields a satisfactory straight line (Figure 3), from which an

Figure 1. Plot of k_{obsd} vs $[Co(phen)_3^{3+}]$ for the oxidation of *P. aeruginosa* azurin(I) [25°, pH = 7.0 (phosphate), μ = 0.2 M (NaCl)]. All reactions were followed at 625 nm. Points plotted in all figures represent averages of at least four data points.

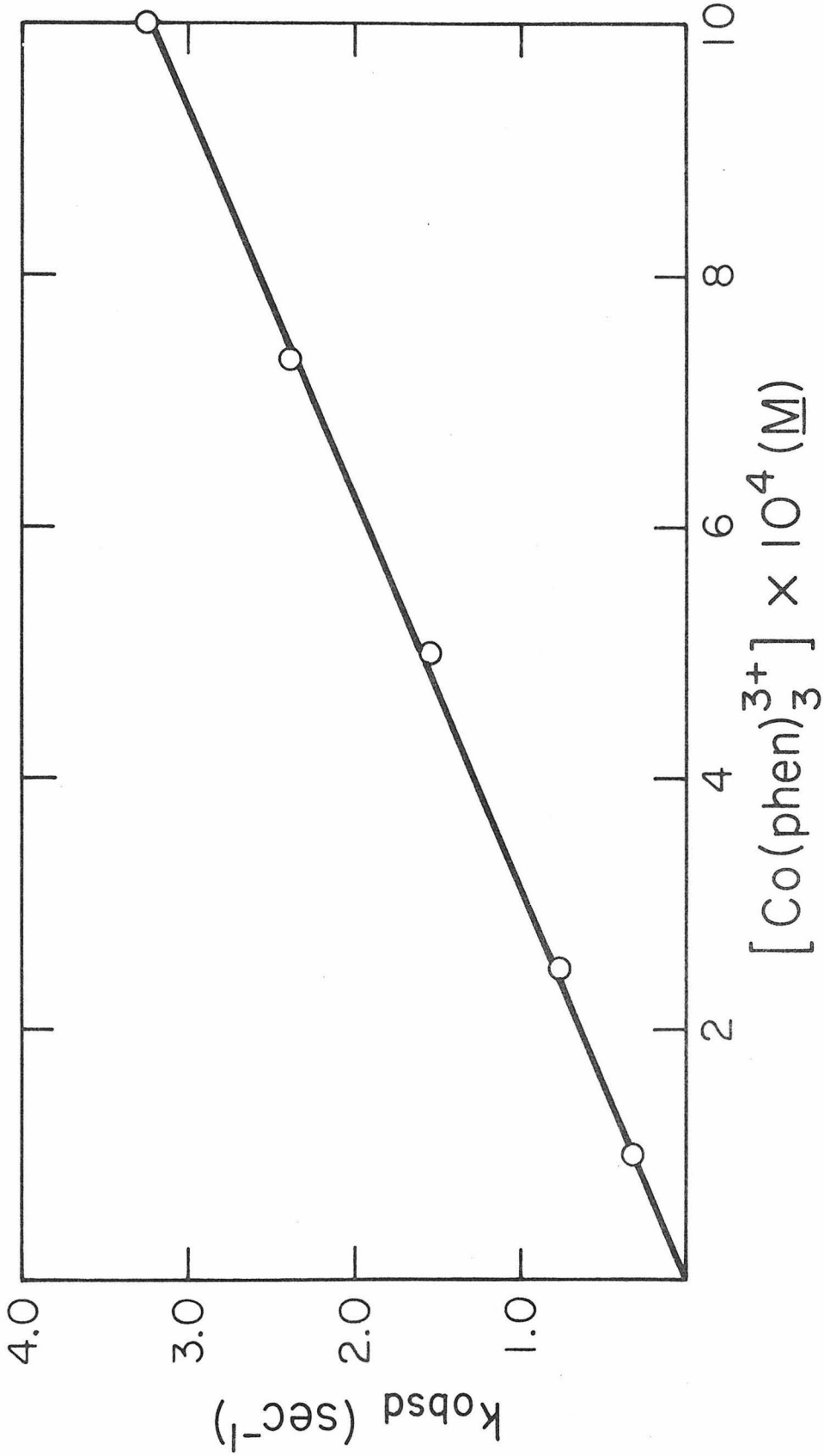

4.0
3.0
2.0
1.0
k_{obsd} (sec^{-1})
2
4
6
8
10
$[Co(phen)_3^{3+}] \times 10^4$ (M)

Figure 2. Eyring plot of rate data for the oxidation of P. aeruginosa azurin(I) by $Co(phen)_3^{3+}$ [pH = 7.0 (phosphate), $\mu = 0.2$ (NaCl)]. ○, $[Co(phen)_3^{3+}] = 1.0 \times 10^{-4}$ M; Δ, $[Co(phen)_3^{3+}] = 2.5 \times 10^{-4}$ M; ×, $[Co(phen)_3^{3+}] = 5.0 \times 10^{-4}$ M.

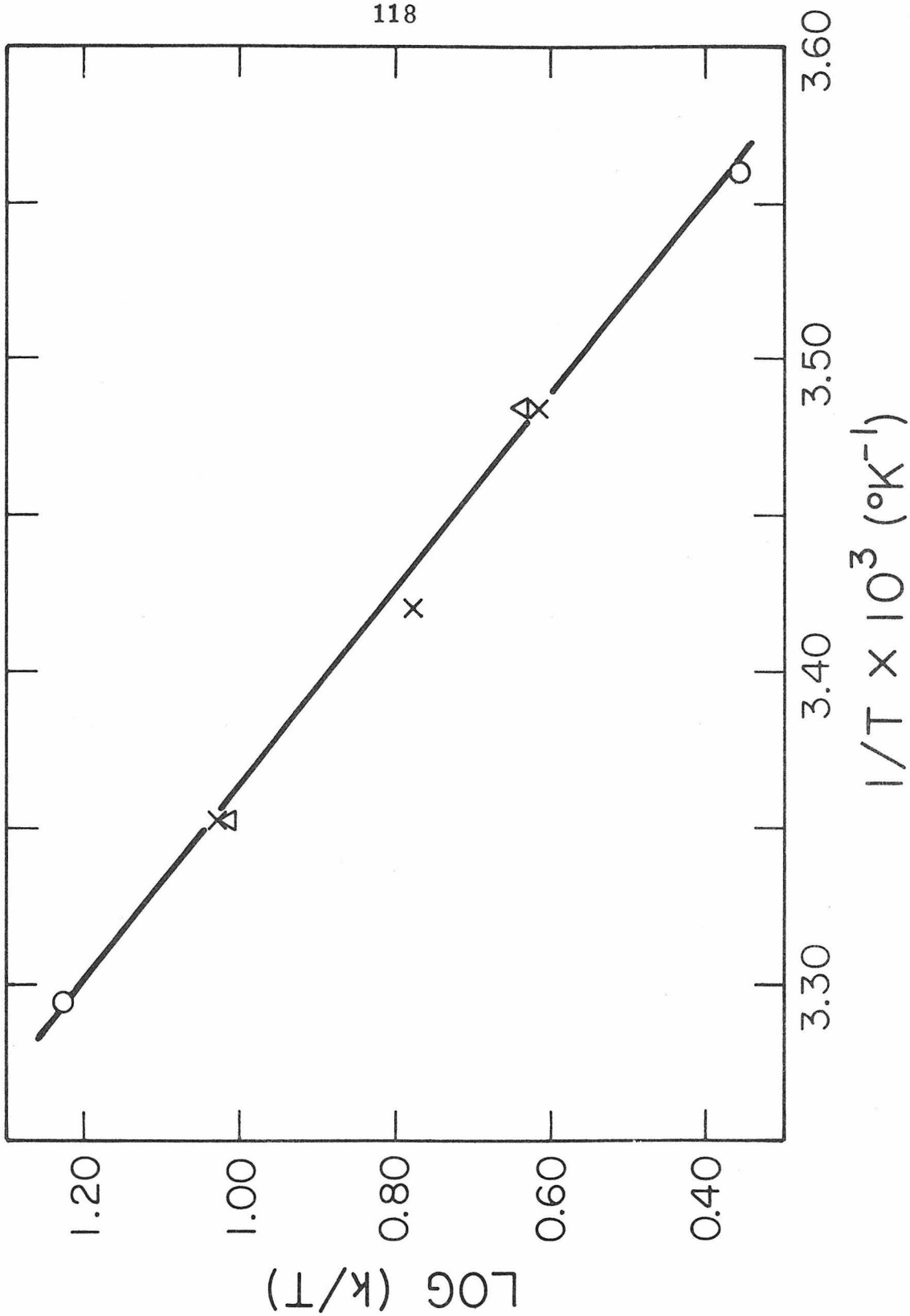
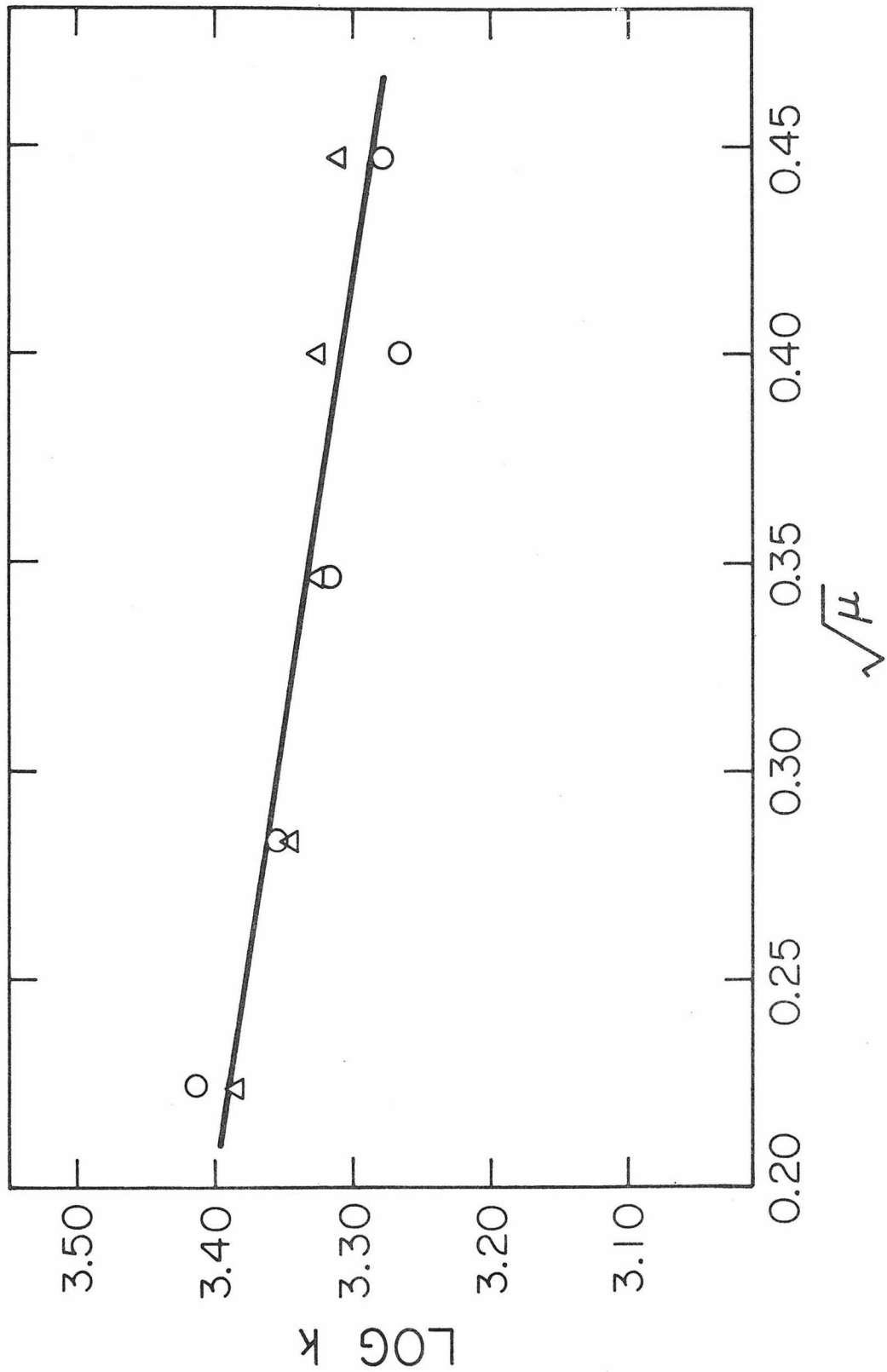

LOG (k/T)
1.20
1.00
0.80
0.60
0.40
3.30
3.40
3.50
3.60
1/T X 10^3 (°K^-1)

Figure 3. Plot of log k vs $\sqrt{\mu}$ for the oxidation of P. aeruginosa azurin(I) by $Co(phen)_3^{3+}$ [25°, pH = 7.0 (phosphate).] ○, [$Co(phen)_3^{3+}$] = 1.0×10^{-3} M; Δ, [$Co(phen)_3^{3+}$] = 2.0×10^{-3} M.


