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SPECTROELECTROCHEMICAL INVESTIGATION OF
CYTOCHROME c AND CYTOCHROME c OXIDASE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Larry Neil Mackey, B.S.

* * * * *

The Ohio State University
1975

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ACKNOWLEDGMENT

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PUBLICATIONS

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LIST OF SYMBOLS

A
ADC
A_N
ATP
b
C
C^0
Cm
Cu_H
Cu_L
D
E
E^0'
EPR
A_e
F
heme a_H
heme a_L

absorbance
analog to digital convertor
normalized absorbance
adenosine triphosphate
cell optical path length in cm
concentration in moles per liter
degrees centigrade
bulk solution concentration in moles per liter
centimeter
copper of cytochrome c oxidase with higher formal potential
copper of cytochrome c oxidase with lower formal potential
diffusion coefficient in cm^2 per sec
potential
formal redox potential for the defined solution conditions
electron paramagnetic resonance
molar absorptivity change in M^{-1}cm^{-1}
Faraday's constant
heme a of cytochrome c oxidase with higher formal potential
heme a of cytochrome c oxidase with lower formal potential
<table>
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<tr>
<td>HMF</td>
<td>1,1'-bis(hydroxy-methyl)ferrocene</td>
</tr>
<tr>
<td>HMF⁺</td>
<td>1,1'-bis(hydroxy-methyl)ferricinium</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz (cycles per second)</td>
</tr>
<tr>
<td>I</td>
<td>ionic strength</td>
</tr>
<tr>
<td>i</td>
<td>current in amperes</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>IRS</td>
<td>internal reflection spectroscopy</td>
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<tr>
<td>k, k⁺, k⁻</td>
<td>second order rate coefficients in ( \text{M}^{-1}\text{s}^{-1} )</td>
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<tr>
<td>( K_{eq} )</td>
<td>equilibrium constant</td>
</tr>
<tr>
<td>M</td>
<td>molarity in moles/liter</td>
</tr>
<tr>
<td>mV</td>
<td>millivolt</td>
</tr>
<tr>
<td>( \text{MV}^{++} )</td>
<td>1,1'-dimethyl-4,4'-bipyridylium dichloride (methyl viologen dication)</td>
</tr>
<tr>
<td>( \text{MV}^{+} )</td>
<td>methyl viologen cation radical</td>
</tr>
<tr>
<td>( n )</td>
<td>number of electrons transferred</td>
</tr>
<tr>
<td>NHE</td>
<td>normal hydrogen electrode</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>OTE</td>
<td>optically transparent electrode</td>
</tr>
<tr>
<td>PMT</td>
<td>photomultiplier tube</td>
</tr>
<tr>
<td>Q</td>
<td>electronic charge</td>
</tr>
<tr>
<td>R</td>
<td>resistance in ohms</td>
</tr>
<tr>
<td>( s )</td>
<td>seconds</td>
</tr>
<tr>
<td>SEC</td>
<td>spectroelectrochemistry</td>
</tr>
<tr>
<td>( t )</td>
<td>time</td>
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\( \tau \) forward step time for double potential step experiment

TMPD \( N,N,N',N'-\text{tetramethyl-}p\text{-phenylene diamine} \)

\( V \) cell volume
I. INTRODUCTION

Biological oxidations provide the cells of higher organisms with the necessary energy to sustain life. The oxidation of foodstuffs, such as glucose, generates high energy molecules, such as adenosine triphosphate (ATP). The hydrolysis of ATP to form adenosine diphosphate (ADP) and inorganic phosphate provides free energy which is utilized for cellular processes.

The primary catabolic processes which generate the high energy molecules are divided into three main categories. The glycolytic pathway functions in the cellular cytoplasm and converts glucose to pyruvate while yielding two molecules of ATP. The Kreb's cycle and the electron transport chain both function in the mitochondrion. The Kreb's cycle oxidizes acetyl CoA to two molecules of CO$_2$ and releases 8 atoms of hydrogen which are utilized in the electron transport chain to reduce molecular O$_2$ to water. The free energy release for the total process is conserved by generating 38 molecules of ATP.$^{1,2}$

Although the stoichiometry of ATP production has been quantitated, the mechanism by which electron transport is coupled to oxidative phosphorylation is still uncertain. Two main hypotheses have been proposed to account for electron transport coupled oxidative phosphorylation. The chemical coupling hypothesis proposes a series of reactions, having high energy intermediates, leading to formation

1
of ATP. Failure to isolate the proposed high energy intermediates has encouraged experimental testing of the chemiosmotic coupling hypothesis of Mitchell. Mitchell proposed a vectorial transfer of reducing equivalents across the mitochondrial membrane which generates either a proton or electric potential gradient which activates a membrane bound ATPase with the resultant formation of ATP from ADP and inorganic phosphate.

Oxidative phosphorylation studies, using uncoupling agents such as 2,4-dinitrophenol, which prevent phosphorylation, and inhibiting agents such as oligomycin which prevent electron transport, have led to proposed sites of energy coupling. The electron transport chain along with proposed coupling sites, is diagrammed in figure 1. Proposed orientation of the electron transport components in the mitochondrial matrix is illustrated in figure 2. The figures are intended to provide an overall view of electron transport and oxidative phosphorylation. Detailed reaction mechanisms have yet to be elucidated.

This investigation has centered on the terminal electron transport component, cytochrome c oxidase, and its physiological reductant, cytochrome c. Starting with the work of D. Keilin in 1925, the cytochrome components of the mitochondrial electron transport chain have been extensively investigated for fifty years. Four basic approaches to the study of cytochromes have been to (1) investigate intact mitochondria, (2) study submitochondrial electron transport particles (ETP), (3) purify cytochromes to
FIGURE 1

Primary components of electron transport chain, along with proposed oxidative phosphorylation coupling sites, from preprint of "The Enzymes, cytochromes c," R.E. Dickerson and R. Timkovich, California Institute of Technology.
FIGURE 2

eliminate enzymes responsible for respiratory activity not directly related to electron transport, and (4) synthesize model compounds to simulate in vivo electron transport and oxidative phosphorylation reactions.

This investigation used approach (3) and concentrated on the study of the electron transport properties of purified cytochrome c and cytochrome c oxidase. Cytochrome c is water soluble and relatively easily extracted from mitochondria and purified. It is a relatively low molecular weight (12,500) enzyme with the Fe(II/III) heme c redox center. The primary structures of numerous cytochromes c have been established by Margoliash and coworkers. The crystalline structure of cytochrome c has been well characterized in both the oxidized and reduced states by the X-ray studies of Dickerson and coworkers. However, numerous kinetic studies of cytochrome c redox reactions using various oxidants and reductants have yet to elucidate the mechanism for transfer of electrons into cytochrome c from cytochrome c reductase and out of cytochrome c to cytochrome c oxidase.

Contrasted to cytochrome c, cytochrome c oxidase is a much larger enzyme (molecular weight of approximately 150,000). The enzyme is lipophilic, being located in mitochondrial membranes, and must be treated with detergents for purification and solubilization. However, extraction of cytochrome c oxidase from the membrane uncouples the energy conservation process of generating ATP. Also, the detergents, such as cholate, affect the enzyme activity.
primary structure of cytochrome c oxidase has not been determined, nor has it been crystallized for X-ray studies. The enzyme is thought to have four metal redox centers, two iron atoms enclosed in porphyrin ring systems termed heme a's, and two copper atoms. The cytochrome c oxidase used here was a highly purified preparation containing approximately 14 nanomoles of heme a per gram of protein with a copper/heme a ratio of less than 1.1, and less than 5% lipid.\textsuperscript{16}

The study of the purified enzymes was seen as a compromise between relating the relatively simplistic model reactions to reactions of biological significance on the one hand, and interpreting the extremely complex experimental data obtained from intact mitochondrial reactions on the other. The advantages of increased selectivity and sensitivity of physical measurements, as opposed to possible alteration of in vivo reaction mechanisms, have been convincing enough to pursue this line of research. In retrospect, the results from this approach have shown remarkable agreement with those of more complicated systems. The various approaches appear to be complementary.

Determination of the stoichiometry, energetics, and kinetics of cytochrome c and cytochrome c oxidase reactions is a prerequisite to understanding the mechanism of coupling electron transport free energy release to the process of oxidative phosphorylation, which was the ultimate goal of this investigation. The stoichiometric, energetic, and kinetic problems were approached in order. The stoichiometry results were used to guide the energetic's work, and the thermodynamic results guided the kinetic research.
When this work was initiated in 1972, the number of electrons transferred to cytochrome $c$ oxidase from cytochrome $c$ was still a question.\textsuperscript{17} Cytochrome $c$ oxidase was thought to contain 4 metal redox centers, but there was uncertainty as to the number of electrons accepted by each center. At this time, each of the four redox components, two heme $a$'s and two copper atoms, have been shown to accept one electron.\textsuperscript{18} However, it is still uncertain as to whether each component participates in electron transport during \textit{in vivo} operation.

Determination of formal redox potentials has usually been accomplished with potentiometric titrations.\textsuperscript{5} These measurements were complicated by poor coupling of the enzyme potential to the indicator electrode. This problem was somewhat alleviated by adding chemical mediators which couple with the enzyme and with the indicating electrode. However, mediators do not always maintain poised potentials,\textsuperscript{19} and sometimes affect the enzyme activity or energetics.\textsuperscript{20} Early experimental results on cytochrome $c$ oxidase, which were often controversial, were reviewed by Lemberg\textsuperscript{17} in 1969. More recent literature is summarized in "Oxidases and Related Redox Systems."\textsuperscript{21}

In addition to the electrode coupling problems, there are several problems in quantitating the concentrations of oxidized and reduced forms of the cytochrome $c$ oxidase components. The copper centers do not have well defined optical absorption bands, although the 830 nm absorption band is partially attributed to one copper.\textsuperscript{22} An electron paramagnetic resonance signal in cytochrome $c$ oxidase
observed at \( g = 2 \) has been attributed to the same copper. However, background signals due to low spin heme \( a \) and adventitious copper make quantitation of that signal very difficult. Both heme \( a \) components have strong optical absorption bands arising from the excitation of delocalized \( \pi \) electrons of the porphyrin rings. However, the absorption bands overlap making differentiation of the individual heme \( a \) components very difficult. When this investigation was initiated in 1972, both heme \( a \) components were thought to have formal potentials around 280 mV vs NHE in the absence of cytochrome \( c \). At this time, general agreement exists that the heme \( a \) components are split into high and low potential components, where \( H \) and \( L \) stand for high and low potential. However, Van Gelder and coworkers still report both heme \( a \)'s to have formal potentials around 280 mV in the absence of cytochrome \( c \). One copper of cytochrome \( c \) oxidase was thought to have a formal potential of 285 mV vs NHE in 1966. However, our results have shown that the copper atoms are split in potential along with the heme \( a \) components (\( E^{\circ}_{CuH} = 350 \text{ mV vs NHE and } E^{\circ}_{CuL} = 215 \text{ mV vs NHE} \)). These results have been substantiated by Wilson and coworkers.

Very few kinetic studies of cytochrome \( c \) and cytochrome \( c \) oxidase electron transfers had been attempted when this investigation was undertaken. The most notable works were the flash photolysis experiments of Gibson and coworkers in 1965, and the stopped flow experiments of Malmstrom and coworkers in 1970.
More recently, cytochrome c and cytochrome c derivatives have been investigated with a variety of oxidants and reductants. Inner sphere oxidants appear to be relatively inert at physiological pH. Outer sphere reactants have been used to probe the steric requirements of ferrocytochrome c oxidation. Experimental rates have agreed well with rate coefficients calculated from Marcus theory.

Reduction of cytochrome c oxidase by ferrocytochrome c has been studied by a number of workers. Malmstrom and coworkers in 1972 indicated the reaction was biphasic with one mole of ferricytochrome c being formed per mole of cytochrome c oxidase in the rapid phase of the anaerobic reaction. Van Gelder and coworkers in 1974 again indicated the reaction to be biphasic, but found two equivalents of cytochrome c to be oxidized per equivalent of heme a reduced during the rapid phase of the reaction. Wilson et al. in 1975 again indicated the reaction was biphasic, but found the stoichiometry in the rapid phase depended on the concentration of ferrocytochrome c.

Recent evidence indicated electron transfer in the solubilized cytochrome c oxidase, cytochrome c solutions followed the pathway; cytochrome c to cytochrome a, cytochrome a to cytochrome a3, and from cytochrome a3 to molecular oxygen. However, the in vivo electron transport pathway is yet to be determined.

The principle physical method of choice in this study has been spectroelectrochemistry (SEC) at optically transparent electrodes (OTE). The method applied to this biological system involved using mediator-titrants such as 1,1'-dimethyl-4,4'-bipyridylium
dichloride, commonly termed methyl viologen (abbreviated MV\textsuperscript{++}), N,N,N',N'-tetramethyl-p-phenylene diamine (TMPD), or ferricyanide to transfer charge from the OTE to the electroinactive enzyme, and monitoring the transmission of light through the OTE and solution. For thermodynamic studies, the electrogenerated titrant is allowed to equilibrate with the enzyme in solution, followed by optical monitoring of the enzyme's visible spectrum. For kinetic experiments, the time dependent absorption of the titrant, the enzyme, or a combination of the two is monitored concurrently with the electrogeneration of the titrant. Mechanisms and kinetic rates of reaction are established by comparing the experimental data to digitally simulated Absorbance (A) \textit{vs} time (t) curves.

Hawkridge \textit{et al.}\textsuperscript{39} demonstrated the utility of SEC in obtaining the stoichiometric \(n\) value of one electron per heme for cytochrome \(c\). Electric current transferred at the OTE was integrated and compared with the absorbance at 550 nm of cytochrome \(c\) at successive stages of reduction and oxidation. Heineman \textit{et al.} used this approach in 1972 to assess the stoichiometry\textsuperscript{18} and energetics\textsuperscript{40} of the four cytochrome \(c\) oxidase metal redox centers.

This investigation used indirect coulometric titrations to determine the energetics of both the heme \(a\) and copper components of cytochrome \(c\) oxidase. Since the copper centers do not have well defined optical absorption bands, the formal potentials of the copper components were determined indirectly by their effects on experimental \(A_{605}\) \textit{vs} injected charge (Q) curves. The experimental data were
curve fitted to computer generated $A \text{ vs } Q$ curves. Clues as to intramolecular as well as intermolecular electron transfer are obtained from the titrations of cytochrome $c$ oxidase and cytochrome $c$-cytochrome $c$ oxidase mixtures.

The applicability of SEC to assist in the evaluation of kinetic rates and mechanisms involving biological electron transfer components has been previously demonstrated by Ito et al.\textsuperscript{41} for the reduction of triphospho pyridine nucleotide by electrogenerated methyl viologen cation radical, catalyzed by spinach ferredoxin-TPN-reductase. Kinetic rates of electron transfer from electrogenerated mediator-titrants to the heme proteins have been evaluated in this work using SEC under chronocamperometric conditions. The digital simulation technique described by Feldberg\textsuperscript{12} is used to relate calculated normalized absorbances to kinetic parameters. Since the heme proteins are electroinactive, mediators are employed to transfer charge from the electrode to the enzymes and in doing so, the initial form of the mediator is regenerated. This catalytic regeneration mechanism has been previously demonstrated by Blount et al.\textsuperscript{43,44} for the ferricyanide-ascorbic acid reaction. However for the biological systems studied here, special experimental precautions were found to be necessary, especially during signal averaging of repetitive SEC experiments.

The experimental sections for the equilibrium and kinetic measurements are presented separately in Chapters 2 and 3. The appendices include the $A-Q$ and Digital Simulation computer programs.
Chapter 2 reviews the SEC indirect coulometric titration studies for analyzing the energetics of cytochrome c, cytochrome c oxidase, and mixtures of the two. Formal potentials of the cytochrome redox components are established, and intermolecular and intramolecular electron transfer pathways are discussed.

Chapter 3 summarizes the potential step, double potential step, and potential step-relaxation SEC experiments applied to the evaluation rates and mechanisms of cytochrome c and cytochrome c oxidase reactions. Spectroelectrochemistry is shown to be of significant importance in analyzing the energetics and kinetics of both optically monitorable and non-monitorable redox species.
LIST OF REFERENCES


II. EVALUATION OF FORMAL POTENTIALS OF CYTOCHROME c, CYTOCHROME c OXIDASE, AND THEIR MIXTURES

Three primary methods are used to evaluate the energetics of the transfer of electrons in and out of the redox centers of cytochrome c oxidase. The most common method is the potentiometric titration. A second method, first used by Minnaert, is the log-log plot. The third method, developed by Kuwana and coworkers in 1972, is curve fitting of experimental absorbance (A) vs charge (Q) curves to computer simulated A vs Q plots.

For the potentiometric titration, the log of the ratio of concentrations of oxidized and reduced species (log(ox/red)), determined by some physical technique such as visible spectroscopy or electron paramagnetic resonance, is plotted versus the potential of a platinum indicator electrode dipped into solution. Using the potentiometric titration method, Muijsers et al. reported the potentials of the hemes of cytochrome c oxidase in the presence of cytochrome c (E° heme a₃ = 333-360 mV vs NHE and E° heme a = 200-250 mV vs NHE) and asserted the hemes titrated as indistinguishable entities in the absence of cytochrome c (E° heme a₃ = 280 mV vs NHE). Potentiometric mediators were employed in that work to insure potentiometric coupling to the platinum indicator electrode. The mediators must exchange electrons with the enzyme and with the Pt
indicator electrode at a rate sufficient that drifting of the measured potential does not occur. To insure this, normally a variety of mediators are employed with formal potentials ranging over the potential interval of interest. Reports of potential drift are indicative of slow electron exchange with the Pt indicator electrode.

Wharton and Cusanovich presented potentiometric data for purified cytochrome c oxidase in the presence of varying amounts of ferricyanide ranging from zero to $5 \times 10^{-2}$ M. They concluded that the hemes of cytochrome c oxidase are split in potential in the presence of ferricyanide, independent of cytochrome c. Wharton also reported the midpoint of at least one copper of cytochrome c oxidase, spectrally observed at 830 nm, to be 278 mV vs NHE.

The potentiometric results of Tsudzuki and Wilson in 1971 indicated that the hemes of highly purified cytochrome c oxidase titrated as a single component with midpoint potentials of 285 mV, whereas the hemes of partially purified cytochrome c oxidase were resolved into high and low potential components. A copper was shown by both the 830 nm absorption band and EPR measurements to have a midpoint potential of 225 mV.

The kinetic experiments of Malmstrom indicated an initial fast reduction of cytochrome a by cytochrome c. For this to occur, they concluded the potential of cytochrome a must be more positive than cytochrome c. Therefore, they maintained that the separation of
potentials of the hemes of cytochrome c oxidase, even in the presence of cytochrome c, was not manifested in their experiments.

These discrepancies in the reported states of the hemes of cytochrome c oxidase clearly point up the need for alternatives to the potentiometric method of evaluating the energetics of cytochrome c oxidase. One alternative is the method first used by Minnaert of plotting the log(ox/red) of one species versus the log(ox/red) of another species. This method is similar to the potentiometric titration, but instead of determining the solution potential from a platinum indicator electrode, the potential is referenced to the oxidation state of an internal standard whose midpoint potential is known. The oxidation state of the standard is monitored by a physical technique, such as visible spectroscopy or EPR, and the solution potential is calculated from the standard's Nernst equation. This method eliminates the need to add numerous potentiometric mediators which have varied effects on the cytochrome c oxidase.

A second alternative to the potentiometric method is curve fitting of experimental absorbance versus charge curves to computer simulated A vs Q plots. The procedure employed to obtain the experimental data is to monitor the transmission of light through an optically transparent electrode (OTE), usually a tin oxide or indium oxide semiconductor OTE, after an electrochemical injection of reductive or oxidative charge. The charge is transferred from the OTE to the heme protein by way of a chemical mediator. The electroactive mediator is employed since the heme proteins themselves are
essentially electroinactive. Ferricytochrome c has been shown to be slowly reduced electrochemically at a mercury electrode. However, the rate of heterogeneous electron transfer at the presently used semiconductor electrodes is quite slow. Thus, the electron transfer to the heme protein proceeds essentially quantitatively through the electrolytically generated mediator titrant. The charge transferred is monitored with an electronic integrator.

The A vs Q curves are computer generated by calculating the concentrations of all redox components in solution from their respective Nernst equations. The amount of charge needed to reach a given redox state is then plotted versus the absorbance of the monitored species at that redox state. Parameters affecting the shape of the computer generated curve are the number of redox species in solution, the midpoint potentials of the species, and the molar absorptivities of the monitored species.

This study used all three methods to determine the energetics of detergent solubilized purified cytochrome c oxidase. Mixtures of cytochrome c oxidase with ferricyanide or tetramethyl-p-phenylene-diamine (TMPD) were coulometrically titrated while monitoring the visible and near IR spectra.

EXPERIMENTAL:

1. Electrochemical equipment

A conventional three electrode potentiostat (Figure 3) was used for controlled potential generation of titrant during the indirect
A conventional three electrode potentiostat with adjustable positive feedback for IR compensation. All amplifiers are general purpose Teledyne-Philbrick 1026 operational amplifiers, except for the Hewlett Packard 467A booster amplifier.
coulometric titration. The electrochemical cell was designed to isolate any electrolysis products generated at the auxiliary electrode. It was separated from the cell body by a medium frit and the long narrow opening of a Hamilton valve. This isolation in space led to high solution resistance between the auxiliary and working electrodes. A Hewlett Packard 467A booster amplifier which has a peak output capability of 20 V and 0.5 amp was used to overcome the high IR drop.

The injected charge was measured by integrating the current flowing through the 100 ohm load resistor. The integrator used was either a Teledyne-Philbrick 1009 operational amplifier integrator or a Vidar model 240 voltage to frequency convertor used in conjunction with a Beckman model 5311DR counter. A Hewlett Packard 3300-3302 function generator was used for linear sweep voltages in cyclic voltammetric experiments.

2. Optical equipment

Visible spectra were obtained after incremental additions of charge with a rapid scanning spectrophotometer (RSS) similar to one previously described. The modifications have been reported by Hawkridge and Kuwana. The optics were optimized to scan either the 400-650 nm wavelength range or the 600-850 nm wavelength range. For the 400-650 nm range, an Illumination Industries Inc. xenon arc lamp powered by a PEK 401A short arc power supply and EMI 9529B photomultiplier tubes were used. For the 600-850 nm range, a Norma iodine 11 quartz iodine tungsten lamp powered by an Acme 12 V power
supply, and Hammamatsu 7102 photomultiplier tubes were used. Fluke 412 B high voltage power supplies powered the PMT's in both cases. A Teledyne-Philbrick 4361 log ratio amplifier giving a 1.00 ± .005 V/ absorbance unit response replaced the previous electronic circuitry. The RSS could scan at a rate of less than 0.1 Hz to a maximum of 500 Hz with a resolution of ca. 1.5 nm at the optimum wavelength. A Houston Instruments Model 2000 X-Y recorder was used for signal monitoring purposes.

3. Electrochemical cell

The electrochemical cell, machined from a Lucite rod, is similar to one previously described. This cell has several unique features of significance. The cell volume is very small (approximately 1 ml). Therefore only small quantities of "costly" enzyme are required (approximately 10 nanomoles). A vacuum degassing procedure allows molecular oxygen to be reduced to and maintained at less than $5 \times 10^{-7}$ M. Small amounts of titrant can be added quantitatively and repetitively without varying the solution volume or the total mass of reactants. The constant volume and constant total mass greatly simplify the theoretical $A$ vs $Q$ calculations. Both the semiconductor OTE and a Pt microelectrode are available to adjust the redox state of the solution with the mediator-titrants. A silver-silver chloride auxiliary electrode is used to avoid generating molecular oxygen.
4. Reagents

The horse heart cytochrome c was obtained from Sigma (Type VI) and purified according to Margoliash. The beef heart cytochrome c oxidase was provided by C.R. Hartzell. Phosphate buffer (Titrisol) pH 7.00 and sodium chloride (Suprapure) were obtained from E. Merck Co. Potassium chloride was from Matheson, Coleman and Bell. The 1,1'-dimethyl-4,4'-bipyridylium dichloride (methyl viologen) and 1,1'-dibenzyl-4,4'-bipyridylium dichloride (benzyl viologen) were from K and K Laboratories. The ferrocene and 1,1'-bis(hydroxy-methyl)-ferrocene were from Strem Chemical Co. The N,N,N',N'-tetramethyl-p-phenylene diamine (TMPD) was from Aldrich Chemical Co. Prepurified nitrogen gas was from J.T. Baker. Tween 20 from Sigma Chemical Co. was chromatographed on an alumina column. All other reagents were used without further purification. Doubly glass distilled water was used throughout.

5. Procedure

Stock solutions of cytochrome c were prepared by diluting the purified cytochrome c samples 1:25 in 0.067 M pH 7 phosphate buffer (I = 0.15). Cytochrome c stock solutions and phosphate buffer solution were stored at 0°C. Cytochrome c oxidase was stored immersed in liquid nitrogen. Cytochrome c oxidase test solutions were prepared immediately prior to the experiments by purging 0.067 M pH 7 phosphate buffer (I = 0.15) containing 0.1% Tween 20 with nitrogen gas. The electrochemical mediators were then added to the
purged solution in solid form. Finally, the enzyme, which had been thawed slowly, was diluted with the buffer solution. Concentrations of the enzymes were determined spectrophotometrically using the following molar absorptivities: Cytochrome $c$, $E_{550 \text{ nm}} = 18,500 \text{ M}^{-1} \text{ cm}^{-1}$; Cytochrome $c$ oxidase, $E_{605 \text{ nm}} = 12,000 \text{ M}^{-1} \text{ cm}^{-1}$ per heme $a$.

A vacuum degassing train was used to deaerate the solutions and to fill the electrochemical cell. Oxygen was removed from the nitrogen gas by passing the gas through two tubes filled with copper turnings at $500^\circ\text{C}$. Copper oxide was removed by passing hydrogen gas over the hot copper turnings. The nitrogen gas was also bubbled through distilled water to presaturate the nitrogen and avoid concentrating the solutions during the degassing and filling procedure. A liquid nitrogen trap was placed between the vacuum pump and the cell to isolate pump oil.

The tin oxide OTE's, obtained from Corning Glass Company, were washed in isopropanol and distilled water. The OTE's had resistances between 6 and 10 ohms per square and had absorbances of approximately 0.2 units over the entire visible wavelength range. For reductive titrations, MV$^+$ was generated at the tin oxide OTE by stepping the potential to $-0.85 \text{ V vs Ag/AgCl}$. Ferricyanide and ferricinium ions were generated at the OTE for oxidative titrations by stepping the potential to $+0.85 \text{ V vs Ag/AgCl}$. Molecular oxygen was generated at the OTE at $+1.5 \text{ V vs Ag/AgCl}$.
Corrections for background charge, mostly double layer charging, were obtained by stepping the potential from 0.0 V vs Ag/AgCl to a given value for the same time period in the absence of the mediators, as was stepped from open circuit in the presence of mediators. The corrections amounted to 10-20% of the total charge for reductive titrations and 5-10% for oxidative titrations. Corrected charge was estimated to be accurate within ±3%. All experiments were repeated at least twice. The titration procedure was described in detail by Hawkridge and Kuwana. All experiments were run at 23 ± 1°C.

RESULTS AND DISCUSSION:

The coulometric titrations in this study were designed to determine the formal midpoint potentials of all four redox centers in cytochrome c oxidase. Cytochrome c oxidase is known to contain four titratable redox components, however physical probes of all four components are not available. Cytochromes typically have alpha, beta, and Soret visible absorption bands due to the iron heme components. Cytochrome c oxidase has the alpha and Soret bands but lacks a clearly distinguishable beta band. Complications arise in the study of cytochrome c oxidase due to the smallest functional enzyme unit capable of reducing molecular O₂ to H₂O containing two heme a groups. The two heme a absorption bands overlap significantly in both the alpha and Soret regions. Therefore it is impossible to determine specifically which heme a is being monitored by visible absorption spectroscopy alone.
An added complication is that a quantitative probe of the two copper centers of cytochrome \( c \) oxidase does not exist. The 830 nm band of cytochrome \( c \) oxidase has been attributed to one of the copper centers.\(^{15}\) We have SEC experimental data indicating that the 830 nm band cannot be due solely to one copper center. The electron paramagnetic resonance signal in cytochrome \( c \) oxidase observed at \( g = 2 \) has been attributed to copper.\(^{16}\) However, background signals due to low spin heme and adventitious copper make quantitation of that signal very difficult.

Since a quantitative probe of the copper centers is not available and the heme \( a \) absorption bands overlap significantly, attempts have been made to isolate individual redox center signals by blocking electron transfer in and out of optically interfering centers with inhibitors such as cyanide ion, carbon monoxide, or azide ion.\(^{17,18}\) These studies do not necessarily reflect the \textit{in vivo} energetics of cytochrome \( c \) oxidase though, since the inhibitors do affect the physical properties of the redox centers which are not blocked.\(^{19}\) This study has avoided the use of inhibitors and has concentrated on determining the energetics of cytochrome \( c \) oxidase with a minimum of possibly interfering reactants present.

**REDUCTIVE TITRATION OF FERRICYANIDE-CYTOCHROME \( c \) OXIDASE**

Solutions containing 7 to 15 \( \mu M \) cytochrome \( c \) oxidase, 0.1 to 2.0 mM ferricyanide, 0.5 mM methyl viologen dichloride, 0.067 M potassium phosphate buffer, pH 7.0, and 0.1% Tween 20 in doubly
distilled water were indirectly coulometrically titrated while simultaneously monitoring the 605 nm band. The cytochrome c oxidase mixtures were initially in the completely oxidized forms, and were reduced with electrochemically generated methyl viologen cation radical (MV⁺). The mixtures were reoxidized with either electrochemically generated ferricyanide or oxygen. Ferricyanide is electrochemically irreversible on SnO₂ electrodes possibly due to interferences caused by the oxide nature of the surface. However ferrocyanide can be oxidized at +0.8 V vs NHE quantitatively (see Figure 4). Water can be quantitatively oxidized to molecular oxygen at +1.5 V vs NHE without any apparent change in the electrochemical characteristics of SnO₂. The ferricyanide acts as both a coulometric titrant and as a mediator. The formal potential of ferricyanide (E° = +424 mV vs NHE in pH 7 phosphate buffer) is some 80 mV higher than that of heme a₇ so that the equilibrium constant for its reaction with heme a₇ is around 23. In order to quantitatively oxidize heme a₇ with ferricyanide in the indirect coulometric titrations, a large excess of ferricyanide over heme a must be generated since the ferri-ferrocyanide couple is present in approximately a 100 fold higher concentration. The equilibrium constant can be accurately determined by relating the change in optical absorbance of heme a to the amount of electrochemically injected charge. Hence it is possible to determine the potentials of the two heme a's of cytochrome c oxidase relative to the potential of ferricyanide. The potentials of the cytochrome c oxidase redox centers are actually assessed by curve
fitting the experimental optical absorbance vs injected charge plots to computer generated A vs Q curves. The computer simulated A vs Q curves were derived by treating the ferricyanide-cytochrome c oxidase system as a five component system of two hemes, two coppers, and ferricyanide. The electrochemically injected charge is related to the concentration of each of these components by the equation $Q = nFVC$ where $Q$ is charge in milli-coulombs, $F$ is Faraday's constant (96,487 coulombs/equivalent), $V$ is the cell volume in milliliters, and $C$ is concentration in moles per liter. The amount of titrant was monitored with nanoequivalent accuracy by measuring the amount of charge injected with an electronic integrator. The concentration of the hemes was calculated from the absorbance change at 605 nm, taking the molar absorptivity per heme as $12 \text{ mM}^{-1}\text{cm}^{-1}$. The A vs Q curves were simulated by (1) assuming a solution potential, (2) calculating the concentrations of the five solution species from the Nernst equations:

$$E = E^0_{\text{heme a}_H} + 59 \log\left(\frac{\text{heme a}_H^{(\text{ox})}}{\text{heme a}_H^{(\text{red})}}\right)$$

$$E = E^0_{\text{Cu}_H} + 59 \log\left(\frac{\text{Cu}_H^{(\text{ox})}}{\text{Cu}_H^{(\text{red})}}\right)$$

$$E = E^0_{\text{heme a}_L} + 59 \log\left(\frac{\text{heme a}_L^{(\text{ox})}}{\text{heme a}_L^{(\text{red})}}\right)$$

$$E = E^0_{\text{Cu}_L} + 59 \log\left(\frac{\text{Cu}_L^{(\text{ox})}}{\text{Cu}_L^{(\text{red})}}\right)$$

$$E = E^0_{\text{ferri}} + 59 \log(\text{ferri}/\text{ferro})$$

and (3) calculating the amount of charge needed to reach the redox
Cyclic voltammogram of ferrocyanide and methyl viologen at Sb doped tin oxide semiconductor electrode. Solution contains 0.5 mM methyl viologen dichloride, 1.0 mM potassium ferrocyanide and 0.067 M pH 7 phosphate buffer.
states of all species in solution corresponding to the assumed potential. A sample computer program is given in Appendix I. Account was taken for the weak charge-transfer complex between methyl viologen dication and ferrocyanide. The formation constant for the reaction

\[ \text{MV}^{2+} + \text{Fe(CN)}_6^{4-} = \text{MVFe(CN)}_6^{2-} \]

was 52 M\(^{-1}\). The significant advantage of the A vs Q method of determining formal potentials compared to the potentiometric and Minnaert methods is that the energetics of redox components for which optical probes are not available, such as the copper components of cytochrome c oxidase, can be evaluated indirectly by their effects on the A vs Q curves of optically monitorable species.