LOG K
3.50
3.40
3.30
3.20
3.10
0.20
0.25
0.30
0.35
0.40
0.45
$\sqrt{\mu}$

active site charge of azurin(I) may be calculated to be -0.2.[1] Since the isoelectric point of azurin is 5.70 (reference 2), it is clear that, analogous to results obtained with horse heart cytochrome c and P. aeruginosa cytochrome c_{551}, the calculated active site charge of azurin is near zero and much less than the overall charge of the protein. Even though this result agrees with an earlier value of -0.1 for the active site charge of azurin(II),[3] it is mentioned once again that caution must be used in the interpretation or application of this parameter.

The self-exchange rate of azurin has not been determined, except that H. A. O. Hill (private communication) has demonstrated the rate to be too slow to observe on the NMR time scale ($\leq 10^3$ $\underline{M}^{-1}$ sec^{-1}). However, a self-exchange rate may be calculated by employing the Marcus equation and the known cross reaction rate of azurin with its physiological partner cytochrome c_{551}. The Marcus equation is

$$\log k_{12} = 0.5[\log k_{11} + \log k_{22} + 16.9\ \Delta E^\circ] \qquad (2)$$

The cross reaction rate, k_{12}, has been measured as 6.2×10^6 $\underline{M}^{-1}$ sec^{-1}.[4] The self-exchange rate, k_{11}, of cytochrome c_{551} was calculated above to be 2.40×10^7 $\underline{M}^{-1}$ sec^{-1}. Taking the reduction potential of cytochrome c_{551} to be +0.26 (reference 5) and that of azurin to be +0.33 (reference 2), the self-exchange rate of azurin is calculated to be 1.17×10^5 $\underline{M}^{-1}$ sec^{-1}. This parameter is in total disagreement with the experimental observation of Hill. Nevertheless, we may extend the calculations. Using the Marcus equation again, a cross-reaction rate for the oxidation of azurin(I) may now be calculated.

The self-exchange rate of $Co(phen)_3^{3+}$ extrapolated to 25° from the data of Baker, et al.,[6] is 2.1×10^1 $\underline{M}^{-1}$ sec^{-1}, and the reduction potential is +0.42V.[7] The calculated rate of oxidation of azurin(I) by $Co(phen)_3^{3+}$ is 9.4×10^3, in remarkable agreement with experiment.

The large discrepancy between the calculated self-exchange rate of azurin and the upper limit of the rate set by experiment may at first appear discouraging. Cytochrome $\underline{c}_{551}$ and $Co(phen)_3^{3+}$ both "see" an "effective" self-exchange rate of azurin of $\sim 1 \times 10^5$ $\underline{M}^{-1}$ sec^{-1}, while in fact azurin(I) may not transfer an electron to azurin(II) at a rate greater than 10^3 $\underline{M}^{-1}$ sec^{-1}. Apparently, the site of electron transfer of one azurin molecule is not well approached by that of another azurin molecule. Further, the similarity of reactivity of cytochrome $\underline{c}_{551}$ and $Co(phen)_3^{3+}$ toward azurin is strong evidence that electron transfer involving cytochrome $\underline{c}_{551}$ occurs through the heme edge.

The Marcus calculations may be repeated to include electrostatic work terms according to the equation given by Haim and Sutin[8]:

$$\Delta G_{12}^{*} = 0.5[\Delta G_{11}^{*} - w_{11} + \Delta G_{22}^{*} - w_{22} + \Delta G_{12}^{\circ} + w_{12} + w_{21}]$$

Including charge effects, the self-exchange rate of azurin is calculated to be 1.21×10^{5}, and the rate of oxidation of azurin by $Co(phen)_3^{3+}$ is calculated to be 18.0×10^{3} $\underline{M}^{-1}sec^{-1}$. Corrections for charge effects are seen to be small but not to improve agreement between calculated and observed parameters. Again, the validity of the protein active site charge is suspect.

The agreement of Marcus theory and experiment for the oxidation of azurin by $Co(phen)_3^{3+}$ suggests the occurrence of ordinary outer-sphere electron transfer processes. Considering the derivation of the azurin self-exchange rate, simple electron transfer is also suggested for the reaction between cytochrome $\underline{c}_{551}$ and azurin, in agreement with a recent proposal.[9] Further, it is apparent that the reaction between azurin and cytochrome $\underline{c}_{551}$ or $Co(phen)_3^{3+}$ involves electron transfer through the conjugated ring system of either of the latter two reagents. Agreement of Marcus theory and experiment also indicates that electron transfer into and out of azurin occurs at the same site. That this site is near to the buried hydrophobic copper-binding environment of azurin is evidenced by the large

enthalpy of activation and positive entropy of activation required for either reduction by cytochrome $\underline{c}_{551}$ ($\Delta H^{\ddagger}$ = 10.6 kcal mol^{-1}, $\Delta S^{\ddagger}$ = +8.6 e.u.[10]) or oxidation by $Co(phen)_3^{3+}$ ($\Delta H^{\ddagger}$ = 14.3 kcal mol^{-1}, $\Delta S^{\ddagger}$ = +7 e.u.). Apparently a considerable degree of protein activation is required for azurin to efficiently accept electron transfer agents.

II Oxidation of Pseudomonas aeruginosa Azurin(I) by Tris Complexes of Modified 1,10-Phenanthroline and Cobalt(III)

If electron transfer to $Co(phen)_3^{3+}$ from azurin(I) involves attack of the active site of azurin and electron conductance through the π-system to cobalt(III), then steric hindrance of the phenanthroline rings should markedly affect the parameters of electron transfer. Therefore, kinetic studies were performed on the oxidation of azurin(I) by tris complexes of modified 1,10-phenanthroline with cobalt(III). The important variations of the properties of the oxidants with the different ring substitutions have been presented above in Part III of the Results and Discussion section of Chapter I of this thesis and should be kept in mind here. For convenient reference Table IV of Chapter I containing kinetic parameters for the oxidation of $Co(terpy)_2^{2+}$ and $Ru(NH_3)_5py^{2+}$ by the various oxidants is repeated here as Table I. Table II contains the kinetic parameters for the oxidation of azurin(I).

First-order plots of the absorbance-time data observed at 625 nm are linear for about 90% of the reaction of azurin(I) and $Co(5\text{-Cl-phen})_3^{3+}$. The rate law obtained is first-order in each reagent and gives a second-order rate constant of $(4.21 \pm 0.05) \times 10^2 \underline{M}^{-1} sec^{-1}$.

Table Ia. Kinetic Parameters for the Oxidation of $Co(terpy)_2^{2+}$ [25°, pH = 7.0 (phosphate), μ = 0.5 (NaCl)].

Oxidant	$k \times 10^{-2}$, $M^{-1}sec^{-1}$	$\Delta H^{\ddagger}$, kcal mol^{-1}	$\Delta S^{\ddagger}$ cal deg^{-1} mol^{-1}
$Co(phen)_3^{3+}$	4.16 (8)[a]	6.6(5)	-24(1)
$Co(5\text{-Cl-phen})_3^{3+}$	0.77	10.9	-13
$Co(5,6\text{-Me}_2\text{-phen})_3^{3+}$	1.22	6.7	-26
$Co(4,7\text{-Me}_2\text{-phen})_3^{3+}$	1.04	11.6	-11
$Co(4,7\text{-}[\phi\text{-SO}_3]_2\text{-phen})_3^{3-}$	265	2.6	-29

[a] Figure in parentheses marks the error of the last digit given.

Table Ib. Kinetic Parameters for the Oxidation of $Ru(NH_3)_5py^{2+}$ [25°, pH = 7.0 (phosphate), μ = 0.5 (NaCl)]

Oxidant	$k_1 \times 10^{-2}$, $M^{-1}sec^{-1}$	k_2, sec^{-1}	$\Delta H^{\ddagger}$, kacl mol^{-1}	$\Delta S^{\ddagger}$ cal deg^{-1} mol^{-1}
$Co(phen)_3^{3+}$	19.1(7)[a]	0.04(5)	5(1)	-24(2)
$Co(5\text{-Cl-phen})_3^{3+}$	9.0	0.15	3	-35
$Co(5,6\text{-Me}_2\text{-phen})_3^{3+}$	3.3	0.05	7	-24
$Co(4,7\text{-Me}_2\text{-phen})_3^{3+}$	3.0	0.02	8	-17
$Co(4,7\text{-}[\phi\text{-SO}_3]_2\text{-phen})_3^{3-}$	200	--	2	-32

[a] Figure in parentheses marks the error of the last digit given.

Table II. Kinetic Parameters for the Oxidation of P. aeruginosa Azurin(I). [25°, pH = 7.0 (phosphate), μ = 0.2 (NaCl)].

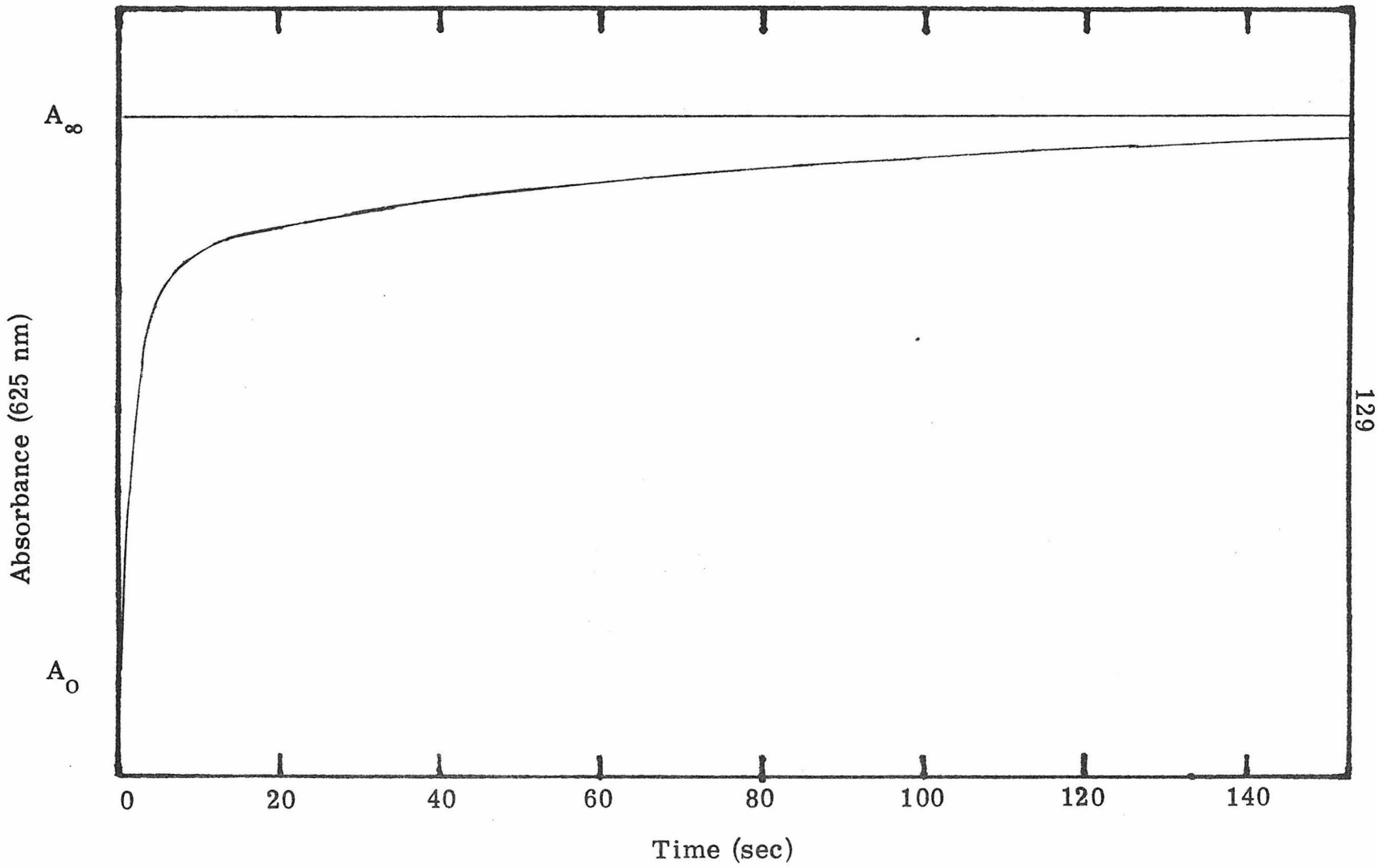
Oxidant	$k \times 10^{-3}$, $M^{-1}sec^{-1}$	$\Delta H^{\ddagger}$, kcal mol^{-1}	$\Delta S^{\ddagger}$, cal $deg^{-1}mol^{-1}$
$Co(phen)_3^{3+}$	3.20(5)[a]	14.3(5)	+7(1)
$Co(5\text{-Cl-phen})_3^{3+}$	0.421	8.0	-17
$Co(5,6\text{-Me}_2\text{-phen})_3^{3+}$	1.54	11.6	-5
$Co(4,7\text{-Me}_2\text{-phen})_3^{3+}$	0.0841	9.9	-17

[a] Figure in parentheses marks the error of the last digit given.

The rate is thus reduced by a little more than a factor of seven compared to oxidation by $Co(phen)_3^{3+}$. Quite large changes in the activation parameters are observed. The value of $\Delta H^{\ddagger}$ decreases by more than 5 kcal mol^{-1}, and $\Delta S^{\ddagger}$ decreases by about 24 e.u. These changes are large even considering the anomalous behavior of the $Co(5\text{-Cl-phen})_3^{3+}$ oxidant (see Table IV and discussion in Part III of Chapter I above). Considering the proposed nature of the active site of azurin, a reasonable explanation of the present data is that $Co(5\text{-Cl-phen})_3^{3+}$ cannot be accommodated as near to the electron transfer site as $Co(phen)_3^{3+}$ so that electron exchange takes place over a larger distance resulting in lowered rate, and lowered enthalpy and entropy of activation.

Oxidation of azurin(I) by either $Co(5,6\text{-Me}_2\text{-phen})_3^{3+}$ or $Co(4,7\text{-Me}_2\text{-phen})_3^{3+}$ displays kinetics which are slightly complicated. Spectral changes observed at 625 nm shows two distinct phases: an oxidant concentration dependent phase which accounts for about 65% of the observed change in absorbance, and an oxidant concentration independent phase (Figure 4). The oxidant concentration dependent phase gives k_{obsd} values which when plotted against oxidant concentration yield straight lines with zero intercepts. The purity of the relevant azurin(I) solution was checked by observing monophasic kinetics upon oxidation with $Co(phen)_3^{3+}$ and acquiring an accurate k_{obsd} value. A similar test was performed using the oxidant and $Co(terpy)_2^{2+}$. Despite these tests, the observed biphasic kinetics may be attributable to impurities.

Figure 4. Experimental plot of absorbance vs time for the oxidation of azurin(I) by 2×10^{-4} M $Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$.


A∞
A0
Absorbance (625 nm)
0
20
40
60
80
100
120
140
Time (sec)

The second phase of the reaction was difficult to characterize because of its slowness and the small absorbance change involved (Figure 4). The first-order rate obtained for the second phase is $0.01\ sec^{-1}$ with $\Delta H^{\ddagger} = 4 \pm 1$ kcal and $\Delta S^{\ddagger} = -54 \pm 2$ cal $deg^{-1} mol^{-1}$. A mechanism which explains the observed results is as follows:

$$Az(I) \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} Az'(I)$$

$$Az(I) + ox^{3+} \xrightarrow{k_1} Az(II) + ox^{2+}$$

Az'(I) is some isomerized form of reduced azurin which is not oxidizable. The proposed mechanism is identical to that proposed by Wilson, et al.,[4] but the rates of isomerization do not agree at all ($40\ sec^{-1}$ vs $0.01\ sec^{-1}$). The complication observed with the cobalt oxidants must not be related to that experienced by Wilson, and will not be considered further.

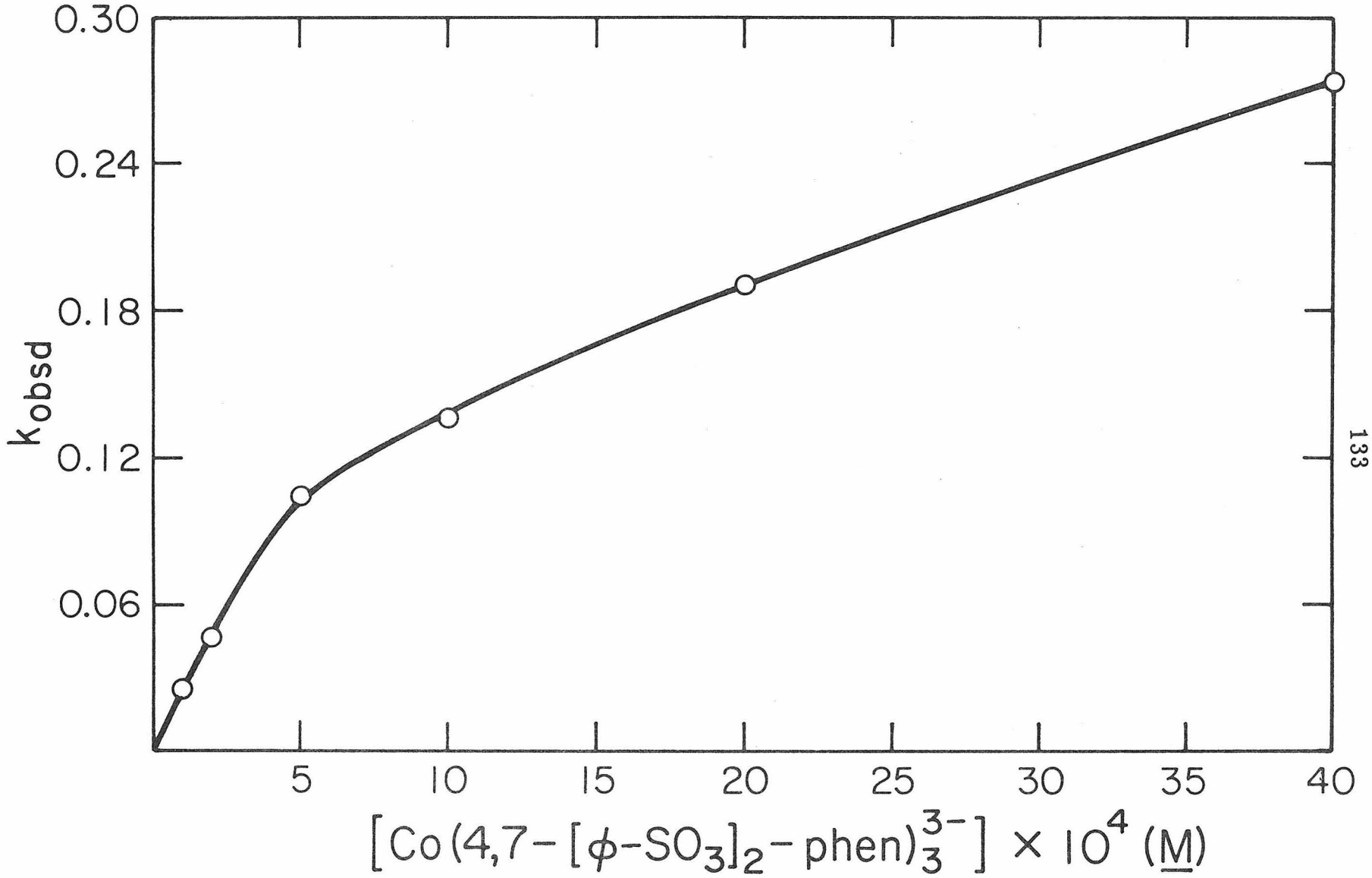
Oxidation of azurin(I) by $Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$ shows a decrease in rate of only a factor of two. Similar rate decreases were observed in other systems involving $Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$. However, the changes in the activation parameters for the oxidation of azurin(I) are quite striking. A decrease of about 2.5 kcal mol^{-1} is seen in $\Delta H^{\ddagger}$, and a decrease of about 12 e.u. is seen in $\Delta S^{\ddagger}$. For all other systems involved in this study, oxidation by $Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$ shows only small increases in activation parameters. The unique behavior of azurin(I) toward $Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$ may indicate that access to the active site of the protein is very restricted. The

5,6-dimethyl substitution on the phenanthroline rings may cause electron transfer to $Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$ to occur over a greater distance, yet oxidation by $Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$ proceeds at a rate nearly equal to that of oxidation by $Co(phen)_3^{3+}$.

The kinetic parameters of oxidation of azurin(I) by $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ may be interpreted in a manner similar to the above. The rate of electron transfer falls by a factor of forty, while $\Delta H^{\ddagger}$ decreases by 4 kcal mol^{-1} and $\Delta S^{\ddagger}$ decreases by about 24 e.u. The activation parameters may reflect the inaccessibility of the azurin(I) active site as well as the necessity of meeting the increased steric requirements for electron transfer to the $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ oxidant.

Oxidation of azurin(I) by $Co(4,7\text{-}[\phi\text{-}SO_3]_2\text{-}phen)_3^{3-}$ yields first-order plots that are linear for about 90% of the reaction. However, a plot of k_{obsd} vs $Co(4,7\text{-}[\phi\text{-}SO_3]_2\text{-}phen)_3^{3-}$ concentration is not linear. Figure 5 shows such a plot. The data of Figure 5 were accumulated at an ionic strength of 0.5 M to try to limit charge effects. The plot remains non-linear even at an ionic strength of 1.0M. The value of the second-order rate constant obtained at low $Co(4,7\text{-}[\phi\text{-}SO_3]_2\text{-}phen)_3^{3-}$ concentration is $2.5 \times 10^2\ M^{-1}sec^{-1}$, more than an order of magnitude lower than the value found for oxidation by $Co(phen)_3^{3+}$. The six concentrated areas of negative charge on the oxidant may be responsible for both the reduction in rate and the non-linear behavior.

Figure 5. Plot of k_{obsd} vs $[Co(4,7\text{-}[\phi\text{-}SO_3]_2\text{-}phen)_3^{3-}]$ for the oxidation of P. aeruginosa azurin(I) [25°, pH = 7.0 (phosphate), μ = 0.5 M (NaCl)].


0.30
0.24
0.18
0.12
0.06
k_{obsd}
5
10
15
20
25
30
35
40
$[Co(4,7-[\phi-SO_3]_2-phen)_3^{3-}] \times 10^4$ (M)

Meaningful activation parameters could not be obtained.

The description of azurin obtained from a study of the oxidation of P. aeruginosa azurin(I) by tris complexes of 1,10-phenanthroline and modified 1,10-phenanthroline with cobalt(III) essentially agrees with other descriptions in the literature. The site of electron transfer for azurin is not very accessible and probably included in or adjacent to the hydrophobic environment of the copper atom. Thus the active site may be specific for a hydrophobic "bridge" or "probe" from the in vivo redox partners of azurin. Electron transfer most likely occurs through an outer-sphere type mechanism but requires considerable protein activation. The sulfur ligand may be involved in the electron transfer mechanism. If the other copper ligands are only oxygen and nitrogen, the path of approach to the hydrophobic environment for a redox reagent should lead to the sulfur-ligated side of copper. Confirmation of the above description and further details will have to await the completion of the X-ray structure determination of azurin.