Since this method depends upon an equilibrium between the mediator-titrant and the cytochrome c oxidase components when ferricyanide (\(E^0' = 424 \text{ mV vs NHE}\)) is used as mediator titrant, the method is most sensitive to the high potential components of cytochrome c oxidase. The potentials of all four redox centers of cytochrome c oxidase in the presence of ferricyanide were determined from the A vs Q plots to be split into a high potential heme a-Cu pair (\(E^0'_{\text{heme a}} = E^0'_{\text{CuH}} = 350 \pm 10 \text{ mV vs NHE}\)) and a low potential heme a-Cu pair (\(E^0'_{\text{heme a}} = E^0'_{\text{CuL}} = 220 \pm 30 \text{ mV vs NHE}\)). Figure 5 illustrates the experimental A vs Q points and the computer simulated best fit for a representative experiment. The dashed line illustrates how a \(\text{CuH}\) potential of 280 mV would affect the computer simulation. Solutions
FIGURE 5

Plot of Absorbance (A/cm) vs Charge (nanoequivalents/ml) for first reduction titration of cytochrome c oxidase-ferricyanide mixture. Solution contained 0.5 mM Fe(CN)$_6^{3-}$, 0.5 mM methyl viologen dichloride, and 9.2 uM cytochrome c oxidase. (O) - data points for reduction of cytochrome c oxidase with electrochemically generated methyl viologen cation radical. (---) computer simulation for

$E^{o'}_\text{heme a}_H = 350 \text{ mV}, E^{o'}_{Cu_H} = 350 \text{ mV},$  
$E^{o'}_\text{heme a}_L = 220 \text{ mV}, E^{o'}_{Cu_L} = 220 \text{ mV},$  
$E^{o'}_{\text{ferricyanide}} = 424 \text{ mV}.$ (----) computer simulation for  

$E^{o'}_\text{heme a}_H = 350 \text{ mV}, E^{o'}_{Cu_H} = 280 \text{ mV},$  
$E^{o'}_\text{heme a}_L = 220 \text{ mV}, E^{o'}_{Cu_L} = 220 \text{ mV}, E^{o'}_{\text{ferricyanide}} = 424 \text{ mV}.$ Computer simulations are adjusted for 15% charge correction.
containing 0.1, 1.0, and 2.0 mM ferricyanide produced similar results. Lindsay and Wilson \(^{23}\) have recently verified these results.

The main assumptions made for quantitatively analyzing the indirect coulometric titration data are: (1) The change in absorbance at 605 nm is due to the iron porphyrin systems of cytochrome \(c\) oxidase. (2) The change in absorbance at 605 nm is 50% due to one heme moiety and 50% to the other heme moiety. Both of these assumptions are points of contention.\(^{24,25}\) The copper moieties could possibly absorb in the 605 nm region, such as with blue copper oxidases, although the hemes are most certainly the primary contributors to the 605 nm absorbance. As regards assumption (2), during a coulometric titration, the 560 nm isosbestic remained stationary through 50% of the titration and then shifted 4 nm to a shorter wavelength for the remaining 50% of the titration. The rapid shift in isosbestic at 50% of the absorbance change is strong evidence for the validity of assumption (2). The two isosbestic were also evaluated as being direct probes on which heme was being titrated. Another possible source of error involves background absorbance changes. The 605 nm band lies on the sloping tail absorbance of either the Soret or beta absorption bands. The absorption maximum at 605 nm appears to shift to longer wavelengths during the course of a titration. However, difference spectra\(^{25,a}\) (reduced-oxidized) of cytochrome \(c\) oxidase show the \(A\) maxima to be stationary at 605 nm. The question remains whether the background
absorbance is changing. For this reason, it is dangerous to make quantitative evaluations based on very small absorbance changes.

Potentiometric measurements were taken simultaneously with the absorbance and charge measurements to provide supportive and comparative data on the thermodynamic status of the cytochrome $c$ oxidase redox centers. The potential of a platinum microelectrode epoxied into the electrochemical cell versus the silver/silver chloride reference electrode was measured with a Corning model 17 pH meter. The exchange current between the cytochrome $c$ oxidase redox centers and the platinum electrode was not great enough to maintain a poised potential. Therefore, potentiometric measurements could not be taken for the methyl viologen-cytochrome $c$ oxidase system, when cytochrome $c$ oxidase was oxidized with electrogenerated molecular oxygen. However, when ferricyanide was used as the electrochemically generated titrant, the ferricyanide at 1.0 mM concentration served as both the titrant and as potentiometric mediator. A poised potential could be maintained in the 250 mV to 450 mV vs NHE range. A plot of potential vs absorbance is shown in Figure 6. These data correspond to a reductive titration with the absorbance changes due to incremental additions of 0.5 millicoulombs of charge. At the higher potentials, more data points are available since ferricyanide is in equilibrium with the cytochrome $c$ oxidase and is accepting a portion of the injected charge. At lower potentials, the heme $a_l$-Cu$_l$ pair is accepting the entire charge injection. If each heme $a$ is assumed to contribute 50% to the absorbance change, a Nernst type
Potential vs Absorbance $_{605\ nm}$ plot of reductive titration data. Solution contained 1.0 mM ferricyanide, 0.5 mM methyl viologen dichloride, 8.6 $\mu$M cytochrome c oxidase and pH 7 phosphate buffer. Indicator electrode is a platinum microelectrode.
plot of potential vs log(ox/red) yields midpoint potentials of $E^\circ_{\text{heme } a_H} = 359 \text{ mV vs NHE}$ and $E^\circ_{\text{heme } a_L} = 219 \text{ mV vs NHE}$ (see Figure 7). These potentials agree quite well with the indirect coulometric titration determinations. The $n$ values calculated from the Nernst slopes are 0.87 and 0.83 for the high and low potential hemes, respectively. Quantitation of $n$ values from Nernst slopes are generally considered much less accurate than coulometrically determined $n$ values.\(^{11}\) The crux of Van Gelder's assertion of the potentiometric equivalence of the hemes in purified cytochrome $c$ oxidase\(^3,25\) in his value of $n = 1$ determined from the slopes of a Nernst plot.

**TMPD-CYTOCHROME $c$ OXIDASE MIXTURE**

Determination of an unknown midpoint potential by the $A$ vs $Q$ method depends upon an equilibrium with a species with a known midpoint potential. When ferricyanide is used as the mediator titrant, the method is most sensitive to the high potential heme $a$-$Cu$ pair and less sensitive to the low potential heme $a$-$Cu$ pair. Tetramethyl-$p$-phenylenediamine (TMPD) was used to obtain more accurate values of the midpoint potentials of the low potential cytochrome $c$ oxidase components.

The spectral and electrochemical characteristics of TMPD were initially studied to determine the suitability of TMPD as a mediator-titrant. TMPD shows two well defined one electron oxidations over
Nernst type plot of potential vs Log(ox/red).

Heme $a_H$ and heme $a_L$ components are resolved by attributing 50% of $A_{605}$ nm to one heme, and the other 50% of $A_{605}$ nm to the other heme. Midpoint potentials calculated from the x-axis intercept are $E^0_{heme \, a_H} = 359 \text{ mV vs NHE}$ and $E^0_{heme \, a_L} = 219 \text{ mV vs NHE}$. $n$ values calculated from the slopes are 0.87 and 0.83 for heme $a_H$ and heme $a_L$, respectively.
most of the aqueous pH range. The first one electron transfer is reversible, but the second electron transfer is complicated by a complex follow up reaction. A potentiometric titration was performed to determine the midpoint potential of the reversible one electron reaction in pH 7.0 potassium phosphate buffer. The concentration of oxidized TMPD was determined by monitoring its optical absorbance in the 530 to 630 nm range. Figure 8 shows the TMPD spectra taken during an indirect coulometric titration. The TMPD was initially oxidized with ferricyanide and was re-reduced with electrochemically generated methyl viologen cation radical.

The potentiometric plot of absorbance vs potential is shown in Figure 9. The midpoint potential for the one electron oxidation of TMPD in pH 7.0 phosphate buffer was determined to be +300 mV vs NHE. Since TMPD air oxidizes and decomposes slowly, the concentration of TMPD was determined spectrophotometrically at 605 nm. The molar absorptivity at 605 nm was determined from the slope of an A vs Q plot. This method has the advantage that the concentration of TMPD in solution need not be known to determine the molar absorptivity. The molar absorptivity is determined from the ratio of A/Q and the concentration terms in the numerator and denominator cancel out. Figure 10 shows the A vs Q plot for TMPD. The slope corresponds to a molar absorptivity of 7140 M⁻¹ cm⁻¹.

A solution containing 10 μM TMPD, 0.5 mM methyl viologen dichloride, 10.6 μM cytochrome c oxidase, and 0.1% Tween 20 in pH 7.0 phosphate buffer was indirectly coulometrically titrated to more
FIGURE 8

Spectra of one electron oxidation product of TMPD taken after incremental additions of reductive charge during an indirect coulometric titration. Solution contained $10^{-4}$ M TMPD, 0.5 mM methyl viologen dichloride, and pH 7 phosphate buffer. All of the TMPD was not initially oxidized. (---) corresponds to excess generation of methyl viologen cation radical.
ABSORBANCE

WAVELENGTH (nm)

0.1 a.u.
FIGURE 9

Potentiometric plot of Absorbance vs Potential for the one electron oxidation of TMPD in pH 7 phosphate buffer. The midpoint potential is determined to be +300 mV vs NHE.
FIGURE 10

Absorbance vs Charge plot for reductive titration of TMPD. The molar absorptivity of the one electron oxidation product of TMPD at 615 nm is determined from the slope of the A vs Q plot to be 7140 M\(^{-1}\)cm\(^{-1}\) (\(E_{605\,\text{nm}} = 7140\,\text{M}^{-1}\text{cm}^{-1}\)).
accurately determine the midpoint potentials of the low potential cytochrome c oxidase components. The spectral bands of oxidized TMPD and heme a at 605 nm overlapped. The absorption bands were deconvoluted by taking the total absorbance change at 560 nm, the heme a isosbestic, to be due to TMPD (see Figure 11). The ratio $A_{605}/A_{560}$ for TMPD was calculated from the Coulometric titration data to be 1.19. The $A_{560}$ was multiplied by 1.19 and the value added to the $A_{605}$ to compensate for the spectral overlap. Treating the data in this manner, an $A_{heme a} \text{ vs } Q$ plot is shown in Figure 12. The best fit computer simulation was calculated for $E^{o'}_{heme a_H} = 340 \text{ mV}$, $E^{o'}_{Cu_H} = 350 \text{ mV}$, $E^{o'}_{heme a_L} = 215 \text{ mV}$, $E^{o'}_{Cu_L} = 215 \text{ mV}$, and $E^{o'}_{TMPD} = 300 \text{ mV}$. The dashed line is drawn for $E^{o'}_{Cu_H} = 280 \text{ mV}$ and the midpoint potentials of the other components the same as for the best fit. It is estimated that the Cu potentials, determined indirectly by their effect on the heme a absorbance vs charge plot, are accurate to within $\pm 10 \text{ mV}$.

The potentials of heme $a_H$ and heme $a_L$ of cytochrome c oxidase obtained from $A \text{ vs } Q$ data were substantiated by Minnaert\textsuperscript{1} plots of $\log(TMPD(\text{ox})/\text{TMPD(\text{red})}) \text{ vs } \log((\text{heme } a_H + \text{heme } a_L)\text{ox}/(\text{heme } a_H + \text{heme } a_L)\text{red})$. Experimental data were again fitted with a computer simulated curve which is shown in Figure 13. The potentials of hemes $a_H$ and $a_L$ agreed with the potentials determined by the $A \text{ vs } Q$ method.

Minnaert plots for the ferricyanide-cytochrome c oxidase mixture were not calculated since the small ferricyanide absorption band could not be deconvoluted from the intense Soret band.
FIGURE 11

Spectra of TMPD-Cytochrome c Oxidase mixture taken after incremental additions of reductive charge during an indirect coulometric titration. Absorbance changes at 560 nm, the cytochrome c oxidase isosbestic, are due to TMPD. 1.19 times the absorbance change at 560 nm plus the absorbance change at 605 nm is the deconvoluted absorbance change for cytochrome c oxidase. The numbers 0 through 9 correspond to successive stages of reduction. A slight excess of MV⁺ has been generated at 9.
FIGURE 12

Plot of Absorbance (A/cm) vs Charge (nanoequivalents/ml) for first reduction titration of cytochrome c oxidase-TMPD mixture. Solution contained 10 μM TMPD, 0.5 mM methyl viologen dichloride, 10.6 μM cytochrome c oxidase and pH 7 phosphate buffer. (○) – data points for reduction of cytochrome c oxidase with electrochemically generated methyl viologen cation radical. (____) computer simulation for

\[ E^{o'}_{\text{heme } a_H} = 340 \text{ mV}, \ E^{o'}_{\text{Cu}_H} = 350 \text{ mV}, \ E^{o'}_{\text{heme } a_L} = 215 \text{ mV}, \ E^{o'}_{\text{Cu}_L} = 215 \text{ mV}, \text{ and } E^{o'}_{\text{TMPD}} = 300 \text{ mV}. \]

(----) computer simulation for

\[ E^{o'}_{\text{heme } a_H} = 340 \text{ mV}, \ E^{o'}_{\text{Cu}_H} = 280 \text{ mV}, \ E^{o'}_{\text{heme } a_L} = 215 \text{ mV}, \ E^{o'}_{\text{Cu}_L} = 215 \text{ mV}, \ E^{o'}_{\text{TMPD}} = 300 \text{ mV}. \]
FIGURE 13

Plot of \( \log((\text{heme } s_H + \text{heme } s_L)_\text{ox}/(\text{heme } s_H + \text{heme } s_L)_\text{red}) \) vs \( \log(\text{TMPD(ox)}/\text{TMPD(red)}) \).

Solution contained 10 \( \mu \text{M} \) TMPD, 0.5 \( \text{mM} \) methyl viologen dichloride, 10.6 \( \mu \text{M} \) cytochrome \( \text{c} \) oxidase, and pH 7 phosphate buffer. (O) – data points for reduction of cytochrome \( \text{c} \) oxidase with electrochemically generated methyl viologen cation radical. (——) computer simulation for \( E^o_\text{heme } s_H = 335 \text{ mV}, E^o_\text{heme } s_L = 220 \text{ mV}, \) and \( E^o_{\text{TMPD}} = 300 \text{ mV} \). (—-) computer simulation for \( E^o_\text{heme } s_H = E^o_\text{heme } s_L = 280 \text{ mV}, \) and \( E^o_{\text{TMPD}} = 300 \text{ mV} \) for comparison.
These values for the potentials of heme $a_H$ and heme $a_L$ in the presence of ferricyanide and TMPD agree with the values previously reported in the presence of cytochrome $c$.\textsuperscript{27}

**CYTOCHROME c OXIDASE 830 nm BAND**

No general consensus as to the origin of the 830 nm cytochrome $c$ oxidase absorption band exists at this time. The 830 nm band has been considered to be due to the copper components\textsuperscript{28,29} but whether it is due to one or both of the coppers is not yet certain.\textsuperscript{30} A possible heme $a$ contribution at 830 nm has also been mentioned.\textsuperscript{31} Various midpoint potentials and $n$ values have been attributed to the 830 nm component(s). Tzagoloff et al.\textsuperscript{32} reported this component to have an $n$ value of 1.0 and $E^{\circ'} = 284$ mV. Wilson\textsuperscript{6} reported this component to have an $n$ value of 0.9 and $E^{\circ'} = +250$ mV. Van Gelder\textsuperscript{25} reported an $n$ value of 1.6 and $E^{\circ'} = +280$ mV in the absence of cytochrome $c$. In the presence of cytochrome $c$, the $n$ value is 1.0 and $E^{\circ'} = +280$ mV. He attributed the $n$ value of 1.6 to a copper-copper interaction.

The 605 nm and 830 nm absorption bands of cytochrome $c$ oxidase were simultaneously monitored during an indirect coulometric titration of a solution containing 10.9 uM cytochrome $c$ oxidase, pH 7.0 phosphate buffer, 0.5 mM methyl viologen dichloride, and 0.1% Tween 20. The cytochrome $c$ oxidase was repetitively cycled between its redox states by reducing with electrogenerated methyl viologen cation radical, and oxidizing with electrogenerated molecular oxygen. Figure
14 shows the $A$ vs $Q$ curves for the repetitive titrations. The low molar absorptivity of $1.4 \text{ mM}^{-1}\text{cm}^{-1}$ at 830 nm $^{33}$ makes quantitation of that signal very difficult. Baseline drift of the spectrometer and background absorbance corrections, due to other solution components become very important at the small absorbance changes (ca 0.05 units total change) measured in this experiment.