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CONCLUDING REMARKS

Much of the foregoing discussion and interpretation of experimental results was centered upon calculations involving Marcus theory. It must be pointed out that all calculations were performed with only one experimentally determined self-exchange rate constant (that of horse heart cytochrome c). Protein-protein cross reactions were then used to obtain values of self-exchange rates for P. aeruginosa cytochrome c_{551} and azurin. The self-exchange rate of azurin may then be considered a "second-generation" value. Using k_{11} values derived from protein-protein reactions, the agreement found between experiment and theory for the $Co(phen)_3^{3+}$ oxidations is extraordinary. Most importantly, $Co(phen)_3^{3+}$ seems to be confirmed as an excellent reagent to investigate the mechanisms of electron transfer proteins. Further confirmation is taken from the similarity of activation parameters found for protein-protein redox reactions and those found for $Co(phen)_3^{3+}$ oxidation of proteins (large $\Delta H^{\ddagger}$, near zero $\Delta S^{\ddagger}$). Similar results are obtained for the oxidation of Chromatium high potential iron-sulfur protein.[1]

Marcus calculations which were performed to include charge interactions of the reagents based on the Haim-Sutin suggestion were seen not to improve the calculations. The calculations based on the Haim-Sutin equations may in a sense be regarded as a test of the active site charge parameters experimentally derived. If so viewed, the Haim-Sutin equations demonstrate the truth of the statement contained in the first report of ionic strength dependence studies that

active site charge "value(s) cannot be taken seriously."[2] The equations do support the idea that protein active site charges are small.

A totally contrasting picture may be presented for the reagent $Fe(EDTA)^{2-}$. Reduction of cytochrome $\underline{c}_{551}$ by $Fe(EDTA)^{2-}$ is experimentally observed to occur with a rate constant of 5.6×10^3 $\underline{M}^{-1}sec^{-1}$ (reference 3), while Marcus theory predicts a rate of 1.3×10^6. Reduction of azurin by $Fe(EDTA)^{2-}$ proceeds at a rate of 1.3×10^3 $\underline{M}^{-1}sec^{-1}$ (reference 4), while Marcus theory predicts a rate of 3.6×10^5 $\underline{M}^{-1}sec^{-1}$. Similarly disagreeable results are obtained for Chromatium HiPIP.[1] Calculations for electrostatic effects based on the present values of active site charges do little to improve correlation of theory and experiment. Charge interactions are not expected to be of importance in the calculations because all of the proteins are acidic. In addition, activation parameters for the reduction of azurin and HiPIP yield low values of $\Delta H^{\ddagger}$ and large negative values of $\Delta S^{\ddagger}$. Activation parameters have not yet been acquired for the reduction of cytochrome $\underline{c}_{551}$ by $Fe(EDTA)^{2-}$. The results have been interpreted in terms of the inability of $Fe(EDTA)^{2-}$ to attack buried protein redox centers.[1] In contrast to $Co(phen)_3^{3+}$, $Fe(EDTA)^{2-}$ lacks a suitable hydrophobic area to approach the active site.

A contradiction immediately arises from this explanation. The reduction of horse heart cytochrome $\underline{c}$ by $Fe(EDTA)^{2-}$ gives excellent correlation of simple Marcus theory and experiment, although the

reaction proceeds with a fairly low value of $\Delta H^{\ddagger}$. P. aeruginosa cytochrome c_{551} was proposed to have an electron transfer site of increased accessibility or increased flexibility relative to horse heart cytochrome c. Therefore, one would expect good correlation of theory and experiment for the $Fe(EDTA)^{2-}$ reduction of the bacterial protein also. Alternatively, the results of the $Fe(EDTA)^{2-}$ reduction of cytochrome c_{551} indicate that the active site of the bacterial protein is less accessible.

It appears that an explanation is needed to account for the slowness of the reduction of cytochrome c_{551} by $Fe(EDTA)^{2-}$. However, it must emphatically be pointed out that even the "flexible" cytochrome c_{551} in order to transfer an electron to $Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$ still requires 11 kcal mol^{-1} in activation enthalpy. The site of electron transfer is still not approached without considerable protein activation. The terms "more flexible"and "more accessible" should be applied only in reference to mammalian cytochrome c. Perhaps the real explanation required is that to account for the facility of the reduction of horse heart cytochrome c by $Fe(EDTA)^{2-}$.

Oxidized horse heart cytochrome c is the only protein of those studied whose active site charge (+1.7) is calculated to be greater than one unit.[2] Further, the difference of 10^3 in rate of reaction of mammalian and bacterial cytochrome toward azurin has been attributed to charge effects.[5] Perhaps, if the true value of the active site charge of horse heart cytochrome c were known, theory and experiment for reduction by $Fe(EDTA)^{2-}$ would disagree by the same order of magnitude

that is found for the reduction of cytochrome $\underline{c}_{551}$. Indeed, if the true active site charge of horse heart cytochrome $\underline{c}$ is +3.4 (which should be considered within the error limits of the measured value), the rate of reduction is calculated to be 1.1×10^6 $\underline{M}^{-1}\text{sec}^{-1}$, compared to the value of $2.0 \times 10^4 \underline{M}^{-1}\text{sec}^{-1}$ calculated from simple Marcus theory, and 2.6×10^4 $\underline{M}^{-1}\text{sec}^{-1}$ which is the observed value. Allowance should be made for charge interactions for the reduction by $Fe(EDTA)^{2-}$ for horse heart cytochrome $\underline{c}$ rather than cytochrome $\underline{c}_{551}$ because the mammalian protein is more likely to be highly positvely charged based on pI values (10 for horse heart cytochrome $\underline{c}$[6] and 5.70 for the bacterial protein[7]). Thus it is possible that $Fe(EDTA)^{2-}$ reduction of the various proteins also follows a consistent pattern.

Agreement with a statement made by Rosenberg, et al.,[8] is found for all the data in this work. Efficient electron transfer occurs with close approach of the redox sites, high values of $\Delta H^{\ddagger}$, and near zero values of $\Delta S^{\ddagger}$. Inefficient redox reactions are not permitted close approach of the reactive centers; therefore, electron transfer occurs over a larger distance with very negative values of $\Delta S^{\ddagger}$ but with low values of $\Delta H^{\ddagger}$. A single exception to this statement may be seen in the reaction of $Co(4,7\text{-}Me_2\text{-phen})_3^{3+}$ with cytochrome $\underline{c}_{551}$. Electron transfer remains "efficient" (relative to the corresponding parameters obtained with horse heart cytochrome $\underline{c}$) through a reduction in $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. An explanation has been adequately given in the text above.

It is interesting to speculate about the fact that small hydrophilic reagents have poor accessibility to the active sites of electron transfer proteins. Physiological efficiency may gain from having small water soluble molecules be poor mimics of the *in vivo* redox reagents.

Lastly, I should like to introduce a very sensitive subject. Some workers in the field of electron transfer proteins, not only at the California Institute of Technology, but probably in laboratories all over the world, have customarily referred to the removal of an electron from these proteins as the process of "*re*-oxidation." Of course, workers in foreign countries would use a corresponding term of their own language. I feel the term is degrading to those of us who have studied the oxidative mechanism because it implies that the primary purpose of these proteins is to be reduced. Thus it further implies that the process of oxidation is secondary. In fact, I believe the term has been propagated by the reductive bioinorganic chemists as a means of keeping their work at the forefront of attention of the world's scientific community, thereby ensuring themselves of a larger portion of the pie of scientific supporters. I hereby call for the unification and liberation of oxidative bioinorganic chemists and for the independence of oxidative processes from anything to do with reduction. Furthermore, I call for help in thinking up a catchy name with a meaningful abbreviation for our group. I shall end with a proposed slogan: Power from Oxidation!

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PROPOSITION I

Oxidative and Reductive Cleavage of Alkylcobalamins

Since the 1920's when it became obvious that animal liver contained a factor capable of curing megaloblastic anaemias,[1,2] intensive work to isolate, characterize, and synthesize that factor, now termed vitamin B_{12}, has been performed. To date vitamin B_{12} and its derivatives have been implicated in a large number of biochemical isomerization reactions, the deoxygenation of ribonucleotides, and the biosynthesis of methionine, as well as numerous other biochemical functions. The subject has recently been excellently reviewed by Hill[3] and Brown.[4]

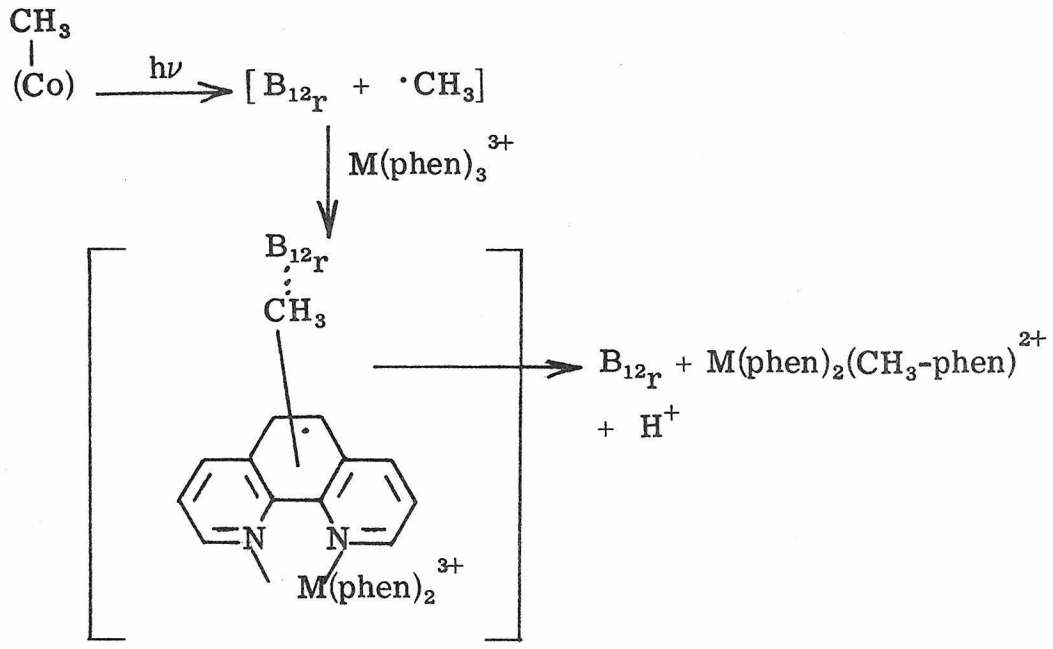
The complex and widely varied chemistry of vitamin B_{12} continues to evolve today. A great deal of current research focuses on the photolytic and thermolytic cleavage of the Co-C bond. Thus Pratt[5] found that the half-life of methylcobalamin could be increased from one minute to twenty hours by excluding oxygen during photolysis. Experimenting with the same complex labeled at the methyl ligand, Hogenkamp[6] reported that ^{14}C could be found reacted with the corrin ligand under high intensity of incident light. Further, it is thought that the photolysis of higher alkyls may proceed with the reduction of B_{12_r} by the alkyl radical following homolytic cleavage to give the observed product olefins.[4] All of these observations are consistent with an equilibrium of the type

$$\begin{matrix} R \\ | \\ (Co) \end{matrix} \underset{\longleftarrow}{\overset{h\nu}{\longrightarrow}} (\dot{C}o) + R\cdot$$

In the absence of another reagent, the equilibrium is stabilized by the solvent cage effect. However, if a suitable reagent is available for removing the alkyl radical, the equilibrium is destroyed and the reaction proceeds quickly to the right.

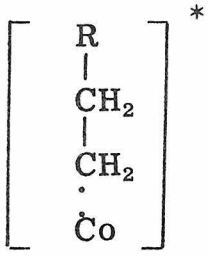
Nearly all the photolysis of alkylcobalamins has been performed in the presence of reducing agents. Oxidation of alkylcobalamins seems to have been nearly completely neglected. The only notable exceptions are the work of Johnson[7,8] and Halpern.[9] Halpern has suggested that oxidative cleavage of alkylcobalamins may also occur homolytically when $IrCl_6^{2-}$ is used as the oxidant.

If an oxidant or reductant that has the ability to react with an alkyl radical could be used in conjunction with the photolysis of alkylcobalamins, much information may be gained concerning the mechanism of alkyl transfer reactions. To study the photolysis of alkylcobalamins in the presence of oxidizing agents, complexes of the type $M(phen)_3^{3+}$ may be employed where M = Co, Rh, and Ru, and phen = 1,10-phenanthroline. Previous research has shown the great utility of using $Co(phen)_3^{3+}$ as an oxidant,[10] while $Ru(phen)_3^{3+}$ may prove too difficult to handle under the conditions normally found in the study of alkylcobalamins.[11] In analogy to the formation of toluene from the photolysis of methylcobalamin in the presence of benzene,[12] one very interesting sequence of reaction steps might be as follows:



It seems reasonable that $M(phen)_3^{3+}$ would find the lowest Franck-Condon energy barrier for electron transfer by approaching the Co near the alkyl bound position (for small R at least). Thus a "free" radical would have a high chance of encountering $M(phen)_3^{3+}$ before another reactive species. Alkylcobalamins other than methyl could also be tested.

Similarly, if photolytic decomposition of higher alkyls proceeds through some activated complex



before β-hydride elimination occurs (as suggested by Schrauzer[13]), substitution in an aromatic ring of $M(phen)_3^{3+}$ may result. In either

case, nucleophilic substitution in the aromatic ligand may be induced by the strongly electron withdrawing M^{3+}.

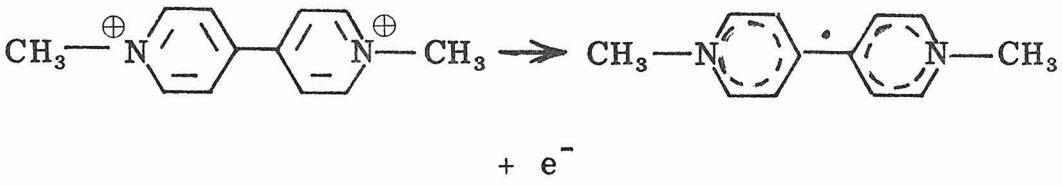
Specificity of ring position substitution as well as alkyl group configuration may be examined.[14] If ring substitution were specific for a certain position, that position could be blocked by a previous methyl substitution and the experiment could be repeated.

On the other hand, the correct reaction sequence (and the sequence that might be expected in the dark) could be

$$\overset{R}{\underset{|}{(Co)}} + M(phen)_3^{3+} \longrightarrow B_{12_a} + M(phen)_3^{2+} + R'$$

Electron transfer would then seem more likely to be occurring through the conjugated system of the corrin ligand. This mechanism is subject to investigation of the effect of increasing alkyl chain length upon electron transfer. When the chain increases to sufficient length to interfere with the electron transfer site, the rate of the redox reaction should decrease drastically. A locus of possible electron transfer sites could then be determined statistically.

Analogous considerations may be made for the reducing agent viologen and the various substituted viologens which yield fairly stable free radicals upon oxidation



Reductive cleavage therefore may also be studied in the presence of a free radical trap.

The reaction products of both oxidative and reductive cleavage may be analyzed stereochemically.[14] Details of the mechanism of either process thus appear to be accessible.

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PROPOSITION II

Synthesis of Complexes for the Study of Intramolecular Electron Transfer

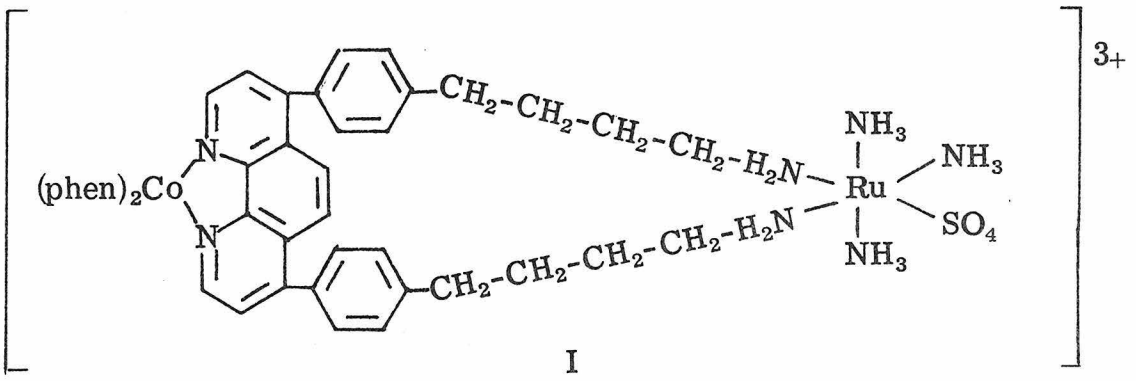
The process of electron transfer has been intensely investigated in the last decade. Much of the interest centers on the fact that electron transfer is intimately involved in ATP synthesis, the process of energy conservation that sustains life. Experimentally, the oxidation-reduction reactions which have been studied have been those reactions which occur intermolecularly. Intermolecular electron transfer appears, from considerations of the synthesis of the reagents, to be more convenient to study. However, rate and activation parameters are complicated by the necessity of rearranging to some degree the solvent and the reagents in a reactive collision.[1]

Intramolecular electron transfer, involving electron transfer from one metal center to another through a stable bridging system, thus is of special interest. Intramolecular electron transfer has proven difficult to observe experimentally, and results have been reported for only a few systems (see, for example, refs. 2,3,4). Ambiguities, however, are difficult to avoid without both metal centers having very substitution-inert bonds to the bridging ligand.

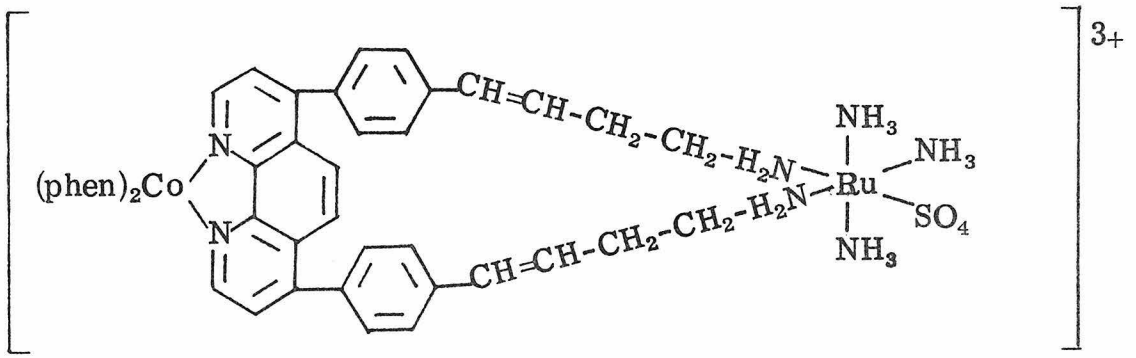
One successful method for preparing complexes for the study of intramolecular electron transfer has been described recently.[5] An extension of that method to the synthesis of novel binuclear complexes suitable for the study of intramolecular electron transfer is presented in this work. Two of these complexes (I and II) are of

value since the relative positions of the metal centers are known with certainty, while the group of complexes may provide information about the effect of conjugation on the rate of electron transfer.

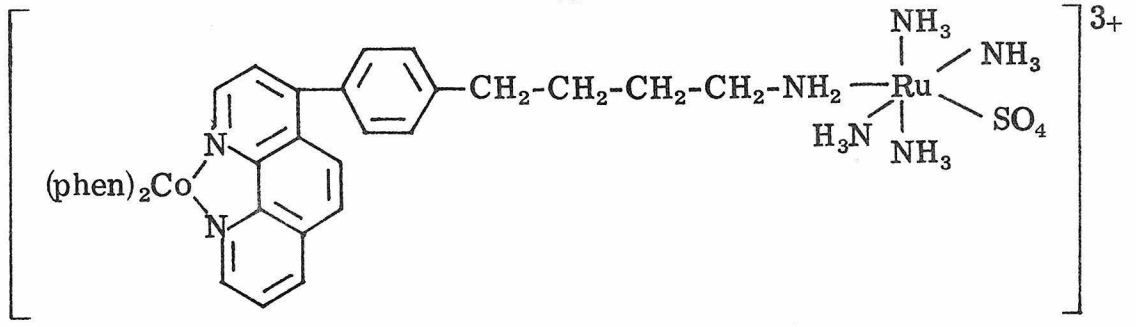
The final structures of the complexes to be prepared are given below:



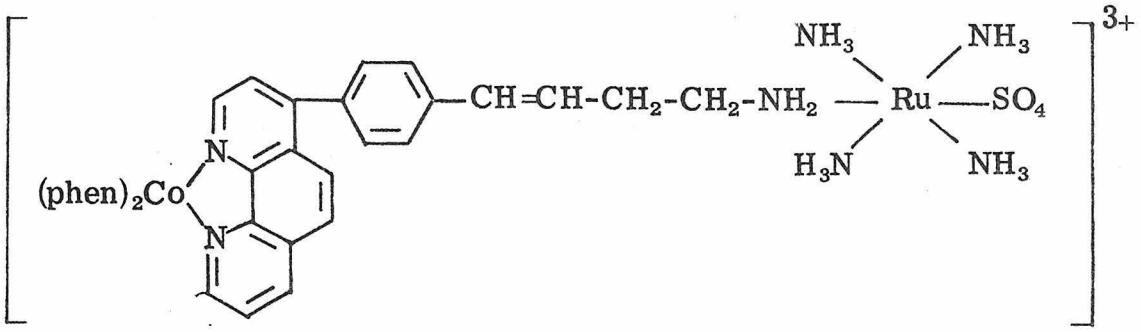
I



II



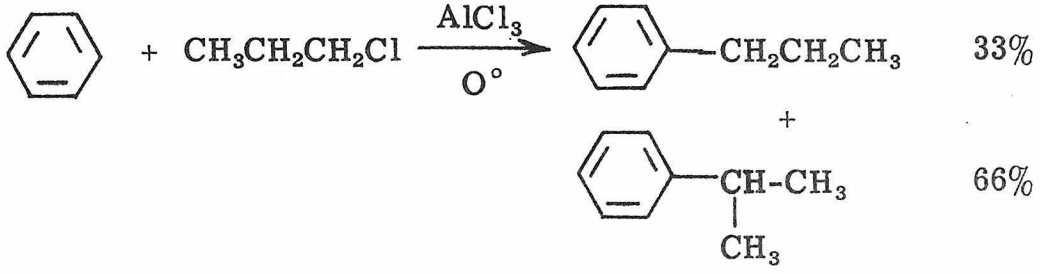
III

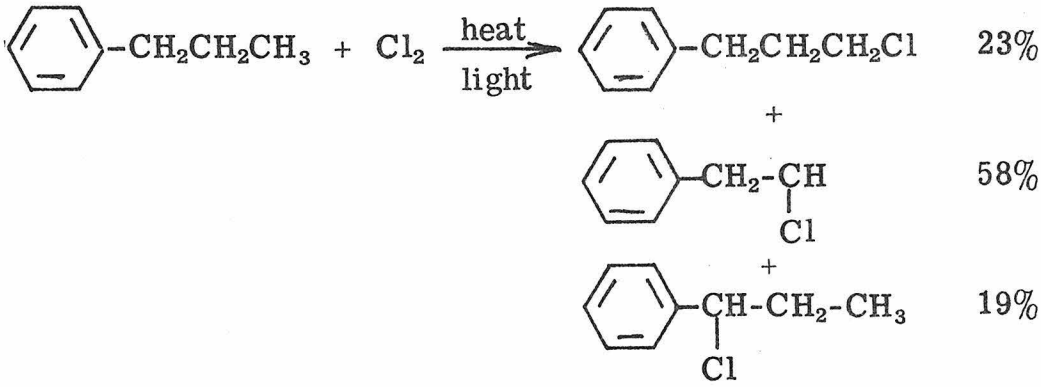


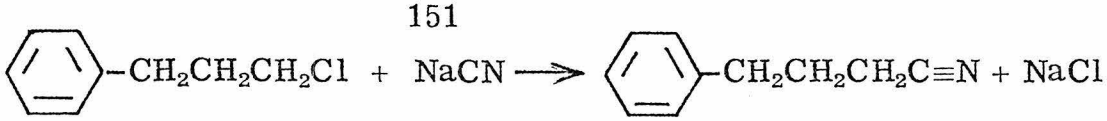
IV

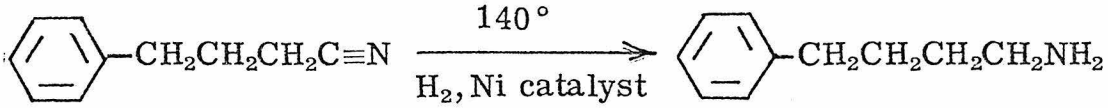
Molecular models indicate that the correct alkyl chain length in complexes I and II corresponds to four carbons.