Therefore only qualitative observations of the 830 nm band $A$ vs $Q$ plots have been attempted. The 830 nm band moves parallel with the 605 nm band. Both $A$ vs $Q$ plots are essentially linear for both oxidations and reductions. Four electrons per molecule of cytochrome $c$ oxidase are taken up during the reductions. Since each heme $a$ contributes 50% to the total absorbance at 605 nm, the linearity of the $A_{605}$ vs $Q$ plot indicates two possibilities as to the energetics of the four redox centers. Either all four redox centers have the same midpoint potentials, in which case all four components would be titrated simultaneously, with the resulting linear $A$ vs $Q$ plot, or the redox components are split into two heme $a$-Cu pairs, also leading to a linear $A$ vs $Q$ plot. The combination of four electrons per molecule of cytochrome $c$ oxidase being taken up, and the linear $A$ vs $Q$ plot, exclude all other midpoint potential possibilities. The ferricyanide-cytochrome $c$ oxidase and TMPD-cytochrome $c$ oxidase indirect coulometric titration experiments then confirmed the four redox centers to be split into two heme $a$-Cu pairs.
Absorbance vs Charge plot for repetitive indirect coulometric titration of cytochrome c oxidase with electrochemically generated methyl viologen cation radical as reductant and electrochemically generated molecular oxygen as oxidant. ▼- corresponds to 605 nm absorbance (left hand scale). ◦- corresponds to 830 nm absorbance (right hand scale).
Similarly, the linearity of the 830 nm absorption band and its parallelism with the 605 nm band allows one to exclude certain possibilities as to the origin of the 830 nm band, and the midpoint potentials of the 830 nm component(s).

Since the 830 nm band moves linearly with charge and is fully reduced after four equivalents per cytochrome c oxidase molecule are added, the 830 nm band cannot be due to any one component alone. The parallelism with the 605 nm band also suggests that the 830 nm band is not due to one heme and one copper unless the molar absorptivities of the two components at 830 nm are approximately equal. This is a possibility, however, since the kinetic experiments of Malmström indicated that heme a contributed 40% to the total absorbance change at 830 nm. He reduced cytochrome c oxidase with ferrocytochrome c and monitored the 550 nm band of cytochrome c and the 445, 605, and 830 nm bands of cytochrome c oxidase. An initial rapid phase of the reaction corresponded to the formation of one mole of ferricytochrome c per mole of cytochrome c oxidase. Subsequent reactions were slow enough that the rapid phase was completely isolated. The one to one stoichiometry indicated that a single component of cytochrome c oxidase was accepting electrons rapidly. Therefore, the absorbance changes during the rapid phase of the reaction at 445, 605, and 830 nm were attributed to cytochrome a. Present data at 830 nm are not precise enough to discount the possibility of a 40% heme a and 60% copper contribution to the 830 nm band.
The kinetic results of Malmström are in disagreement with the pulse radiolysis kinetic results of Van Gelder and coworkers\textsuperscript{34} for the reduction of cytochrome c. Van Gelder also reports a biphasic reaction, but with the initial rapid phase of the reaction corresponding to a 2:1 stoichiometry of ferricytochrome c generated to cytochrome c oxidase reduced. Van Gelder reported that one of the copper components was accepting an electron in his experiments during the rapid phase of the reaction, but he did not report any 830 nm observations.

Another possibility which one cannot exclude at this time is that the 830 nm band is due purely to the two heme a's without any copper contribution. The indirect coulometric titration data clearly indicate, however, that copper midpoint potentials determined solely from the 830 nm band without any correction for spectral contribution from other redox components are inaccurate.

A great deal of controversy still remains as to the condition under which the hemes of cytochrome c are split in potential. Minnaert,\textsuperscript{1} Slater and coworkers\textsuperscript{35} and Tzagoloff and Wharton\textsuperscript{28} used log-log plots to determine the equilibrium constant of the reaction between cytochrome c and cytochrome c oxidase and reported the midpoint potential of cytochrome a to be +285 mV with an \( n \) value of 0.5. Tsudzuki and Wilson\textsuperscript{6} reported the same midpoint potential and \( n \) value using the potentiometric titration technique. These last authors attributed the unusual \( n \) value to changes in the heme protein caused by the harsh procedures to which the cytochrome c oxidase is
necessarily subjected during purification. Partially purified and intact mitochondrial cytochrome c oxidase apparently gave titration curves with the heme components resolved into a high potential cytochrome(a') \((E^0' = 375 \text{ mV and } n = 1.0)\) and a low potential cytochrome (a) \((E^0' = +225 \text{ mV and } n = 1.0)\). Wilson later reported that the two heme moieties were split in potential for the highly purified state.\(^{36}\) The anomalous \(n\) values and potentiometric equivalences of the hemes for Minnaert's, Slater's, Tzagoloff and Wharton's, and Wilson's works were apparently due to monitoring only the intermediate regions of the potentiometric titrations. If the -2 to +2 Log(heme a-(ox)/heme a(red)) potentiometric region were monitored, a plot of \(E\) vs Log(ox/red) would exhibit a sigmoid shape. This is characteristic of a potentiometric plot when more than one species is being physically monitored. Therefore, we suggest that much of the previous data are consistent with our indirect coulometric titration results.

In conflict with our results are Wilson's results, Van Gelder and coworkers reported that the hemes of his cytochrome c oxidase preparation titrated as indistinguishable entities in the absence of cytochrome c, each having \(E^0' = 280 \text{ mV and } n = 1.0\). However, in the presence of cytochrome c, the hemes were resolved into a high potential component \((E^0' = 335-360 \text{ mV and } n = 1.2)\) and a low potential component \((E^0' = 200-250 \text{ mV and } n = 1.0)\) with the high potential component contributing 45% of the absorbance change at 605 nm and the low potential component contributing 55%. Potentiometric titrations of cytochrome c oxidase, followed by circular dichroism\(^{37}\)
in the absence of cytochrome c, gave further confirmation of the
potentiometric equivalence of the two hemes of Van Gelder's preparation.

Assuming the experimental data of the different research groups
has been correctly evaluated, the differences in the reported midpotentials of the heme a's could most reasonably be attributed to
differences in experimental conditions. A wide variety of potentio-
metric mediators, chemical titrants, solubilizing detergents, and
stabilizing agents have been employed. The possibility that these
reagents could affect the thermodynamic properties of cytochrome c
oxidase has led us to simplify our solution conditions as much as
possible.

The indirect coulometric titration solutions contained only a
pH 7.0 phosphate buffer, 0.1% Tween 20, 0.5 mM methyl viologen
dichloride, and 0.1 to 2.0 mM ferricyanide. Wilson's solutions36
contained 0.2 mM mannitol, 0.05 M sucrose, 30 mM morpholinopropane
sulfonate, pH 7.2, 80 uM phenazine methosulfate, 120 uM ferrous
sulfate, 20 mM ethylene dinitrilotetraacetate, 100 uM diaminodurene,
300 uM to 2 mM ferricyanide, and NADH as reductant. Van Gelder's
solutions3 contained 100 mM phosphate buffer (pH 7.1), 0.5% Tween 80,
30-50 uM phenazine methosulfate, 30-50 uM diaminodurene, 120-200
uM ferricyanide, and NADH as reductant. The potentiometric mediators
of Van Gelder are common to those contained in Wilson's solution so
it is unlikely that they are causing the discrepancy. The phosphate
buffer and Tween 80 have been used in the indirect coulometric titra-
tions and have not been found to affect the heme a thermodynamics.
However, detergents are known to affect the activity of cytochrome \( c \) oxidase.\(^{38}\) Surface active agents are needed for the solubilization of cytochrome \( c \) oxidase and both nonionic detergents and sodium cholate are present in Hartzell's prep. The 0.2% sodium cholate present in Hartzell's prep inhibits the enzymic activity of cytochrome \( c \) oxidase,\(^{39}\) but does not necessarily alter the thermodynamics of the redox components. Also, cholate is common to all cytochrome \( c \) oxidase preparations and therefore does not explain the differences in the reported heme \( a \) potentials. This leaves the purification methods as one of the most possible sources for the reported discrepancies. However, at this time, a definitive explanation of the discrepancies cannot be given.

One point should be made and that is the experimentally observed split in potential of the hemes and coppers of cytochrome \( c \) oxidase is not proof that they are distinguishable in the fully oxidized or fully reduced states. Keilin and Hartree\(^{40}\) originally conceived cytochrome \( c \) oxidase to consist of "two distinct but closely allied and intimately connected compounds, \( a \) and \( a_2 \)." They also theorized the possible interconvertibility of the two compounds. This concept infers that cytochrome \( c \) oxidase is a symmetrical dimeric molecule with two equivalent hemes and two equivalent coppers. Van Gelder takes this concept and states "which one of these two equivalent heme \( a \) groups is first reduced is governed only by statistics, not by thermodynamics". He pictures cytochrome \( c \) inducing conformational changes in cytochrome \( c \) oxidase which alter the midpoint potentials...
of the redox components, effectively splitting the components into high and low potential groups. They term this idea negative cooperativity between the two heme $a$ groups of cytochrome $c$ oxidase, and also between the two copper centers.

An alternative reasonable explanation, not invoking ligand conformational changes, for the difference in potentials observed experimentally in that each electron transferred alters the charge and electron density distribution within the cytochrome $c$ oxidase molecule which consequently affects the thermodynamics for the next electron transferred. Ligand effects are considered less likely to cause the split in potential since the potentials of the four redox components of cytochrome $c$ oxidase are reproducible and independent of the mediators used in this study. Wilson and coworkers$^{36}$ dispute the "identical heme" hypothesis, maintaining the experimental observed split in potential proves the distinction of the hemes in the fully oxidized and fully reduced states. However, the methyl viologen used in this study is a good chemical example of how the first electron transferred to the bipyridylium compound alters the thermodynamics of the second electron transferred.$^{41}$

OXIDATIVE TITRATIONS OF FERROCYANIDE-CYTOCHROME $c$ OXIDASE

Few oxidative titrations have been reported in the literature,$^{2,24}$ and except for Heineman et al.'s oxidative titration$^2$ with electro-generated molecular oxygen, have involved the use of inhibitors. Oxidative titrations of cytochrome $c$ oxidase with electro-generated
ferricyanide have been attempted in this study to determine the reversibility of repetitively cycling cytochrome c oxidase between its oxidized and reduced states. The redox titrations of Heineman gave reversible oxidations and reductions as judged by the A vs Q plots. Wilson et al. report potentiometrically reversible titrations both in the presence and absence of carbon monoxide for chemical reductions with NADH and oxidations with ferricyanide. These titrations were judged to be reversible from plots of potential vs A605-624 nm.

Our oxidations were not mirror images of the reductive A vs Q plots. Figure 15 shows the A vs Q plot for the initial reduction of cytochrome c oxidase, and the reoxidation with electrochemically generated ferricyanide. The slope of the reductive points is 0.0034 A/ml/nanoequivalent which corresponds to an n value of two electrons per heme a. One copper and one heme, having the same midpoint potentials, titrate simultaneously, which accounts for the n value of 2. The slope of the oxidative titration points, however, is 0.0074 A/ml/nanoequivalent, corresponding to an n value of one electron per heme a. This indicates that the low potential heme a is initially oxidized but that the low potential copper is not accepting electrons. Whether this is attributable to kinetic blocking of the copper site or to a change in the CuL midpoint potential has not yet been determined. If the copper site is kinetically inhibited from accepting electrons, the inhibition could most likely be attributed to ferricyanide or dissociated cyanide ion,
FIGURE 15

Absorbance vs Charge (nanoequivalents/ml) plot for reduction of cytochrome c oxidase with electrogeneated methyl viologen cation radical and oxidation with electrogeneated ferricyanide. Heme a absorbance is monitored at 605 nm. Solid line drawn through initial reduction points has a slope of 0.0034 corresponding to an n value of 2. Solid line drawn through oxidation points has a slope of 0.0074 corresponding to an n value of 1.
since all other solution species were present in Heineman et al.'s reversible titrations. It is puzzling why the ferricyanide, which was present at the start of the experiment, did not affect the reductive part of the titration. However, if cyanide were responsible for the inhibition, it is known that cyanide reacts very slowly with oxidized cytochrome c oxidase (approximately 15 hours, independent of the concentrations of cyanide and cytochrome c oxidase) but reacts rapidly with reduced heme a \( (k = 1.3 \times 10^2 \text{ M}^{-1}\text{s}^{-1}) \).\(^{18}\)

Other problems associated with the oxidative titration of cytochrome c oxidase with ferricyanide were slow equilibration after charge injections, and slow rereduction of the cytochrome c oxidase upon standing, probably due to the Tween 20 detergent. Slow reduction of the enzyme does not occur when Triton QS-30 detergent is used to solubilize the enzyme. However, these problems do not account for the \( n = 1 \) slope of the \( A \) vs \( Q \) plots.

**OXIDATIVE TITRATIONS WITH DETERGENT SOLUBILIZED FERROCENE**

Since dissociated cyanide could account for the nonreversible oxidative titration with ferricyanide present, an oxidative titration with detergent solubilized ferrocene was attempted. The electrochemistry of detergent solubilized ferrocene in pH 7 aqueous solution has been demonstrated by Yeh and Kuwana\(^{42}\) to be a clean, 1 electron reversible process at a platinum electrode. Fujihira et al.\(^{43}\) used electrogenerated detergent solubilized ferricinium to demonstrate reversible oxidative and reductive titrations of cytochrome c-cytochrome c oxidase mixtures. He determined the potentials of the heme a
components relative to the cytochrome $c$ and found the midpoint potentials to be relatively unchanged from those determined by oxidation with electrogenerated molecular oxygen.\textsuperscript{27}

A solution containing 0.98 mM ferrocene, 2.5% Tween 20, 13.6 uM cytochrome $c$ oxidase, 0.5 mM methyl viologen dichloride, and pH 7 phosphate buffer was indirectly coulometrically titrated. The oxidative and reductive titrations were reversible as judged by the $A$ vs $Q$ plots being mirror images. The midpoint potentials of the heme $a$'s and coppers were determined relative to ferrocene ($E^\circ_{\text{ferrocene}} = +437$ mV vs NHE) by curve fitting the experimental data to computer simulated $A$ vs $Q$ plots. Figure 16 illustrates the $A$ vs $Q$ plot for the first oxidation. The best fit line is simulated for $E^\circ_{\text{heme } a_1} = 350$ mV, $E^\circ_{\text{CuH}_1} = 350$ mV, $E^\circ_{\text{CuH}_2} = 350$ mV, $E^\circ_{\text{heme } a_2} = 215$ mV, $E^\circ_{\text{CuL}_1} = 215$ mV, and $E^\circ_{\text{ferrocene}} = 437$ mV. The comparison lines are simulated for $E^\circ_{\text{CuH}_1} = 330$ mV and 370 mV and all other midpoint potentials the same as for the best fit. The computer simulation is less sensitive to the high potential copper than in the TMPD-cytochrome $c$ oxidase system since the potential of ferrocene is further removed from the copper potential than that of TMPD. However, the oxidation indicates all four redox center midpoint potentials are relatively unchanged from the potentials determined during the reduction.

TITRATIONS OF CYTOCHROME $c$-CYTOCHROME $c$ OXIDASE MIXTURES

Cytochrome $c$ is the precursor of cytochrome $c$ oxidase in the electron transport chain. Adding this enzyme to cytochrome $c$ oxidase
FIGURE 16

Plot of Absorbance vs Charge (millicoulombs/ml) for the first oxidative titration of cytochrome c oxidase-ferrocene mixture. Solution contained 0.98 mM ferrocene, 2.5% Tween 20, 0.5 mM methyl viologen dichloride, 13.6 µM cytochrome c oxidase, and pH 7 phosphate buffer. (0) – data points for oxidation of cytochrome c oxidase with electrochemically generated ferricinium. (___) – computer simulation for $E^{o'}_{heme} = E^{o'}_{CuH} = 350$ mV, $E^{o'}_{CuL} = 215$ mV, $E^{o'}_{ferrocene} = 437$ mV. Comparison simulations are for $E^{o'}_{CuH} = 330$ and 370 mV for lower and upper curves, respectively.
adds more complexity to the experiment, but comes one step closer to evaluating the \textit{in vivo} mechanisms of electron transport and oxidative phosphorylation. Heineman et al.\textsuperscript{27} previously evaluated the midpoint potentials of both heme a's relative to cytochrome c for oxidations and reductions with log-log plots. The midpoint potentials were determined to be $E_{\text{heme}}^{0} = 350$ mV and $E_{\text{heme}}^{0} = 210$ mV for the reductive titration, and $E_{\text{heme}}^{0} = 350$ mV and $E_{\text{heme}}^{0} = 225$ mV for the oxidative titration with molecular oxygen. Fujihira et al.\textsuperscript{43} used log-log plots to evaluate the heme potentials in cytochrome c-cytochrome c oxidase mixtures for oxidations with electrogenerated ferricinium. The potentials were determined to be $E_{\text{heme}}^{0} = 340$ mV and $E_{\text{heme}}^{0} = 209$ mV for both oxidations and reductions. The heme a midpoint potentials were essentially the same within experimental error for both studies even though the titrations were irreversible in one case and reversible in the other case. Since the heme a potentials were the same in both studies, the differences in reversibility of the A vs Q plots must be due to either kinetic inhibition of electron transport to the copper centers, or to changes in the midpoint potentials of the copper centers. However, the copper potentials were not monitored in either study.