The synthesis of each of the phenyl-alkylamines will now be given. The phenyl-alkylamines will subsequently be used in the synthesis of the various substituted phenanthrolines.

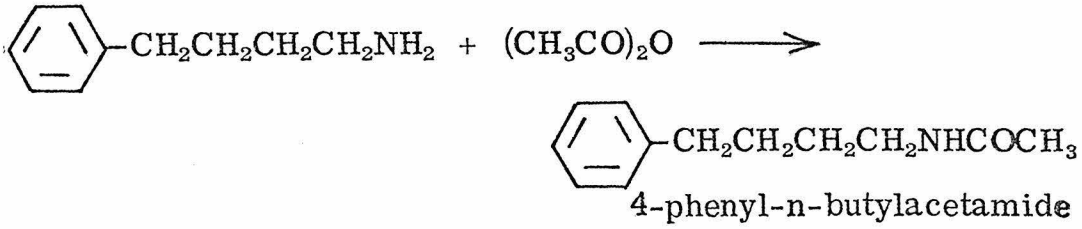
Ia. 

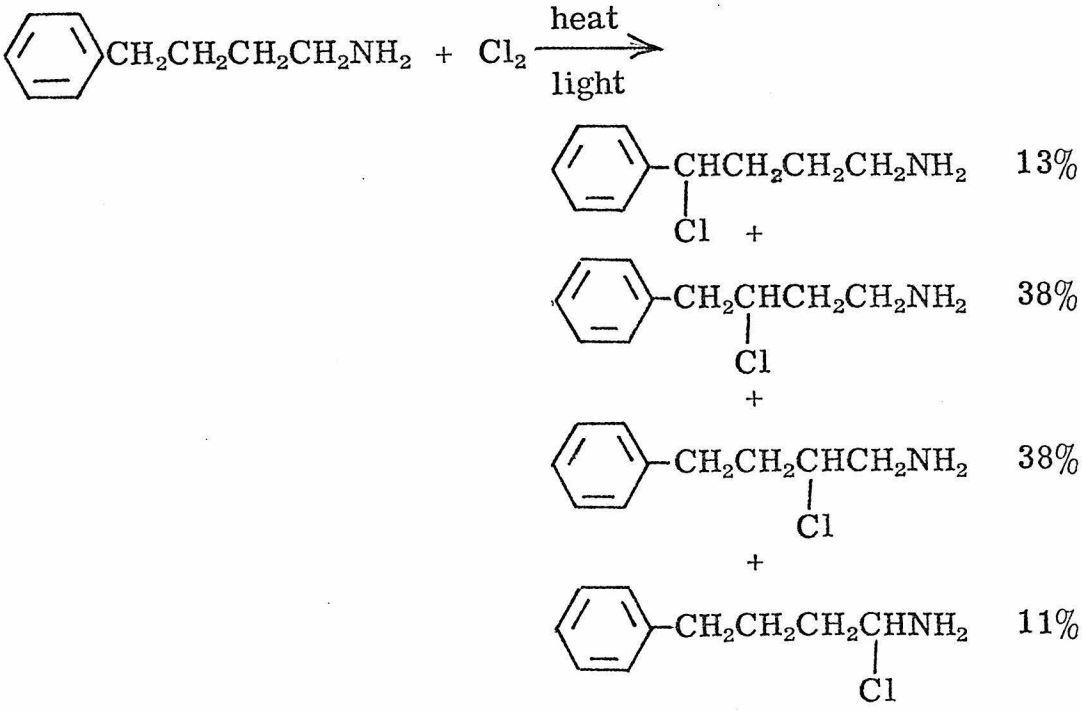
Ib. 

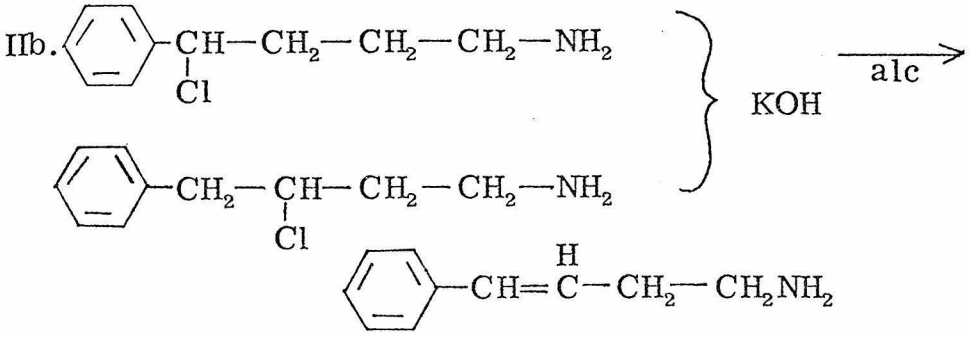
Ic. 

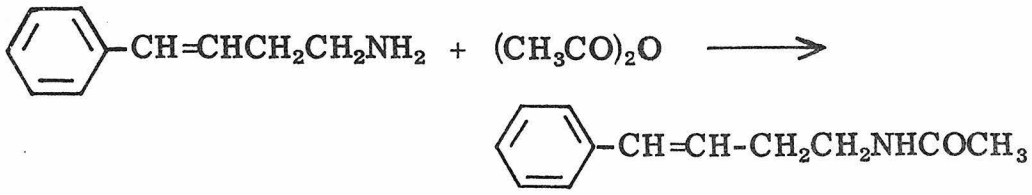
Id. 

The amino function must be protected from reaction in later steps.

Ie. 

IIa. 

IIb. 

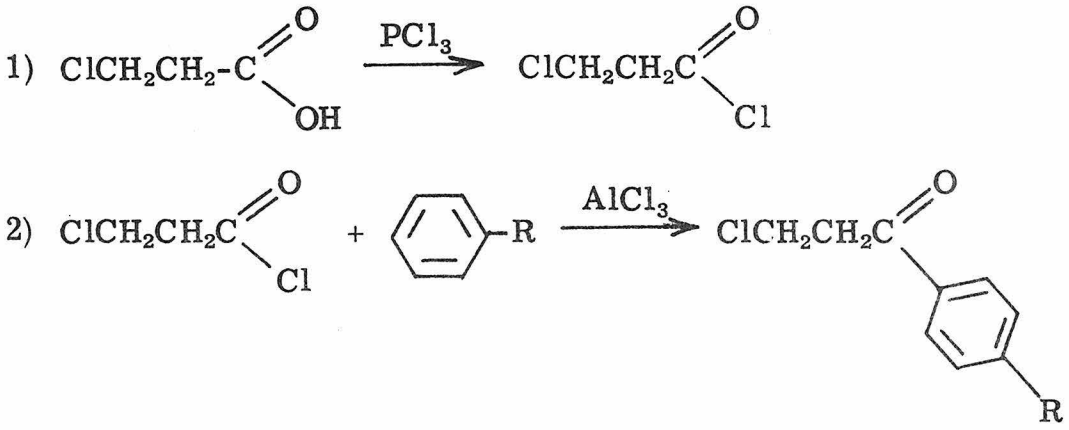
IIc. 

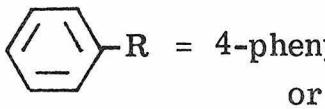
4-phenyl-3-butenylacetamide

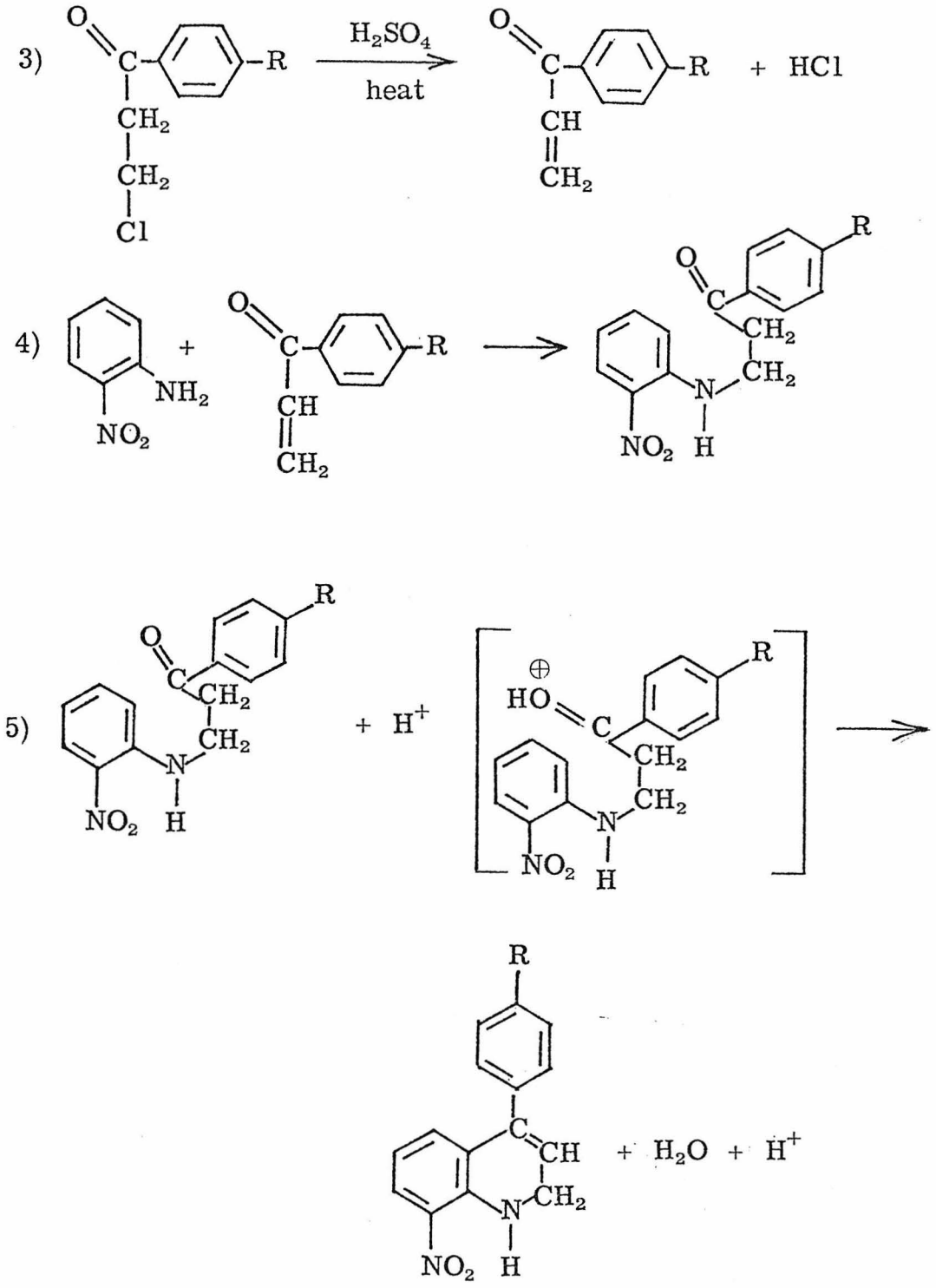
III. 4-phenyl-n-butylacetamide is prepared as in procedure I.

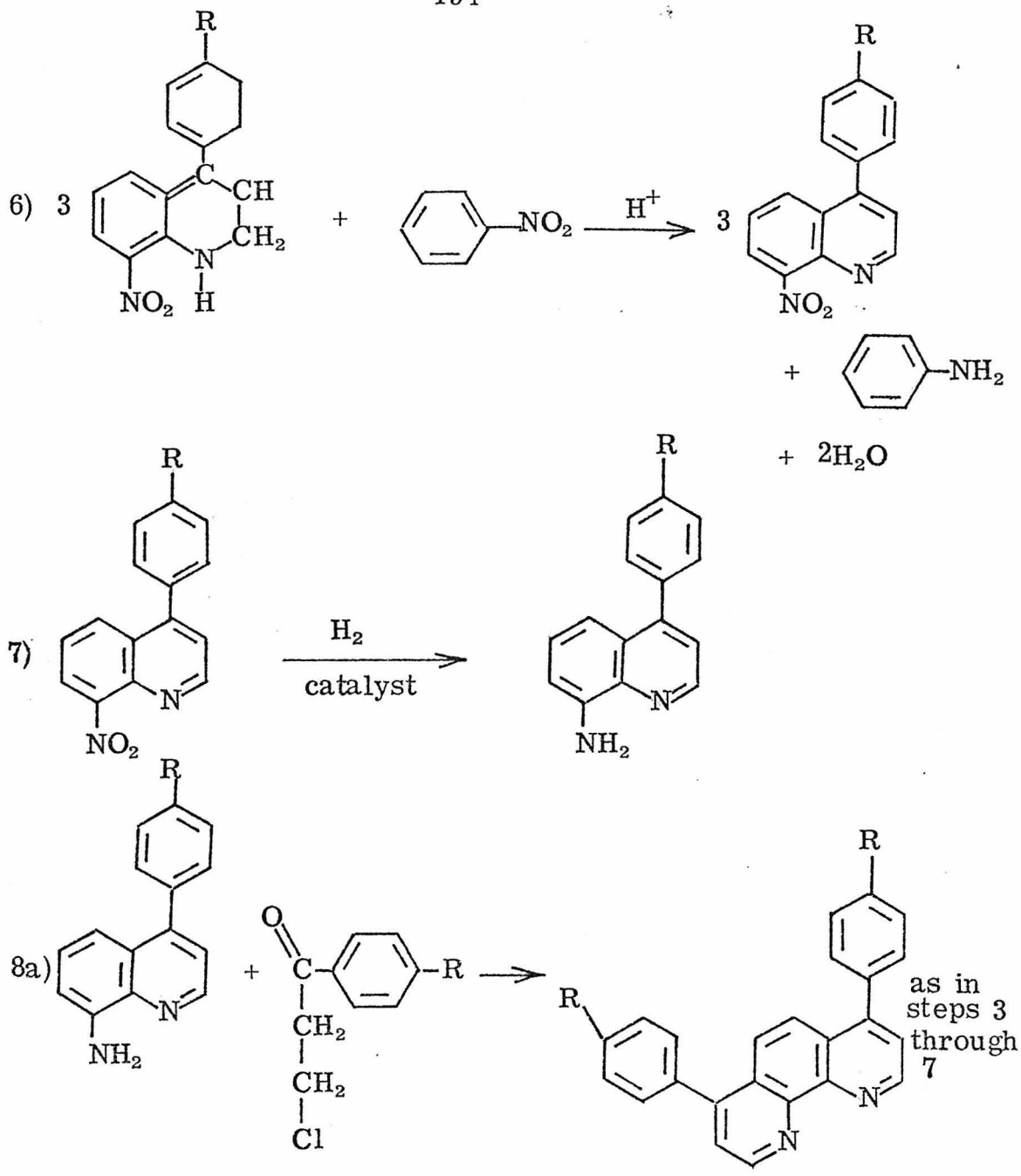
IV. 4-phenyl-3-butenylacetamide is prepared as in procedure II.

The synthesis of the substituted phenanthroline ligands will now be given. The substituted β-chloropropiophenone synthesis (steps 1 and 2) is analogous to that described by Conant and Kirner,[6] and the modified Skraup synthesis (steps 3 through 8) is similar to that described by Case.[7]

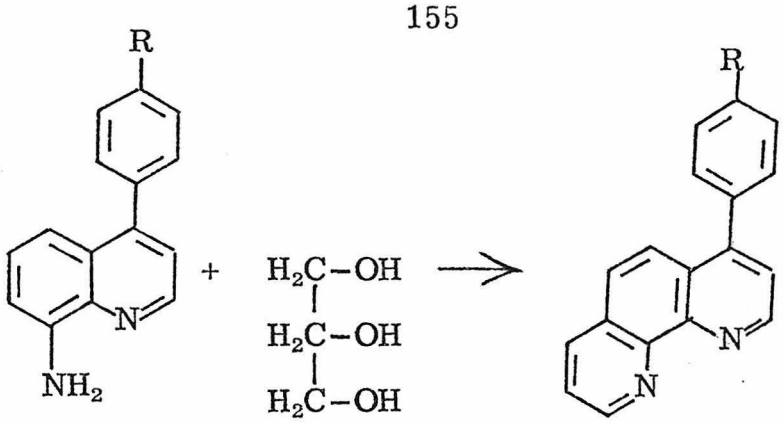


 = 4-phenyl-n-butylacetamide
or
4-phenyl-3-butenylacetamide


3)
H_2SO_4
heat
+ HCl
4)
5)
+ H^+
+ H_2O + H^+



1, 10-phenanthroline modified at the 4, 7 positions

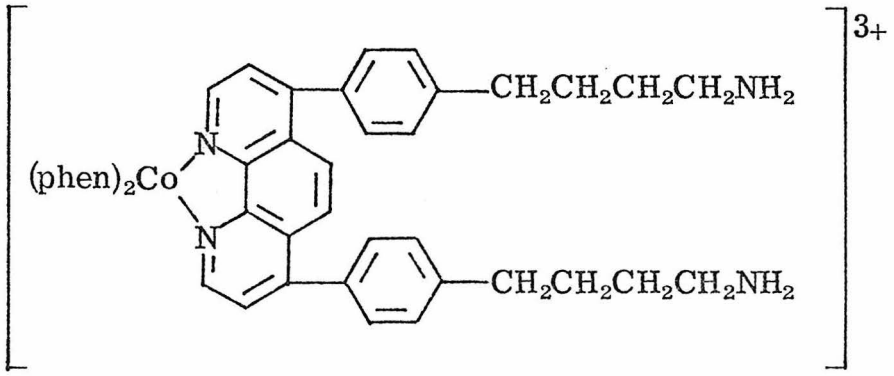
8b) 

analogous to steps 3 through 7

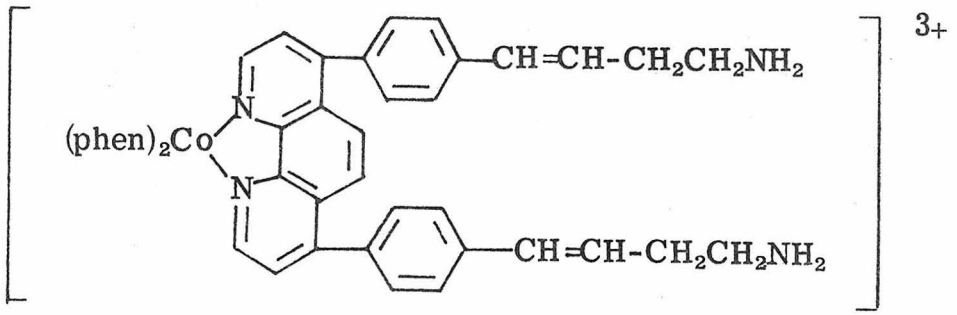
1,10-phenanthroline modified at the 4 position.

The products of steps 8a and 8b are recrystallized from benzene.

Each of the four modified phenanthroline ligands may then separately be refluxed with an excess of $[Co(phen)_2Cl_2]Cl \cdot 4H_2O$, prepared according to Pfeiffer and Werdelmann.[8] The products are recrystallized from methanol and water, redissolved in methanol and water, and treated with acid to replace the acetamide function with the amine function. Complexes V through VIII may then be recovered.



V



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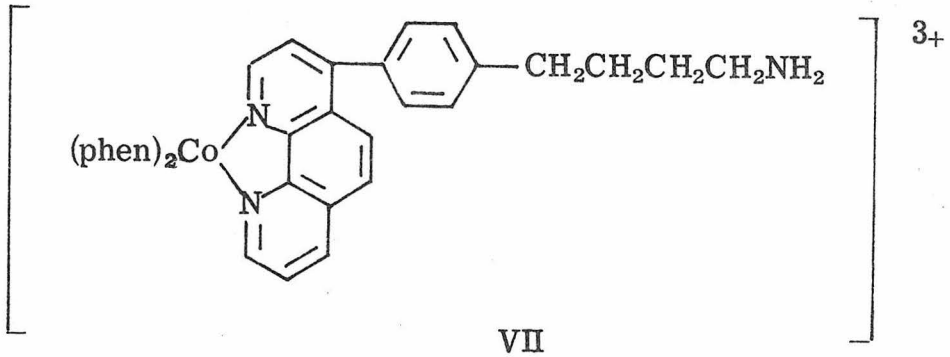
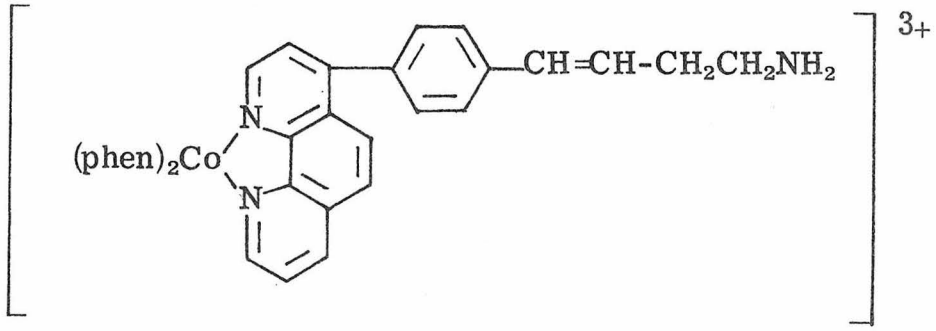


VII



VIII

Complexes V and VI are mixed with $Ru(NH_3)_3Cl_2(SO_2)$ in equimolar quantities, as are complexes VII and VIII with $[Ru(NH_3)_4Cl(SO_2)]Cl$. The ruthenium complexes are prepared according to the method detailed by Vogt, et al.[9] The SO_2 ligand of the ruthenium renders it very weakly reducing, so that intramolecular

electron transfer is very slow.[5] Following recrystallization of the Co(III)-Ru(II) complex, SO_2 is converted to SO_4^{2-} and Ru(II) is oxidized to Ru(III) by dissolving the complex in 1 M HCl and adding H_2O_2. The chloride salts of complexes I through IV may then be crystallized.

The complexes I through IV may be reduced by $Ru(NH_3)_6^{2+}$. The Ru(III) center is expected to be reduced much more rapidly than the Co(III) center (approximately 10^4 times faster).[5,10,11,12] The first order process of intramolecular electron transfer will then become spectrophotometrically observable.

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PROPOSITION III

Hindrance of Mycobacterium tuberculosis

Iron almost certainly is required by all living cells. Yet the solubility of Fe(II) and Fe(III) at physiological conditions is very poor. Microorganisms accomplish the uptake and transport of inorganic iron by a group of closely related chelating agents called siderochromes. The structural feature found throughout the siderochromes is that the iron is usually complexed to the ligand through three hydroxamic acid groups, through three phenolate groups, or some combination of the two. A class of siderochrome which displays binding of iron by more than one type of functional group is mycobactin, whose structure is shown in Figure 1.[1]

Mycobacteria have been recognized for a long time as pathogens. Despite the severity of some mycobacteria infections, certain strains of mycobacteria are not able to produce their own mycobactin, and are thus dependent upon other mycobacteria for this growth factor. One such mycobacteria is the cause of leprosy, and another is the cause of Johne's disease. The pathogen Mycobacterium tuberculosis has been implicated as the supplier of mycobactin for other mycobacteria.[1]

Raffel[2] first suggested that a virulent strain of tubercle bacillus may be distinguished from an avirulent strain only by being better able to use the nutrients of the host, and not by being better able to interfere with the host's defense mechanism. Kochan has summarized in a recent review[3] the relationships among

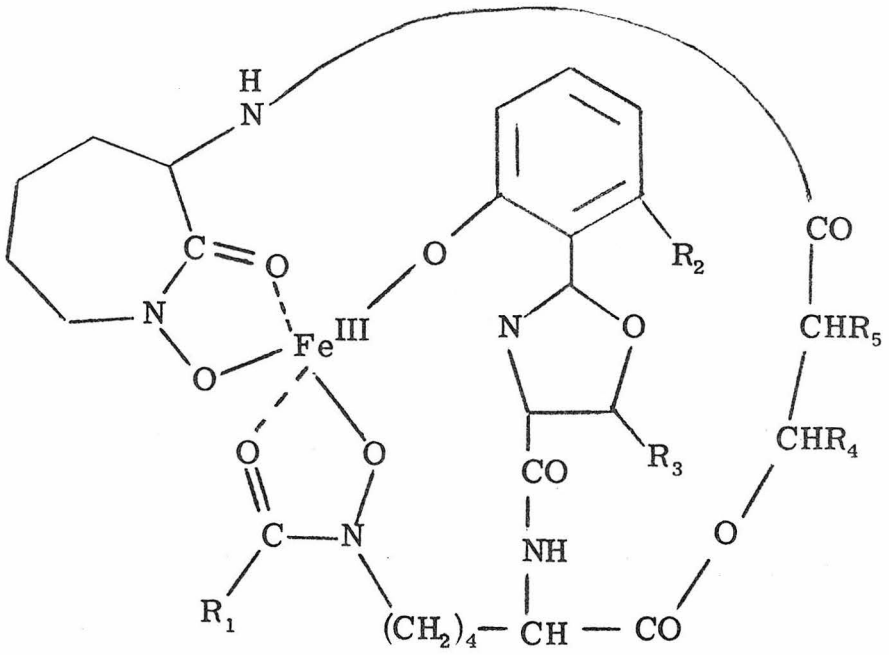

Figure 1. The structure of ferric mycobactin. R_1 through R_5 may vary depending upon the specific mycobacteria from which the mycobactin is extracted.

iron concentration, transferrin concentration, and the tuberculostasis properties of animal blood sera. To summarize briefly, when the concentration of iron exceeds the transferrin iron binding capacity, free iron exists and infection by Mycobacterium tuberculosis becomes more likely. In addition, virulent tubercle bacilli possess on their surface more lipoidal material which is necessary for the association of mycobactin. Mycobactin, unlike other siderochromes, is not extractable in water and is membrane bound.[4] Thus it appears essential that Mycobacterium tuberculosis receive a regular supply of iron.