Cytochrome c-cytochrome c oxidase mixtures were indirectly coulometrically titrated by reducing with methyl viologen cation radical and oxidizing with ferricinium to evaluate the midpoint potentials of the copper centers. The heme a midpoint potentials were
initially evaluated by curve fitting experimental log-log plots to computer simulated curves. Figure 17 shows the Log(Cyt c(ox)/Cyt c(red)) vs Log((heme aH + heme aL)ox/(heme aH + heme aL)red) data points and the computer simulation best fit. The simulation is for $E^0_{\text{heme } a_H} = 352 \text{ mV}$, $E^0_{\text{heme } a_L} = 213 \text{ mV}$, and $E^0_{\text{Cyt } c} = 255 \text{ mV}$. The comparison curves are for the midpoint potentials of the heme a components being $\pm 5 \text{ mV}$ from the best fit simulation. The potential evaluations are judged to be accurate within $\pm 5 \text{ mV}$. The cytochrome c absorbance at 550 nm was corrected for heme aL absorbing with a molar absorptivity of 2200 M$^{-1}$cm$^{-1}$. The heme a absorbance at 605 nm was corrected for cytochrome c absorbing with a molar absorptivity of 930 M$^{-1}$cm$^{-1}$.

The midpoint potentials of the hemes determined from the log-log plot were then used in the A vs Q plot. The midpoint potentials of the coppers were varied in the computer simulation to obtain the best fit in Figure 18. The midpoint potentials for the best fit are $E^0_{\text{heme } a_H} = 352 \text{ mV}$, $E^0_{\text{heme } a_L} = 213 \text{ mV}$, $E^0_{\text{Cyt } c} = 255 \text{ mV}$, $E^0_{\text{Cu}_H} = 350 \text{ mV}$, and $E^0_{\text{Cu}_L} = 215 \text{ mV}$. The 550 nm absorbance was again corrected for heme aL absorbance. The midpoint potentials of the copper atoms are the same within experimental error as in the absence of cytochrome c.

An indirect coulometric titration of cytochrome c-cytochrome c oxidase mixture in the absence of ferrocene was then performed to evaluate the copper midpoint potentials in that situation. The solution contained 0.5 mM methyl viologen dichloride, 13.3 mM...
FIGURE 17

Plot of \( \log((\text{heme } a_H + \text{heme } a_L)_{\text{ox}}/\text{(heme } a_H + \text{heme } a_L)_{\text{red}}) \) vs \( \log(\text{Cyt } c_{\text{ox}}/\text{Cyt } c_{\text{red}}) \). Solution contained 21.9 mM cytochrome \( c \), 13.3 mM cytochrome \( c \) oxidase, 0.98 mM ferrocene, 0.5 mM methyl viologen dichloride, 2.5% Tween 20, and pH 7 phosphate buffer. (O) - data points for oxidation with electrochemically generated ferricinium. (-) - computer simulation for \( E^0'_{\text{heme } a_H} = 352 \text{ mV} \), \( E^0'_{\text{heme } a_L} = 213 \text{ mV} \), and \( E^0'_{\text{Cyt } c} = 255 \text{ mV} \). Comparison curves are for the midpoint potentials of the heme \( a \) components being \( +5 \text{ mV} \) from the best fit. Upper curve is simulated for \( E^0'_{\text{heme } a_L} = 208 \text{ mV} \). Lower curve is simulated for \( E^0'_{\text{heme } a_H} = 347 \text{ mV} \) and \( E^0'_{\text{heme } a_L} = 218 \text{ mV} \).
Normalized Absorbance vs Charge (millicoulombs/ml) plot for cytochrome c-cytochrome c oxidase mixture. Solution contained 21.9 µM cytochrome c, 13.3 µM cytochrome c oxidase, 0.98 mM ferrocene, 0.5 mM methyl viologen dichloride, 2.5% Tween 20, and pH 7 phosphate buffer. (O) - data points for absorbance of cytochrome c at 550 nm and heme a at 605 nm. (- -) - computer simulation for $E^{\circ}_{heme-H} = 352$ mV, $E^{\circ}_{CuH} = 350$ mV, $E^{\circ}_{heme-L} = 213$ mV, $E^{\circ}_{CuL} = 215$ mV, $E^{\circ}_{Cyt c} = 255$ mV. A indicates Cytochrome c. B indicates Cytochrome c oxidase.
cytochrome c oxidase, 21.9 μM cytochrome c, 2.5% Tween 20, and pH 7 phosphate buffer. The computer simulation best fit for the A vs Q plot, shown in Figure 19, corresponds to $E_{heme a}^{°} = 350$ mV, $E_{CuH}^{°} = 270$ mV, $E_{heme aL}^{°} = 200$ mV, $E_{CuL}^{°} = 190$ mV, and $E_{Cyt c}^{°} = 250$ mV. This indicates that the midpoint potential of the high potential copper has been lowered by approximately 70 or 80 mV. This agrees well with a qualitative evaluation of Heineman et al.'s reductive titration of the cytochrome c-cytochrome c oxidase mixture.

The binding studies of Schejter have shown that the midpoint potential of cytochrome c is ionic strength dependent at low ionic strength (ca. 0.004 M). The binding studies of Hartzell have shown that cytochrome c binds to cytochrome c oxidase in a one to one ratio at low ionic strength (ca. 0.01 M). A supporting electrolyte is necessary to conduct electrochemical experiments, however, a 0.1 M glycine solution was shown to have no effect on the binding. Therefore, an indirect coulometric titration of a cytochrome c-cytochrome c oxidase mixture at low ionic strength was performed to determine the effect of the binding of cytochrome c to cytochrome c oxidase on the midpoint potentials of the four redox centers. The solution contained 0.1 M glycine, 0.01 M phosphate buffer, 2.5% Tween 20, 0.98 mM ferrocene, 21.9 μM cytochrome c, 13.3 μM cytochrome c oxidase, and 0.5 mM methyl viologen dichloride. Within the experimental error of the measurements, the binding of cytochrome c to cytochrome c oxidase was found to have no effect on the midpoint potentials of the cytochrome c oxidase heme a redox components.
Normalized Absorbance vs Charge (nanoequivalents/ml) plot for cytochrome c-cytochrome c oxidase mixture. Solution contained 21.9 μM cytochrome c, 13.3 μM cytochrome c oxidase, 0.5 mM methyl viologen dichloride, and pH 7 phosphate buffer. (o) - data points for absorbance of cytochrome c at 550 nm, and heme a at 605 nm. (____) - computer simulation for $E^o_{\text{heme aH}} = 350 \text{ mV}$, $E^o_{\text{CuH}} = 270 \text{ mV}$, $E^o_{\text{heme aL}} = 200 \text{ mV}$, $E^o_{\text{CuL}} = 190 \text{ mV}$, and $E^o_{\text{Cyt c}} = 250 \text{ mV}$. A indicates Cytochrome c. B indicates Cytochrome c oxidase.
CHARGE (nanoequiv/ml)

NORM ABSORBANCE

CHARGE (nanoequiv/ml)
CONCLUSION

Quantitation of the mass balance conditions during the redox titrations of electron transport components has been shown to be of significant importance in analyzing the redox states of both optically monitorable and non-monitorable species. Specifically, the monitoring of charge in indirect coulometric titrations has allowed us to probe both the hemes and coppers of cytochrome c oxidase. In addition, the indirect coulometric titration allows the rapid, repetitive cycling of the electron transport enzymes between their oxidized and reduced states.

The indirect coulometric titration of ferricyanide-cytochrome c oxidase mixtures allowed the determination of the energetics of the four redox centers of cytochrome c oxidase during the initial reductive titration. The potentials were evaluated as $E^{O'}_{\text{heme } a_H} = 350 \text{ mV}$, $E^{O'}_{\text{heme } a_L} = 220 \text{ mV}$, $E^{O'}_{\text{Cu_H}} = 350 \text{ mV}$, and $E^{O'}_{\text{Cu_L}} = 220 \text{ mV}$. The indirect coulometric titration of TMPD-cytochrome c oxidase mixtures allowed a more accurate determination of the heme potentials from the log-log plots without interference from the copper components. A more accurate determination of the midpoint potentials of the low potential components was also possible from the $A$ vs $Q$ plots.

The oxidative and reductive $A$ vs $Q$ plots were not seen to be mirror images for the ferricyanide cytochrome c oxidase system. Inhibition by cyanide of electron transfer to cytochrome c oxidase after the initial reduction was suggested as a possible explanation for this observation.
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<td>352 ± 5</td>
<td>ferricyanide present</td>
</tr>
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<td></td>
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<td>200 ± 20</td>
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<td></td>
<td>215 ± 10</td>
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<td>350 ± 10</td>
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<td>215 ± 30</td>
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<td></td>
<td>350 ± 10</td>
<td>cytochrome c present</td>
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<tr>
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<td></td>
<td></td>
<td>190 ± 20</td>
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<td>270 ± 10</td>
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<tr>
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<td>210 ± 15</td>
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<td>Van Gelder (1972)(^3)</td>
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<td>225</td>
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<td></td>
<td>350</td>
<td>285(^c)</td>
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<td>Wilson (1971)(^6)</td>
<td>225</td>
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<td></td>
<td>375</td>
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<tr>
<td></td>
<td>(1974)(^3)(^6)</td>
<td>205</td>
<td>360</td>
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<td>(1974)(^3)(^6)</td>
<td>315</td>
<td>245</td>
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<td>(1974)(^2)(^3)</td>
<td></td>
<td>350 ± 20</td>
<td>highly purified</td>
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\(^a\) frequently designated cyt a  
\(^b\) frequently designated cyt a\(^\alpha\)  
\(^c\) titrated as single component  
\(^d\) based on 830 nm band and EPR
<table>
<thead>
<tr>
<th>NAME</th>
<th>WAVELENGTH</th>
<th>$\text{M}^{-1}\text{cm}^{-1}$</th>
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<td>18,500</td>
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<td>930</td>
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<td>Cytochrome $c$ Oxidase</td>
<td>604</td>
<td>24,000</td>
</tr>
<tr>
<td>Cytochrome $c$ Oxidase</td>
<td>830</td>
<td>1,400</td>
</tr>
<tr>
<td>heme $a_H$</td>
<td>604</td>
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<td>heme $a_L$</td>
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<td>2,200</td>
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<td>Ferricinium</td>
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<td>Methyl Viologen Cation Radical</td>
<td>605</td>
<td>12,500</td>
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<tr>
<td>Methyl Viologen Cation Radical</td>
<td>550</td>
<td>8,200</td>
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A vs Q plots for the detergent solubilized ferrocene-cytochrome c oxidase system confirmed the existence of two heme a-Cu pairs for both oxidation and reduction. Extending this system to include cytochrome c, A vs Q plots showed the reversibility of cytochrome c-cytochrome c oxidase indirect coulometric titrations with one electron oxidants and reductants. The reversibility probably resulted from the fact that the ferrocene-ferricinium couple could act to produce externally mediated intramolecular electron transfer in cytochrome c oxidase.

Heineman et al. 27 presented non-reversible oxidations and reductions of cytochrome c-cytochrome c oxidase mixtures when using a one electron reductant and a four electron oxidant, molecular oxygen. The A vs Q plot simulations for that system indicated that CuH had a midpoint potential of 270 mV during the reductive titration, which was probably the result of applying thermodynamic equations to a nonequilibrium situation. Although the A vs Q plots indicated the oxidations and reductions of cytochrome c oxidase as a whole were non-reversible, the log-log plots indicated the heme a oxidations and reductions were reversible. Since this system does not contain anything that was not present in the ferrocene-cytochrome c-cytochrome c oxidase system, it is unlikely that inhibitors are causing the irreversibility. A more likely explanation for the difference in copper potentials is the absence of the ferrocene titrant, which can also act as a mediator for intramolecular transfer between the four redox centers in cytochrome c oxidase. Methyl viologen is present in Heineman's system, but it cannot act as a mediator because its
midpoint potential is approximately 700 mV removed from the cytochrome c oxidase components. A reasonable explanation for the $A \text{ vs } Q$ behavior of the reductive titration of the cytochrome c-cytochrome c oxidase system in the absence of mediators is that methyl viologen initially transfers charge rapidly to heme $a_H$ but heme $a_H$ does not rapidly transfer charge intramolecularly to the copper center of equal energetics. Once heme $a_H$ is completely titrated, charge is transferred to the low potential components which can then rapidly transfer charge intramolecularly to the high potential copper. This would account for the midway plateau in the cytochrome c oxidase $A \text{ vs } Q$ plot and the associated 270 mV apparent $E^o$ for Cu$_H$. 
LIST OF REFERENCES

5. D.C. Wharton and M.A. Cusanovitch, private communication.


CHAPTER III

SPECTROELECTROCHEMICAL INVESTIGATION OF KINETIC RATES OF CYTOCHROME c AND CYTOCHROME c OXIDASE ELECTRON TRANSFERS

Kinetic studies of the heme proteins, cytochrome c and cytochrome c oxidase, were undertaken to determine if inferences as to the mechanistic pathways of electron transfer to and from the hemes using various reductants and oxidants could be made. Mechanistic questions concerning cytochrome c charge transfer include whether electron transfer occurs by an outer sphere mechanism, possibly through the \( \pi \) electron system of the prophyrin ring, or by an inner sphere process at the heme crevice. It is also unclear whether cytochrome c reductase and cytochrome c oxidase react at the same or different parts of the cytochrome c protein.

Cytochrome c oxidase is thought to contain four metal redox centers and reduction of molecular \( \text{O}_2 \) to \( \text{H}_2\text{O} \) is a four electron process overall. However, it is uncertain whether all four cytochrome c oxidase redox centers are directly involved in the reduction of molecular \( \text{O}_2 \). Also, the specificity of enzymic reactions makes it unlikely that cytochrome c transfers directly to more than one redox center of cytochrome c oxidase. Therefore, intramolecular electron transfer is almost certainly an important aspect of the cytochrome c oxidase catalytic function.
Recent approaches have aimed at developing substrate designs that probe the effective protein conformation for specific transfer mechanisms. For example, Gray and coworkers\(^2\) have used the tris complexes of 1,10-phenanthroline and its derivatives with cobalt(III) to study the steric requirements for oxidation of ferrocytochrome \(c\). Marcus theory calculations for outer sphere electron transfer, using self-exchange rates for the cytochrome \(c^3\) and the inorganic complex, have agreed very well with experimental data, indicating the electron transfer follows the same pathway as in the self-exchange reaction. Castro and coworkers\(^4,5\) have used the axial inner sphere oxidant, bromomalononitrile, to investigate the effect of protein conformation of cytochrome \(c\), cytochrome \(a\), and cytochrome \(a_3\) at various pH values on the rate of oxidation.

Margoliash and coworkers\(^6\) have taken a different approach. Instead of using various oxidants and reductants, they have modified individual amino-acid residues of cytochromes \(c\), and attempted to determine the effects of these changes on the physiological functions of the protein.

Kinetic investigations of cytochrome \(c\) oxidase charge transfer have concentrated either on reduction of completely oxidized cytochrome \(c\) oxidase with ferrocytochrome \(c\),\(^7-9\) or on oxidation of completely reduced cytochrome \(c\) oxidase with molecular \(O_2\).\(^10\) The reduction of cytochrome \(c\) oxidase with ferrocytochrome \(c\) was biphasic, with an initial rapid reaction followed by a slower phase. However, results have differed on the number of equivalents of ferrocytochrome \(c\)
oxidized per equivalent of heme \( a \) reduced in the rapid phase of the reaction. Castro and coworkers' results\(^5\) indicated that the approach of the axial inner sphere oxidant, bromomalononitrile, to the cytochrome \( c \) and cytochrome \( a \) heme centers was blocked by their protein conformations at physiological pH. However, the approach of the inner sphere oxidant to the cytochrome \( a_3 \) heme center was not sterically constrained. He, therefore, postulated the reduction of cytochrome \( a \) by cytochrome \( c \) as a peripheral \( \pi \) transfer.

In this investigation, the kinetic rates of oxidation and reduction of cytochrome \( c \) and cytochrome \( a \) oxidase with electrogenerated reactants have been evaluated by spectroelectrochemical methods at optically transparent semiconductor electrodes.\(^\text{11,12}\)

Reduction of the heme proteins with electrogenerated methyl viologen cation radical (\( MV^\dagger \)) and oxidation of the heme proteins with electrogenerated 1,1'-bis(hydroxymethyl)ferricinium were analyzed.