Since the isolation procedure for mycobactin from Mycobacterium tuberculosis is known,[5] the possibility of supplying the bacteria with a chemically modified mycobactin exists. Such a mycobactin would serve as a chemotherapeutic agent against tuberculosis, leprosy, and Johne's disease, based on the inhibition of microbial iron transport. Since animals do not depend on mycobactin, a non-functional mycobactin should not prove toxic to them.

The recent preparation, separation, and identification of the coordination isomers of chromic-substituted deferrisiderochromes[6,7,8] suggests a promising modified mycobactin to serve as a chemotherapeutic agent. The kinetically inert chromium(III) complexes were made to prevent isomerization of the complex or ligand system. In addition, iron(III) does not displace the chromium(III) at a detectable rate.[9] Chromic mycobactin prepared from the non-pathogenic Mycobacterium phlei is the only reported modified mycobactin used in an attempt to inhibit bacterial growth.[9] The chromic mycobactin

was found to have a significant but not complete inhibitory effect on the growth of the mycobacteria. However, the effect is specific for Mycobacterium phlei .

A very serious objection may be raised concerning the chromic mycobactin experiment described in reference 9. Chromic mycobactin was added to the bacteria medium in an ethanol solution. Ethanol is used to extract mycobactin, which is associated with the lipid layer of tubercle bacillus. Therefore, ethanol may have been acting as a surfactant, loosening mycobactin. In an in vivo system, repeated synthesis and release of mycobactin would result in concentrations of mycobactin capable of removing iron from transferrin, thus permitting unhindered growth of the bacteria.[3] Ethanol in growth hindering experiments must be avoided.

A considerable improvement in mycobactin antagonism experiments would result if the chromic mycobactin were first suspended in lipid micelles. The lipid micelles would be saturated with chromic mycobactin, resulting in only exchange of chromic mycobactin for ferric mycobactin within the lipid layer of the bacillary cell. The concentration of chromic mycobactin should exceed that of the ferric mycobactin by a large factor. Thus, advantage is taken of the water insolubility of mycobactin.

Because chromic mycobactin will not be enzymatically reduced, chromic mycobactin will be concentrated within the cell wall. Ferric mycobactin still present in the lipid layer will follow its normal course of entering the cell wall and supplying the cell with iron. The deferrimycobactin will return to the lipid layer, hopefully to be

exchanged with chromic mycobactin. Because no pathway exists for the return of metallo-mycobactin from within the cell wall to the lipid layer of the mycobacteria, concentration of chromic mycobactin within the cell wall results.[10] Strong support for the feasability of this approach is given by the demonstration of the uptake of a chromic siderochrome by Ustilago sphaerogena.[7]

Parameters which will have to be determined experimentally are the exact composition of the lipid micelles and the ratio of the concentration of chromic mycobactin to the concentration of ferric mycobactin. Initial studies should be carried out with cultures of non-pathogenic bacteria, with extension of Mycobacterium tuberculosis if initial studies prove successful. The experimental procedure could be that of either Snow[9] or Kochan.[4]

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PROPOSITION IV

Investigation of the Active Site Charge of Horse Heart Cytochrome c

Accumulated evidence thus far has determined that "efficient" electron transfer processes occur with substantial enthalpy of activation, while inefficient processes occur with very low values of $\Delta H^{\ddagger}$. Efficient processes are thought to occur with electron transfer between redox centers that are able to closely approach one another, while inefficient processes are thought to involve electron transfer over large distances.

One possible exception to the pattern of kinetic parameters outlined above has been previously discussed. Briefly, electron transfer from $Fe(EDTA)^{2-}$ to horse heart cytochrome c is efficient unless the active site charge of the protein is large ($\gtrapprox +3.0$). Active site charges thus are of fundamental importance in the interpretation of rate and activation parameters of electron transfer reactions of metalloproteins.

The determination of active site charges has to date been attempted only through the study of rate of electron transfer as a function of ionic strength. However, because of the necessity of buffering and of maintaining adequate reagent concentrations to make absorbance changes observable, it has not been possible to carry out the ionic strength dependency studies at low enough ionic strength to render the calculations reliable. At higher ionic strengths, large changes are brought about in activity coefficients of the ions. The

calculations have been questioned by some authors:

> "However, the relationship has been much abused, experimental data being plotted against the square root of ionic strength for concentrated solutions. Since the limiting law is only valid for solutions below 0.01 molal for 1-1 electrolytes, and for lower concentrations still for higher charged ions, such procedure has no theoretical justification."[1]

Thus an alternative method of investigating the active site charge of horse heart cytochrome c is needed. The fact that charge effects may be important for horse heart cytochrome c reactions is suggested by the fact that cytochrome c_{551} from Pseudomonas, cytochrome f from parsley, and cytochrome c_{553} from Plocamium coccineum all reduce Pseudomonas azurin(II) with rates greater than 1×10^6 M^{-1} sec^{-1} (reference 2), while horse heart cytochrome c reduces azurin with a rate of 3×10^3 M^{-1} sec^{-1} (reference 3).

A positive charge on azurin is required to account for this difference in parameters. Even though the isoelectric point of azurin has been measured to be 5.40 (reference 4), a positive active site charge on azurin is reasonable. In fact, the rate of reaction of reduced azurin with $Co(phen)_3^{3+}$ was shown to be nearly independent of ionic strength, indicating that azurin(II) would indeed have a positive charge.

The recent report[5] of the isolation of azurin from Achromobacter cycloclastes suggests an alternative method of estimating the importance of charge in horse heart cytochrome c electron transfer reactions. The potential of Achromobacter azurin is +0.245 V, indicating a slightly modified hydrophobic copper binding site compared to the site in Pseudomonas[6] which has a potential of +0.328 V (reference 4). In addition, the isoelectric point of Achromobacter azurin is basic (~8.0).[5] The active site charge therefore has the possibility of being more positive for Achromobacter azurin.

Electron transfer rates of cytochrome c_{551}, cytochrome f, cytochrome c_{553}, and horse heart cytochrome c to Achromobacter azurin should be determined. More evidence for a large positive active site charge on horse heart cytochrome c may be obtained if the disparity in rate of reduction by horse heart cytochrome c and the three acidic cytochromes increases with respect to reduction of Pseudomonas azurin.

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PROPOSITION V

The Alchemist in Historical Fiction

In the twentieth century, numerous efforts to trace the progress of alchemy have been made. References 1 and 2 contain large bibliographies of relevance. Most, if not all, of the endeavors have been performed by scientists or by science historians whose works are of a very scholarly nature. Their histories are of unquestioned value, yet lack the quality of popular appeal. Communication of science to the layman has always been recognized as a necessity. No less should the communication of the history of science to the layman be emphasized. The appearance of a fictional novel set accurately within an historical time frame could thus be of considerable utility.

The writer of such a novel would obviously find the venture instructive. The alchemists' contributions to modern day chemistry and the arts could be delineated, as could the alchemists' failures. A lesson may be presented as to what science can and cannot accomplish. The origins of the scientific method may also emerge.

Although the alchemist of the proposed novel probably should not be based on any singular historical character, Abu Musa Jabir ibn Hayyan, commonly known as Geber,[3] could serve as a model. Geber was the son of a man who was beheaded and impaled for supporting opposition to the reigning caliph of Kufa, in Iraq. The then fatherless boy was sent to Arabia to study religion, mysticism, the occult, medicine, and alchemy. Like other scholars of his time,

his education was broad and steeped in magic.

The choice of Geber as a model for a fictional alchemist can be supported for a number of reasons. Geber's life time spans an appropriate era (~722 ~810). Nomadic Arabs had conquered Egypt, Syria, Palestine, Persia, and all of the land extending to Spain during the 5th, 6th, and 7th centuries.[4] The beginning of the 8th century saw the translation from Greek to Arabic of many of the alchemical books contained in the library at Alexandria. Alexandria had served as the center of the Western world and contained many of the works of the early alchemists Maria, Hermes, Posidonius, Apollonius, and Zosimos. The distinction between magician and alchemist may not be clear for some of the earlier contributors to the alchemical library. Greco-Egyptian alchemy as practiced at Alexandria was short-lived and subject less to experimentation than to superstition, imagination, speculation, religious theory, and later, fraudulent impostors. The formative years of the medieval world, Geber's lifetime, thus saw the Arabic language and religion exert its influence on what was previously the Greco-Roman empire.

Geber further appears to be a judicious choice as the model alchemist because of the contributions to alchemical theory credited to him.[3,5] Accepting the theory of Aristotle that all things are composed of the elements earth, air, fire, and water, Geber proposed that fire acting on air produces a principle called sulphur, and that air acting on water produces a principle called mercury. Sulfur and mercury were proposed to form the immediate substance of all

metals, while the four elements formed the remote substance of metals. It was accepted that the principles could be formed only from the elements and only by God. Combination of the principles in various ratios and purities was given as the explanation of the variety of metals found in nature. The purest principles combining in the "most complete natural equilibrium"[3] were thought to form gold.

That all things have a common substance or principle is a basic tenet of alchemy and its religious origins. Only the quality and not the substance of the principal was thought to vary from object to object. All things were also thought to possess a soul or spirit. The soul extracted and purified was variously known as the Great Elixir, the Elixir of Life, the Spirit, or perhaps by the best known name of the Philosopher's Stone. Lives and treasures of alchemists over a period of more than a thousand years were devoted to obtaining the Philosopher's Stone, which was considered the ultimate goal of alchemy. The existence and the power of the Stone were unquestioned. "... There abides in nature a certain pure matter, which, being discovered and brought by art to perfection, converts to itself proportionally all imperfect bodies that it touches."[6] Thus base metals could be transmuted to gold, the sick would be made well, the old could be rejuvenated, and the dying could be revived all through a touch of the Philosopher's Stone.

The Stone was thought to be extractable by a process that was constantly likened to death and resurrection. "Death perfects

that which is good; for the good cannot appear on account of that which conceals it."[5] The perfected soul was termed the "Philosopher's Stone" because it was supposed to resist fire, be infinitely purer than the purest gold, yet be impalpable to the touch, sweet to the taste, and fragrant to the smell.

Geber's life was undoubtedly devoted to a quest for the Philosopher's Stone, but his life was also entangled in politics as his father's had been. Geber became favored by the caliph at Baghdad, Harun al-Rashid (the caliph of the Arabian Nights). In his later years (probably within a few years of his death) he fell into disfavor along with some of the caliph's ministers and was expelled from the court.

Thus, present in the life of Geber are many elements required for a popular work of historical fiction: magic, superstition, political intrigue, religious mysticism, and high treason. Potential seems good for the proposed novel.

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