Initial experiments were directed to determining the rate coefficient for the reaction

\[
MV^{++} + e^- \rightarrow MV^\dagger \quad (1)
\]

\[
MV^\dagger + \text{ferricytochrome } c \xrightleftharpoons[k_f]{k_b} MV^{++} + \text{ferro-cytochrome } c \quad (2)
\]

The experiment was performed under chronoamperometric conditions where the electrode potential was stepped to a value such that \( MV^\dagger \) was electrogenerated at a diffusional rate. The reaction of \( MV^\dagger \) with
ferricytochrome c regenerated MV$$^{++}$$, and is referred to as the "catalytic regeneration" scheme by electrochemists.

Previous spectroelectrochemical studies of the kinetics of electron transfer from various bipyridylum radicals to cytochrome c indicated the rate constant was dependent upon the time scale of the experiment.\textsuperscript{13,14} A rate constant for electron transfer from methyl viologen cation radical to oxidized horse heart cytochrome c of $$5 \pm 2 \times 10^{-5} \text{ M}^{-1} \text{s}^{-1}$$ was reported from analysis of only the forward step portions of an 8 msec pulse time chronoabsorptometric experiment using signal averaging with a repetition rate of 1 Hz. Steckhan and Kuwana\textsuperscript{14} examined the rates of electron transfer from three other bipyridylum cation radicals to cytochrome c and found the rates to be in the range of 2 to $$9 \times 10^{-4} \text{ M}^{-1} \text{s}^{-1}$$. The rate constant of the methyl viologen cation radical was given as $$4 \pm 2 \times 10^{-4} \text{ M}^{-1} \text{s}^{-1}$$ and was calculated from analysis of A-t during the forward step of a chronoabsorptometric experiment at times greater than 100 msec.

Wilson\textsuperscript{15} recently examined the rate of electron transfer from methyl viologen cation radical to ferricytochrome c by monitoring both the 602 nm absorbance change due to MV$$^+$$, and the 550 nm change due to a combination of cytochrome c and MV$$^+$$ He separated the MV$$^+$$ absorbance at 550 nm from the cytochrome c absorbance by relying on his results at 602 nm. However, he failed to account for cytochrome c absorbance at 602 nm. He determined the second-order rate coefficient to be $$1.0 \pm 0.3 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$$. 
More recently, Land and Swallow and Simic and coworkers have used pulse radiolysis studies to determine the rate of electron transfer from various bipyridylium salts to cytochrome c. They determined the rate constant for the methyl viologen cation radical to be in the range of $10^8$ to $10^9$ M$^{-1}$s$^{-1}$.

The present work attempted to resolve the discrepancy in the reported rate coefficients and to precisely delineate the problems and necessary experimental precautions which must be taken with spectroelectrochemistry. Problems associated with the determination of the forward rate coefficient for reaction (2) included: (a) ferri-cytochrome c had a high molar absorptivity ($\varepsilon_{550}$ferricyt c = 10,000) at the $\Delta_{\text{max}}$ of cytochrome c so that a high background absorbance was present; (b) the absorbance changes of cytochrome c and MV$^+$ overlapped at 550 nm, the $\Delta_{\text{max}}$ of cytochrome c, so that deconvolution of the signals was necessary before analyzing the data; (c) the effect of the large differences in diffusion coefficients between cytochrome c and methyl viologen on the rate coefficient had to be accounted for; (d) since signal averaging of repetitive experiments was used to increase the signal to noise ratio of the optical signal, it was necessary to determine the appropriate delay time between the repetitive experiments; and (e) extraction of the rate coefficient required an analysis of the absorbance (A) vs time (t) data.

An optically transparent leucite spacer placed in the light path in the electrochemical cell body helped to alleviate problem (a)
so that the alpha band of cytochrome $c$ at 550 nm could be monitored at cytochrome $c$ concentrations between 50 and 100 $\mu$M.

Problems (b) through (e) were solved using a digital technique of simulating Fick's diffusion laws developed by Feldberg. Digital simulation accounted for the spectral overlap of cytochrome $c$ and MV$,^+$, and also included adjustments for the difference in diffusion coefficients between the relatively small organic reactant and the large heme protein. Cytochrome $c$ was essentially electro-inactive at the semiconductor electrodes used in this study. Therefore, the electrode was not involved in the restoration of these species to their initial conditions after electrochemically induced redox reactions. In the absence of a significant back reaction, restoration of the depleted species at the electrode surface was accounted for solely by diffusion from the bulk of the solution.

The digital simulation method was used to quantitatively evaluate the parameters affecting the appropriate delay time between repetitive, signal averaged potential step experiments. The effects of a significant back reaction replenishing the concentration of the depleted species at the electrode surface have also been evaluated.

Qualitative mechanistic implications can be made from the shapes of experimental spectroelectrochemical curves, but determination of kinetic rates requires a quantitative description of the time dependent solution absorbance. Normal kinetic mathematical relationships do not apply to spectroelectrochemistry because concentration gradients from the electrode surface to the bulk solution result from
the electrochemical perturbation. Fick's laws of diffusion, with appropriate kinetic terms, quantitatively describe solution conditions, but analytical solutions for integration of these differential equations have only been evaluated for first order, and pseudo first order reactions. The digital simulation technique is applicable to quantitating diffusion conditions with second order kinetic perturbations. Digital simulation calculates the concentration profiles of all reactant and product species in solution at any time after the electrochemical perturbation. Then calculation of the time dependent absorbance merely involves summing the concentrations over the entire reaction-diffusion layer.

A time independent absorbance change must also be accounted for. This absorbance, due to changes in the optical constants of the electrode-solution interface, has been described theoretically, using the free electron model, as being due to a shift in the plasma frequency. The time dependent and time independent absorbance changes are easily differentiated and pose no serious problems to spectroelectrochemical rate determinations.

EXPERIMENTAL:

1. Electrochemical equipment

All SEC experiments were run under chronoamperometric conditions. Either single pulse, double pulse, or pulse-relaxation experiments were performed, depending on the best way to normalize the absorbance. A conventional three electrode potentiostat
previously shown in Figure 1 was used for controlled potential generation of reactant during the chronoabsorptometric experiments. A Hewlett Packard 475A booster amplifier, which has a peak output capability of 20 V and 0.5 amp, provided the necessary power to generate a fast potentiostat rise time (ca. 20 μsec). A Hewlett Packard 3300-3302 function generator was used for linear sweep voltages for cyclic voltametric experiments, which were run prior to each experiment to verify proper operation of the electrochemical equipment and to set up potential step voltages. IR compensation was provided with positive feedback. For open circuit relaxation experiments, a relay, triggered by an HP function generator, similar in design to Blount et al.'s, was used to disconnect the working electrode.

2. Optical equipment

Set wavelength monitoring of solution species of interest was employed in all SEC kinetic investigations. Rapid scanning capability was available for obtaining complete visible spectra as a function of time, however, manipulation of the large quantities of data obtained is still a problem, especially for signal averaging of repetitive experiments. A 55 W quartz iodide tungsten lamp was used as the light source. However, Winograd has developed a stabilizing circuit for a 75 W xenon arc which would provide a higher light intensity. A Bausch and Lomb grating monochromator (1350 lines/mm) was employed with the slits adjusted for 3 nm resolution. Lenses
were employed after the monochromator to focus the light beam through the electrochemical cell and onto the photomultiplier tube. An EMI 9592B photomultiplier tube in a Pacific PMT housing was used as the detector, with a Fluke Model 412B high voltage power supply adjusted to give 12 microamps PMT current. The anode current was amplified with an operational amplifier in the current follower mode, and the resulting voltage was amplified, biased, and filtered to fall within the \( \pm 5 \) V acceptable to the analog to digital converter. The conventional RC filter was adjusted to 1% of the forward step time of the chronoabsorptometric experiment. The ADC had a maximum time resolution of 5 \( \mu \)sec per point, with between 100 and 1000 points normally employed. The optical experiment was interfaced to a Data General Nova 800 minicomputer with 32 K of memory.

Low frequency noise was virtually eliminated by placing the optical components on a Barry Controls vibrationless table, and using stabilized lamp and PMT power supplies. High frequency noise was primarily the result of PMT shot noise. For short time experiments (less than 50 msec) the signal was virtually buried in noise. The signal to noise ratio was improved with a combination of analog filtering and signal averaging of repetitive experiments. However, extreme precaution must be taken to ensure that experimental conditions are not changing with time during the averaging process.

3. Electrochemical cell

The titration cell, described in Chapter II, was modified to optimize the SEC kinetic parameters. A silver wire was epoxied into
the leucite cell body approximately 1 mm from the working electrode, but out of the light path, and served as the reference electrode. This electrode provided a stable potential, although it was not reproducible from experiment to experiment. Therefore the step potentials were adjusted relative to a cyclic voltammogram taken before each experiment. The silver wire reference electrode provided a lower resistance between the working and reference electrodes which provided for a more stable potentiostat operation.

A platinum wire coil was inserted into the main cell body to serve as an auxiliary electrode for fast potential steps (less than 50 msec). The internal auxiliary reduced the solution resistance between the auxiliary and working electrodes. A transparent leucite spacer was placed in the optical light path, effectively reducing the cell optical path length to 2.5 mm. The spacer reduced solution absorbance, but did not interfere with the absorbance changes in the vicinity of the electrode surface. The spacer reduced background absorbance enough to allow monitoring of the alpha and Soret bands of cytochrome c and cytochrome c oxidase at concentrations between 50 and 100 \( \mu \text{M} \).

Methyl viologen was used at concentrations of less than 100 \( \mu \text{M} \). Methyl viologen cation radical reacts with molecular oxygen at a rate of greater than \( 10^9 \text{ M}^{-1}\text{s}^{-1} \). Therefore 0.1 \( \mu \text{M} \) oxygen has an effect on the chronoabsorptometric experiments. For this reason, the two side Hamilton valves were removed and replaced with ground glass joints to ensure removal of more oxygen during the vacuum degassing procedure.
The reagents were described in Chapter II. The procedure was reviewed in Chapter II and described in detail by Hawkridge and Kuwana.\textsuperscript{23}

RESULTS AND DISCUSSION:

REDUCTION OF CYTOCHROME $c$ WITH METHYL VIOLGEN CATION RADICAL

In the catalytic regeneration reaction sequence,\textsuperscript{24} the

$$\begin{align*}
X + e^- & \rightarrow Y \\
Y + Z & \xrightarrow{k_f} X + Z^* \\
Y + Z & \xrightarrow{k_b} X + Z^*
\end{align*}$$

(3) \hspace{1cm} (4)

electrogenerated reactant ($Y$) serves to transfer charge from the electrode to cytochrome $c$ and in doing so, regenerates the parent reactant ($X$). Thermodynamically, the $K_{eq}$ of the methyl viologen-cytochrome $c$ reaction is much greater than unity since the $E^0'$ of methyl viologen ($-446 \pm 5$ mV vs NHE)$\textsuperscript{25}$ is much more negative in value than the $E^0'$ of cytochrome $c$ ($+258 \pm 17$ mV vs NHE). As such, ferricytochrome $c$ should be more readily reduced than methyl viologen dication when the electrode potential is stepped to a sufficiently negative value that methyl viologen dication is reduced at a diffusion controlled rate (i.e., chronoamperometric conditions). Ferricytochrome $c$ can be slowly reduced electrochemically at a mercury electrode.\textsuperscript{26} However, the rate of heterogeneous electron transfer at the presently used tin oxide electrode is quite slow. Thus, the electron transfer to ferricytochrome $c$ proceeds essentially
quantitatively through reactions (1) and (2) when \( \text{MV}^+ \) is electrolytically generated.

The electrochemistry of methyl viologen has been thoroughly examined for both non-aqueous \(^{27}\) and aqueous \(^{14,25}\) solutions. As studied by cyclic voltammetry, the 1st reduction step (reaction 1) proceeds with a well defined, reversible wave at the tin oxide \( \text{OTE} \) in aqueous, \( \text{pH} \) 7 solutions (phosphate buffer). The second reduction step of the radical cation, \( \text{MV}^+ \), to the fully reduced neutral molecule is also reversible with \( E^0 \) value of \(-772 \text{ mV vs NHE}\).\(^{14}\) The separation of the 1st and 2nd reduction steps is sufficient that the radical cation can be quantitatively generated by coulometry at an applied potential of \(-600 \text{ mV vs NHE}\). However, care must be taken to avoid generating the neutral species which undergoes a disproportionation reaction to form the cation radical. Figure 20 illustrates to what portion of the cyclic voltammetric wave the potential must be stepped to avoid generating the neutral viologen. The \( A-t \) curves are for pulse relaxation experiments on a solution containing 0.36 mM methyl viologen dichloride in \( \text{pH} \) 7 phosphate buffer. The potential was stepped for 50 msec and then open circuited. The dotted line indicated the time at which the disconnection occurred. The positive change in absorbance after disconnection indicated disproportionation of the electrogenerated neutral species. The numbers on the cyclic voltammogram correspond to the numbered \( A-t \) curves, and indicate to what potential the voltage was stepped. Positive deflection occurs for all but curve \#5.
FIGURE 20

A-t curves for open circuit relaxation chronosorptometric experiments on 0.36 mM methyl viologen dichloride in pH 7 phosphate buffer. Forward potential step time was 50 msec. Cyclic voltammogram of MV indicates to which portion of the wave the potential was stepped. The applied potentials vs Ag/AgCl for curves 1 through 5 were -877, -839, -805, -773, and -744 mV, respectively. Dotted line indicates disconnection time. Positive absorbance after disconnection indicates reproportionation of electrogenerated neutral viologen.
With the proper selection of potential, the quantitative
generation of $\text{MV}^+$ during a chronoabsorptometric experiment has been
shown by optically monitoring $\text{MV}^+$ at wavelengths of 550 nm to 630 nm
during an applied potential pulse. The optical absorbance, $A$, 
increased linearly with the square root of time, $t$, as given by
equation (5):

$$A = 2 \varepsilon C^0(Dt)^{1/2}/\pi^{1/2}$$

(5)

where $\varepsilon$ is the molar absorptivity of the MV radical cation; $C^0$ is the
bulk concentration of methyl viologen dication in mole liter$^{-1}$; and $D$
is the diffusion coefficient of methyl viologen dication in cm$^2$ s$^{-1}$.

In the present experimental system, linear $A$-$t^{1/2}$ plots were obtained
to times as short as 25 usec. At these times it is possible to resolve
homogeneous rates of second order reactions on the order of $10^8$ M$^{-1}$ s$^{-1}$.

The i-E curve obtained by cyclic voltammetry for the 1st wave of
methyl viologen in the presence of ferricytochrome $c$ at tin oxide OTE
is shown in Figure 21. There is a well defined, reversible i-E
wave for reaction (1); little or no definable wave for the reduction
of ferricytochrome $c$ at less negative potentials. The small rise in
current at ca. -90 to -180 mV vs NHE is associated primarily with the
tin oxide surface. To ascertain whether the reduction of ferri-
cytochrome $c$ was negligible in the potential regions prior to reduction
of $\text{MV}^{++}$, the optical absorbance was monitored at the cytochrome $c$
absorbance maximum of 550 nm (see spectra Figure 22) while the
potential was kept constant in the 0 to -400 mV region. The change
Cyclic voltammogram of 0.25 mM methyl viologen dichloride, 0.25 mM ferricytochrome c in pH 7 phosphate buffer at a tin oxide optically transparent electrode. The wave at -0.8 V is the 1 e⁻ reduction of MV⁺⁺. Cytochrome c is essentially electroinactive at tin oxide.
$MV^{2+} + e^- = MV^+$

$E_{cytochrome\ C}$

$10\mu A$

$0.2$ $0.0$ $-0.2$ $-0.4$ $-0.6$ $-0.8$

$E / V$ vs. $Ag/AgCl$
Optical spectra of oxidized and reduced forms of methyl viologen and cytochrome $c$.

(a) - spectrum of ferricytochrome $c$
(b) - spectrum of ferrocytochrome $c$
(c) - spectrum of methyl viologen cation radical
(d) - spectrum of methyl viologen dication
in A was less than $10^{-5}$ unit min$^{-1}$ which is negligibly small relative to that which is reduced through reactions (1) and (2) for short termed single and double potential step experiments.

The spectroelectrochemical determination of the rate coefficient involves the diffusion of reactants and products to and from the electrode surface. Previous spectroelectrochemical studies involved reactant molecules of approximately the same size and the diffusion coefficients were assumed to be equal. The effect on the reaction rate of the large difference in diffusion coefficients for cytochrome $c$ and methyl viologen dication, $1.3 \times 10^{-6}$ cm$^2$s$^{-1}$ and $1.0 \times 10^{-5}$ cm$^2$s$^{-1}$ respectively, was accounted for in the working curves by appropriate modification of the digital simulation program. The effect of differences in the diffusion coefficients on the working curve is illustrated in Figure 23 for diffusion coefficient ratios ($D_{c cyt} / D_{reactant}$) equal to 1.0, 0.16, 0.10, and 0.01. The diffusion coefficient of cytochrome $c$ was assumed to be the same for its oxidized and reduced states. This assumption was also made for the reactant. The working curves are affected by the diffusion coefficient difference between cytochrome $c$ and the reactant mainly in the higher $ktCz$ region.

The spectra of ferrocytochrome $c$ and MV$^+$ overlap appreciably at the cytochrome $c$ maximum (see Figure 22). Therefore, deconvolution of the spectra is necessary to analyze the optical signal. Ryan and Wilson$^{15}$ have described a set of circumstances under which two overlapping spectra can be deconvoluted. However, these circumstances depend upon either (a) measuring one species at one wavelength and
FIGURE 23

Working curve for single pulse chronoabsorptometric experiment of catalytic regeneration mechanism (equations (1) and (2)). \( A_N = (A_Y)_{k>0} / (A_Y)_{k=0} \), which are experimentally determined by monitoring the absorbance of \( Y \) in the presence and absence of the enzyme. The various curves illustrate the effect of differences in diffusion coefficients between the reactant and enzyme. The curves from bottom to top correspond to diffusion coefficient ratios \( D_{enzyme} / D_{reactant} \) equal to 1.0, 0.16, 0.1, and 0.01.
correcting the other wavelength with the non-overlapping data, or 
(b) assuming that one species does not absorb appreciably during a 
certain time period of a chronoabsorptometric experiment. Assumption 
(a) limits the number of systems to those which have non-overlapping 
spectra to begin with, and (b) requires prior knowledge as to the 
rate coefficient being determined.

A much more simple and accurate method of deconvolution, which 
did not rely on kinetic data and should be applicable to any SEC 
kinetic determination, was to resolve the optical components using 
the digital simulation method of Feldberg. Digital simulation 
computes the relative concentrations of reactants and products in each 
volume element of solution. Absorbance due to one component is then 
computed by summing the concentrations of that component in all volume 
elements and multiplying by the molar absorptivity. The total 
absorbance at a given wavelength is then the sum of the individual 
asorbances. In actual practice, a normalized absorbance, \( \frac{A_{2T}}{A_T} \), 
or \( \frac{A_T}{A_T(k=0)} \), is used so that only the absorptivity ratio need be 
known. Then, the \( \frac{A_{2T}}{A_T} \) is given by

\[
\frac{A_{2T}}{A_T} = \frac{C_{\text{reactant}_{2T}} + (\Delta \epsilon_{\text{enzyme}}/\Delta \epsilon_{\text{reactant}} \times C_{\text{enzyme}_{2T}})}{C_{\text{reactant}_T} + (\Delta \epsilon_{\text{enzyme}}/\Delta \epsilon_{\text{reactant}} \times C_{\text{enzyme}_T})}
\]

where \( A_{2T} \) is the total absorbance at \( t \) equal to twice the forward step 
time, \( C_{\text{reactant}_{2T}} \) is the concentration of reactant at \( t \) equal to twice
the forward step time, and $\Delta \varepsilon$ refers to the molar absorptivity difference between the oxidized and reduced forms.

Set wavelength data were acquired at 550 nm and 605 nm corresponding to the absorbance maxima of cytochrome c and methyl viologen cation radical. Figure 24 shows the effect of spectral overlap on the experimental $\Delta t$ curve. The curve is simulated for the reduction of ferricytochrome c with electrogenerated MV$^+$, with set wavelength monitoring at 550 nm. Cytochrome c has a molar absorptivity change of 18,500 M$^{-1}$cm$^{-1}$ at 550 nm. MV$^+$ has a molar absorptivity change of 8,200 M$^{-1}$cm$^{-1}$ at 550 nm. Working curves relating kinetic parameters to normalized absorbances at 550 nm and 605 nm are shown in Figure 25. The molar absorptivity ratio $(\Delta \varepsilon_{\text{cyt}c}/\Delta \varepsilon_{\text{MV}^+})$ at 550 nm equals 2.25. The absorptivity ratio at 605 nm equals -0.04. The effect of the spectral overlap at 550 nm is to yield increased sensitivity over the measurement at 605 nm since a larger total absorbance change is monitored.

A previous spectroelectrochemical study$^{21}$ using the catalytic regeneration mechanism had indicated, in a double potential step experiment, reverse step time to forward step time ratios (delay time) in the range of 24:1 at long step time (4 msec) to 200:1 at short step times (500 sec) were appropriate to return the solution to its initial conditions. These delay times were found to be inappropriate for the ferricytochrome c-MV$^+$ reaction, so a thorough examination of the parameters involved in choosing an appropriate delay time was conducted.
Digitally simulated $A-t$ curves illustrating the effect of spectral overlap on the experimental $A-t$ curve for reactions (3) and (4). $K_{eq}$ of reaction (4) is assumed to be large. The curves are simulated for 550 nm monitoring of the reduction of cytochrome $c$ with $MV^+$. $X$ corresponds to absorbance of $MV^+$. $Z'$ corresponds to absorbance change of cytochrome $c$. The absorptivity ratio ($\Delta \varepsilon_{\text{cyt} c}/\Delta \varepsilon_{\text{MV}^+}$) at 550 nm equals 2.25. $D_{Z'}/D_X = 0.10$. 
FIGURE 25

Working curves relating kinetic parameters to normalized absorbances at 550 nm and 605 nm for the upper and lower curve respectively for the reduction of cytochrome c with electrogenerated MV$^+$ by reactions (1) and (2). The molar absorptivity ratio $\frac{\Delta \varepsilon_{\text{cyt} c}}{\Delta \varepsilon_{\text{MV}^+}}$ at 550 nm equals 2.25. The absorptivity ratio at 605 nm equals -0.04. $K_{eq}$ of reaction (2) was assumed large, and $D_x/D_z = 0.10$. $A_N$ is the absorbance during the single step experiment in the presence of the enzyme divided by the absorbance in the absence of the enzyme.
In the catalytic regeneration reaction sequence, \( X \) is rapidly regenerated through reaction (2). However, a large delay time between repetitive experiments is necessary for \( Z \) to diffuse from the bulk solution to the electrode surface. For many mechanisms, all the species of interest in solution are electroactive, so the electrode is involved in restoring the solution to initial conditions by electrolyzing the species during the back step of the double potential step experiment. However, in the catalytic regeneration mechanism, \( Z \) and \( Z' \) (in this case, large heme proteins which are probably sterically blocked from approaching the electrode surface) are electroinactive so the electrode is not involved in replenishing them. The parameters involved in determining the appropriate delay times are the diffusion coefficient ratio \( \left( \frac{D_Z}{D_X} \right) \), the concentration ratio \( \left( \frac{C_Z}{C_X} \right) \), the potential step time \( (t) \), and the rate constant \( (k_r) \). The effects of these parameters were evaluated using digital simulation and are illustrated using concentration vs distance profiles in Figures 26 through 30. Figure 26 shows the concentration vs distance profiles of species \( X \) of reaction (3) after a double potential step experiment in the absence of a following chemical reaction (reaction (4)). Both \( X \) and \( Y \) are electroactive so that \( X \) reacting during the back step of the double potential step experiment regenerates \( X \). \( X \) is regenerated to better than 99% of its initial concentration over the entire diffusion layer \( (2\sqrt{Dt}) \) after a delay time of 30 times the forward potential step time. In Figure 27, the concentration of \( Z \) is plotted versus the distance from the electrode surface. This
Concentration vs distance profiles of species X for reaction (3) in the absence of reaction (4). Both X and Y are electroactive so X is regenerated by Y reacting at the electrode during the back step of the double potential step experiment.

From bottom to top of the left side of the Figure, concentration vs distance profiles are for delay times of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40 times the forward potential step time. This figure illustrates how rapidly the initial conditions are regenerated for an electroactive species compared to a nonelectroactive species (compare Figure 27).
Concentration vs distance profiles after a double potential step experiment for catalytic regeneration mechanism (equations (3) and (4)). The concentration of Z is plotted versus the distance from the electrode surface. $D$ is the diffusion coefficient of species $X$. $t_1$ is the forward potential step time. $2(Dt)^{1/2}$ approximates the reaction-diffusion layer thickness during the forward step. All profiles are simulated for $\log(k_C) = 0.0$ and $C_Z/C_X = 1.0$.

$K_{eq}$ of reaction (4) is assumed large.

(27A) going from bottom to top on the left hand side, concentration vs distance profiles of Z for delay times of 0, 10, ..., 100 times the forward potential step time for $D_Z/D_X = 1.0$.

(27B) same as (27A) except $D_Z/D_X$ equals 0.10.

An equilibrium situation for the reaction of Y with Z does not exist for the 0 time delay profiles. However, after 3 times the forward step time, an equilibrium situation does exist.
figure shows the concentration vs distance profiles of Z for delay times of 0, 10, ..., 100 times the forward potential step time for \(\frac{D_z}{D_x}\) equal to 1.0 and 0.1, respectively. After 100 times the step time, the \(\frac{D_z}{D_x} = 0.1\) profile is virtually flat over the potential step diffusion thickness \((2(Dt)^{1/2})\) and Z has been replenished to only 95% of its initial concentration. For the \(\frac{D_z}{D_x} = 0.1\) profile, corresponding to the cytochrome c-MV\(^+\) reaction, the depletion effect is even more serious with Z being replenished to only 85% of its initial concentration after 100 times the step time.

Figure 28 illustrates the effect of various diffusion coefficient ratios on the concentration vs distance profile with a delay of 100 times the forward potential step time. The curves correspond to diffusion coefficient ratios of \(\frac{D_z}{D_x}\) equal to 1.0, 0.5, 0.2, 0.1, and 0.05. As the \(\frac{D_z}{D_x}\) ratio becomes lower, Z diffuses more slowly and a larger delay time between repetitive experiments is needed to allow Z to diffuse from the bulk of the solution and replace Z which reacted near the electrode surface.

The effects of \(t\) and \(k\) on the concentration vs distance profile for values of Log(ktC\(_z\)) equal to -1.0, -0.5, 0.0, and +1.0 are illustrated in Figure 29. For low values of Log(ktC\(_z\)), very little reaction has occurred within the time of the potential step. Therefore, the amount of Z depleted is small. At larger Log(ktC\(_z\)) values, more Z has reacted and the depletion effect is more serious. Consequently, a larger delay time between repetitive potential steps is needed to allow Z from the bulk solution to diffuse toward the electrode and replace the reacted Z.
FIGURE 28

Concentration vs distance profiles of Z for catalytic regeneration mechanism illustrating the effect of diffusion coefficient differences. All profiles are simulated for $\log(ktC_z) = 0.0$, $C_z/C_x = 1.0$, and a delay time of 100 times the forward potential step time. $K_{eq}$ of reaction (4) is assumed to be large. Curves from top to bottom on the left side of the figure are simulated for $D_z/D_x$ equal to 1.0, 0.5, 0.2, 0.1, and 0.05.
Concentration vs distance profiles of Z for catalytic regeneration mechanism illustrating the effects of $t$ and $k_f$. All profiles are simulated for $C_Z/C_x = 1.0$, $D_Z/D_x = 0.1$, and a delay time of 100 times the forward potential step time. $K_{eq}$ of reaction (4) is assumed to be large. Curves from top to bottom are simulated for $\log(k_tC_Z)$ equal to -1.0, -0.5, 0.0, and 1.0.
Figure 30 shows the depletion of Z when the potential is repetitively stepped with a delay time of 100 times the forward potential step. The concentration vs distance profiles are generated for \( D_z/D_x = 0.1 \) (corresponding to the cytochrome c-MV\(^{++}\) ratio) and \( \log(ktC_z) = 0.0 \) (corresponding to the most sensitive part of the working curve). The profiles from top to bottom correspond to 1, 2, ..., 20 repetitive double potential step experiments. After twenty repetitive steps, the concentration of Z in the reaction-diffusion layer has been depleted to 27% of its initial concentration.

After taking into account the various parameters affecting the appropriate delay time, a minimum delay time of 1000 times the forward step time, coupled with stirring between potential steps, was found necessary for the methyl viologen-cytochrome c system. Since such long delay times were necessary with this system, extensive signal averaging, more than 400 signals, was no longer a viable means of noise reduction. This placed a limit on the minimum detectable absorbance change of about \( 10^{-4} \) absorbance units.

SEC chronoabsorptometric experiments were run on solutions containing 50 \( \mu M \) MV\(^{++}\), 100 \( \mu M \) ferricytochrome c, and pH 7 phosphate buffer. The experiments were repeated twice. Set wavelength data were taken at both 605 nm and 550 nm. The rate of reduction of ferricytochrome c with electrogenerated MV\(^+\) was fast enough that reliable data could not be taken at 605 nm with the presently used equipment. Absorbance at 550 nm was monitored for 50, 10, and 5 msec forward potential step experiments. The normalized absorbance,
Concentration vs distance profiles for catalytic regeneration mechanism illustrating the depletion of Z when the potential is repetitively stepped during a signal averaging experiment, with a delay time of 100 times the forward potential step time. All profiles are simulated for $C_z/C_x = 1.0$, $D_z/D_x = 0.1$, and $\text{Log}(k \text{t}C_z) = 0.0$. After 20 potential steps, the concentration of Z in the reaction-diffusion layer has been depleted to 27% of its initial concentration.
<table>
<thead>
<tr>
<th>Time (s)</th>
<th>$\text{MV}^+ \times 10^{-5}$</th>
<th>$\text{MV}^+ + \text{Cyt} \times 10^{-5}$</th>
<th>$A_{550}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>.0005</td>
<td>.52</td>
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<td>.0010</td>
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</tr>
<tr>
<td>.0050</td>
<td>2.98</td>
<td>11.39</td>
<td>3.82</td>
</tr>
</tbody>
</table>
calculated as the ratio of $A_{550}$ in the presence and absence of cytochrome $c$, was greater than 4 for all times analyzed down to 0.5 msec. The working curve for this reaction is shown in Figure 31 for $C_z/C_x$ equal to 0.5, 1.0, 2.0, 4.0, and 10.0 from bottom to top. The normalized absorbance values of greater than 4 for the experimentally used $C_z/C_x = 2.0$ indicated that the experiments were run on a time scale where $\log(ktC_z)$ was greater than or equal to 1.0. With minimum $t = 0.5$ msec and $C_z = 100 \mu M$, $k$ is calculated to be greater than or equal to $2.0 \times 10^8 M^{-1} s^{-1}$.

**OXIDATION OF FERROCYTOCHROME C WITH HYDROXY-METHYL FERRICINIIUM**

The electrochemistry of 1,1'-bis(hydroxy-methyl)ferrocene (HMF) was examined in aqueous solution (pH 7 phosphate buffer). HMF exhibits a reversible cyclic voltammogram at a platinum electrode with $E^0' = +.465$ volts vs NHE. Quantitative generation of bis(hydroxy-methyl)ferricinium (HMF$^+$) was demonstrated by optically monitoring HMF$^+$ at 635 nm during a chronoabsorptometric experiment. The optical absorbance increased linearly with the square root of time according to equation (5). Figure 32 shows the $A$-$t$ curve for the generation and removal of HMF$^+$ during a double potential step experiment. The background absorbance is due to changes in the optical constants of the electrode during the potential step. The HMF$^+$ oxidation of ferrocytochrome $c$ was analogous to the ferricytochrome $c$-MV$^+$ reaction in that again the diffusion coefficient ratio was approximately 0.1, and the depleted ferrocytochrome $c$ at the electrode surface must be entirely replenished by diffusion from the bulk of the solution.
FIGURE 31

Digital simulation working curves relating normalized absorbance, $A_N$, to the dimensionless kinetic parameter, $\log(ktC_z)$. Normalized absorbance is calculated as the ratio of $A_{550}$ in the presence and absence of cytochrome $c$. The curves from top to bottom correspond to $C_z/C_x = 10.0, 4.0, 2.0, 1.0, 0.5$. $D_z/D_x$ equaled 0.10.
$A-t$ curve for generation and removal of HMF$^+$ during a double potential step chronocoulometric experiment. Absorbance was monitored at 635 nm. Solution contained 0.25 mM HMF in pH 7 phosphate buffer. Potential step time was 50 msec. Dashed line is for background absorbance due to changes in the optical constants of the tin oxide electrode.
Therefore, the delay factors between repetitive, signal averaged experiments applying to the ferricytochrome \( c\text{-}MV^+ \) reaction also apply to the ferrocyanochrome \( c\text{-}HMF^+ \) reaction. Absorbance of HMF\(^+\) at 550 nm is negligible compared to the cytochrome \( c \) absorbance change. Therefore, the absorbance was normalized by performing a double potential step experiment and dividing the absorbance at twice the potential step time by the absorbance at the end of the forward step. A double potential step experiment with a 10 msec forward potential step and with \( C_Z = 86 \mu M \) indicated that the log(ktC\(_Z\)) was greater than 1.0. Therefore \( k \) must be greater than \( 10^7 M^{-1}s^{-1} \). A 2 msec experiment with \( C_Z = 86 \mu M \) indicated that log(ktC\(_Z\)) was less than one, but the signal to noise ratio was such that a more accurate estimation could not be made. Therefore, the rate coefficient was determined to be between \( 10^7 \) and \( 10^8 M^{-1}s^{-1} \).

A notable exception to the necessity of a long delay time between repetitive potential steps is when the \( K_{eq} \) of reaction (4) is between 0.1 and 10. With \( K_{eq} \) in this range, the back reaction is helpful experimentally in that it serves to regenerate the electro-inactive species at the electrode surface so that signal averaged experiments can be performed with much smaller delay times between repetitions. Much smaller absorbance changes can then be monitored. However, if the back reaction rate coefficient is very large, the absorbance change for the forward reaction becomes very small and the experimental advantage due to the back reaction is nullified. For example, determination of the rate of reduction of cytochrome \( c \) with
electrogenerated ferrocyanide was tried unsuccessfully. The back reaction in this case \( K_{eq} = 0.0166 \) overshadowed the forward reaction. The effect of the back reaction on the working curves is demonstrated in Figure 33 for \( K_{eq} \) equal to 0.1, 1.0, and 10.

**REDUCTION OF CYTOCHROME c OXIDASE WITH MV**

As with the reduction of ferricytochrome c, the reduction of fully oxidized cytochrome c oxidase with electrogenerated MV was found to be extremely rapid. Figure 34 presents the A-t curves for the absorbance increase of the 444 nm Soret band which arose from the heme a components of cytochrome c oxidase. The absorbance failed to increase after the potential step, indicating that an equilibrium situation existed at that point, that is, the reaction is fast on the time scale of the experiment. Therefore, knowing the heme a concentration (66 \( \mu \text{M} \)) and the time (5 msec), the rate coefficient was calculated to be greater than \( 4 \times 10^7 \text{ M}^{-1}\text{s}^{-1} \).

**OXIDATION OF CYTOCHROME c OXIDASE WITH HMF**

Several kinetic investigations of the reduction of cytochrome c oxidase with ferrocytochrome c have recently been reported. Biphasic reactions have been observed in all cases. However, the results are in conflict as to whether only one heme or one heme and one copper are reduced in the burst phase. Results were reported only for the reduction of fully oxidized cytochrome c oxidase.
Digital simulation working curve for double potential step experiment relating normalized absorbance, $A_N$, to the dimensionless kinetic parameter, $\log(ktC_z)$. Normalized absorbance is calculated as the ratio of $A_z(2\tau)/A_z(\tau)$. The curves from left to right are for $K_{eq} = 0.1$, $1.0$, and $10$. $C_z/C_x = 1.0$ and $D_z/D_x = 0.1$. 
A-t curve for reduction of heme a of cytochrome c oxidase. Dashed lines indicate beginning and end of potential pulse. Solution contained 66 μM heme a, 66 μM MV++, 0.1% Triton QS-30, and pH 7 phosphate buffer. Curves from top to bottom are for 50, 10, and 5 msec potential pulses. Absorbance was monitored at 444 nm.
The rate of oxidation of fully reduced cytochrome $c$ oxidase with molecular oxygen and with bromomalononitrile have been examined, but again, results at intermediate redox states have not been reported.

Cytochrome $c$ oxidase contains four metal redox centers, but only the heme $a$ components are optically detectable. However, the heme $a$ components are not optically differentiable. Therefore, determining which of the metal centers is reacting on a kinetic time scale is difficult. Kinetic studies of cytochrome $c$ oxidase at various stages of oxidation and reduction should yield more information as to the exact nature of the reacting metal center.

Oxidation of cytochrome $c$ oxidase with HMF$^+$ at heme $a$ redox states of $1/4$, $1/2$, $3/4$, and fully reduced was examined. The solutions contained $0.5\, \text{mM} \, \text{MV}^+$, $45\, \mu\text{M} \, \text{HMF}$, $45\, \mu\text{M} \, \text{heme} \, a$, $0.3\% \, \text{Triton QS-30}$, and pH 7 phosphate buffer. The cytochrome $c$ oxidase was adjusted to the various redox states by reduction with electro-generated $\text{MV}^+$ and heme $a$ oxidation was monitored during the chrono-absorptometric experiments at 444 nm. Since only heme $a$ was optically monitored, the absorbance was normalized by dividing the absorbance at $2\tau$, $A_{2\tau}$, by the absorbance at $\tau$, $A_{\tau}$.

Fast and slow reactions are time resolved with kinetic techniques such as stepped flow, pulse radiolysis, etc. However, a large concentration gradient is formed during an electrochemical potential pulse experiment so that fast and slow reactions are not resolved temporally. Also, digital simulation programs accounting for multiple reactions have yet to be developed.
The SEC chronoabsorptometric data for oxidation of cytochrome c oxidase by HMF+ were evaluated by assuming only one heme a was reacting. With that assumption, the bimolecular rate coefficients at the varying stages of reduction were calculated to be:

<table>
<thead>
<tr>
<th>% heme a reduction</th>
<th>k (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>4.7 x 10⁴</td>
</tr>
<tr>
<td>50</td>
<td>1.0 x 10⁵</td>
</tr>
<tr>
<td>75</td>
<td>8.5 x 10⁴</td>
</tr>
<tr>
<td>100</td>
<td>1.3 x 10⁵</td>
</tr>
</tbody>
</table>

CONCLUSION

Spectroelectrochemistry is of significant utility in studying redox reactions of biomolecules. The catalytic regeneration reaction sequence is useful for studying electroinactive biomolecules. Signal averaging to increase the optical signal to noise ratio is limited with this mechanism due to the large delay time between repetitive experiments, which is necessary to avoid depletion of the nonelectroactive species in the reaction-diffusion layer. Therefore, this mechanism is limited to reactions giving an absorbance change greater than approximately 10⁻⁴ units.

Experiments taking advantage of a back reaction replenishing the electroinactive species could be rapidly signal averaged with a resultant increase in sensitivity. Mediation schemes replenishing the electroinactive species, such as a double-double potential step...
experiment would also enhance the utility of the catalytic regeneration mechanism.
LIST OF REFERENCES


CHAPTER IV. CONCLUSION

Purified cytochrome c and cytochrome c oxidase were examined in this investigation to characterize the stoichiometry, energetics, and kinetics of their reactions individually and as mixtures. This investigation was directed toward determining the in vivo reaction mechanisms of cytochrome c oxidase reduction by cytochrome c, cytochrome c oxidase reduction of molecular oxygen to water, and the coupling of the free energy released during electron transport to the generation of adenosine triphosphate from adenosine diphosphate and inorganic phosphate.

An electrochemical approach was taken for generating oxidative and reductive titrant for the energetic's studies. This approach had a number of advantages over conventional chemical titrations. Only micromoles of enzyme were available. However, nanoequivalents of titrant could be quantitatively generated electrochemically, making it possible to titrate very small quantities of enzyme. Electrochemical cells were designed with a total volume of approximately one milliliter; again reducing the quantity of enzyme required for the experiments. These cells had the additional feature of completely enclosing the solution so that molecular oxygen, which rapidly oxidizes cytochrome c oxidase, could be reduced to less than 0.1 μM and maintained at that level for hours.

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Disadvantages of the electrochemical approach include the lack of well characterized electroactive oxidants and reductants. At this time, only ferricyanide, bis(hydroxy-methyl)ferrocene, TMPD, and a series of viologens are available for use as electrochemically generated titrants.

The formal potentials of the four metal redox centers in cytochrome c oxidase were determined from absorbance and charge data for redox titrations using a variety of titrants, both in the presence and absence of cytochrome c. From data for the reduction of cytochrome c oxidase with electrochemically generated MV$^+$ in the presence of the ferri-ferrocyanide couple, the formal potentials of the cytochrome c oxidase redox components were shown to be $E^{\circ}_{\text{heme } a_H} = 350 \pm 10 \text{ mV}$, $E^{\circ}_{\text{CuH}} = 350 \pm 10 \text{ mV}$, $E^{\circ}_{\text{heme } a_L} = 220 \pm 30 \text{ mV}$, and $E^{\circ}_{\text{CuL}} = 220 \pm 30 \text{ mV}$. Indirect coulometric titrations in the presence of TMPD provided data with which more accurate values for the potentials of heme $a_L$ and Cu$_L$ were obtained ($E^{\circ}_{\text{heme } a_L} = 215 \pm 10 \text{ mV}$, $E^{\circ}_{\text{CuL}} = 215 \pm 10 \text{ mV}$).

Qualitative observations of the 830 nm absorption band of cytochrome c oxidase indicated that the band could not be assigned to only one of the cytochrome c oxidase redox centers. However assignment of the 830 nm band to specific redox components was not attempted.

The $A$ vs $Q$ data for the oxidative titration of the ferri-ferrocyanide-cytochrome c oxidase system was not the mirror image of the $A$ vs $Q$ data for the reductive titration. The slope of the $A$ vs $Q$ curve for the reduction of heme $a_L$ corresponded to an $n$ value of 2. The slope for the oxidation corresponded to an $n$ value of 1.
Dissociated cyanide ion was suggested as possibly inhibiting electron transfer to Cu\textsubscript{L}. When detergent solubilized ferrocene was used in place of ferrocyanide, the reductive and oxidative A vs Q plots were mirror images.

Oxidative titrations of cytochrome \textsubscript{c}-cytochrome \textsubscript{c} oxidase mixtures in the presence of the ferrocene-ferricinium couple were reversible with the reductive titrations. That is, the potentials of the four redox components were determined to be the same during both the reductive and oxidative titrations. Oxidative titrations of cytochrome \textsubscript{c}-cytochrome \textsubscript{c} oxidase mixtures in the absence of the ferrocene-ferricinium couple, or any other mediators, were not reversible with the reductive titrations. From log-log plots, the heme potentials were shown to be 215 mV and 350 mV vs NHE for heme a\textsubscript{L} and heme a\textsubscript{H} during both oxidations and reductions. However, the copper formal potentials did not appear to be reversible. It was suggested in this case that a non-equilibrium situation existed during the titration. Slow intramolecular transfer from heme a\textsubscript{H} to Cu\textsubscript{H}, slow intermolecular transfer from the reductant to Cu\textsubscript{H}, and fast intramolecular transfer from either heme a\textsubscript{L} or Cu\textsubscript{L} to Cu\textsubscript{H} would explain the results of the reductive titration of the mixture. Other possibilities, however, were not ruled out. It was suggested that the titrations were reversible in the presence of the ferrocene-ferricinium couple because that couple acted to externally mediate intramolecular transfer between heme a\textsubscript{H} and Cu\textsubscript{H}. 
Spectroelectrochemical kinetic studies of cytochrome c and cytochrome c oxidase were undertaken to determine if inferences as to the mechanisms of charge transfer between these heme proteins and various oxidants and reductants could be made. Numerous kinetic studies of cytochrome c have recently appeared. The three primary approaches which have been used in probing the mechanistic pathways of cytochrome c charge transfer are: (1) modification of individual amino acid residues of cytochrome c to determine the effects of these changes on the physiological functions of the protein; (2) to evaluate the reactions of a variety of outer sphere reactants to determine the steric requirements for charge transfer with the enzyme; and (3) to investigate the reactions of inner sphere reactants to probe the effects of protein conformations of cytochrome c at various pH values on the charge transfer rates.

Margoliash and coworkers have postulated that mitochondrial cytochrome c reductase and cytochrome c oxidase react at different parts of the cytochrome c protein. Certain of their derivatized cytochrome c reacted normally with cytochrome c oxidase but were unreactive with cytochrome c reductase. Still other derivatized cytochromes c, that were blocked in their reaction with cytochrome c oxidase, reacted normally with cytochrome c reductase. Studies of the antigenic behavior of human cytochrome c in rabbits also indicated that cytochrome c reductase and cytochrome c oxidase reacted with different parts of the cytochrome c molecule.
Investigations of cytochrome c reactions with various inorganic complexes, by Gray and coworkers and Sutin and coworkers, have indicated that these reactions occur primarily through outer sphere mechanisms. Estimates of the "active site charge" of +0.4 for ferrocytochrome c and +1.7 for ferricytochrome c have been obtained based on changes of the rate coefficient with ionic strength. Attempts were made to correlate the experimentally determined rate coefficients with rate constants calculated from Marcus theory for outer sphere charge transfer. The rate coefficients correlated very well for tris(1,10-phenanthroline) cobalt(III) oxidation of ferrocytochrome c and for Ru(NH$_3$)$_6^{2+}$ reduction of ferricytochrome c. Marcus theory would not be expected to predict these rates if the reaction mechanism were significantly different from that employed in the cytochrome c self exchange reaction. Therefore they concluded, in contrast to Margoliash's interpretation, that both oxidation and reduction of cytochrome c proceed at a common active site.

Castro and coworkers have tried to relate protein conformation of well characterized heme enzymes to redox reactivity in order to formulate a basis for assessing the general conformation type of heme proteins of unknown structure. Based on the reactions of the axial inner sphere oxidant bromomalononitrile with both globin and cytochrome heme proteins, they have postulated "G", "C" and "short C" heme protein conformations. Inner sphere reactants were sterically constrained from approaching the "C" conformation heme inner coordination sphere. For the "G" and "short C" conformations, the
axial heme positions are unencumbered. At physiological pH, cytochrome c possessed the "C" conformation.

Kinetic investigations of cytochrome c oxidase have concentrated on reactions involving either the fully oxidized or fully reduced enzyme. However, it is quite possible that its in vivo catalytic reduction of molecular O₂ to H₂O proceeds with the enzyme in an intermediate oxidation state. Castro and coworkers⁶ have indicated that inclusion of the two copper centers of cytochrome c oxidase in the catalytic reaction is not necessary to explain its in vivo mechanism.

In this investigation, the rate coefficients for reduction of the oxidized heme proteins, cytochrome c and cytochrome c oxidase, with electrogenerated MV⁺, and oxidation of the heme proteins with electrogenerated HMF⁺ have been evaluated. Cytochrome c was found to react with MV⁺ at a rate greater than 2 x 10⁸ M⁻¹s⁻¹. A Marcus theory calculation of this reaction rate, using equation (1) was made.⁴

\[ \log(k_{12}) = 0.5(\log(k_{11}) + \log(k_{22}) + 16.9 \Delta E_{12}^o) \]  

(1 x 10³ M⁻¹s⁻¹)⁷ and methyl viologen (3 x 10⁹ M⁻¹s⁻¹)⁸ and E°¹ is the difference in formal potentials between cytochrome c and methyl viologen (0.705 V). Using these values, the rate constant for the ferricytochrome c-MV⁺ reaction was calculated to be diffusion controlled, that is, greater than 10¹⁰ M⁻¹s⁻¹.
Fully oxidized cytochrome c oxidase was found to react with MV$^+$ at a rate greater than $4 \times 10^7$ M$^{-1}$s$^{-1}$. The Soret band at 444 nm was monitored, indicating that a heme a center was reacting. However, the measurement did not distinguish which of the heme a centers was reacting. Nor did the experiment determine if more than one redox center was reacting.

Cytochrome c was oxidized by HMF$^+$ at a rate between $10^7$ and $10^8$ M$^{-1}$s$^{-1}$. A Marcus theory calculation of the ferrocytochrome c-HMF$^+$ reaction rate coefficient using equation (1) yielded a value of $2.9 \times 10^7$ M$^{-1}$s$^{-1}$. The cytochrome c self exchange rate constant was again taken as $1 \times 10^3$ M$^{-1}$s$^{-1}$ and the HMF self exchange rate constant was estimated from the data of Stranks$^9$ to be $3 \times 10^8$ M$^{-1}$s$^{-1}$. The difference in formal potentials between cytochrome c and HMF was 0.204 V.

HMF$^+$ oxidation of cytochrome c oxidase was monitored at the cytochrome c oxidase redox state of fully reduced, as well as at the intermediate redox states of $3/4$, $1/2$, and $1/4$ reduced as judged by the heme a absorbance at 605 nm. The average rate coefficient of the four redox states monitored was $9 \times 10^4$ M$^{-1}$s$^{-1}$. However, this rate was evaluated by assuming only one of the four metal redox centers of cytochrome c oxidase was reacting.

The present kinetic results on cytochrome c oxidase reactions must still be considered preliminary. Further results must be obtained to determine the mechanism of charge transfer from cytochrome c to cytochrome c oxidase to molecular oxygen, and to determine the
mechanism by which the free energy released in this process is coupled to the generation of ATP.
LIST OF REFERENCES


APPENDIX I

The following program simulates and plots Absorbance (A) vs Charge (Q) curves.

0010 INPUT H
0020 CALL 1,2000,2000
0030 INPUT D
0040 CALL 1,4000,4000
0050 INPUT D
0060 CALL 1,0,0
0070 GOTO 0010
0080 CALL 2,0
0090 PRINT "INPUT MIN & MAX X-COORDS"
0100 INPUT Q1,Q2
0110 PRINT
0120 PRINT "INPUT MIN & MAX Y-COORDS"
0130 INPUT A8,A9
0140 PRINT
0150 PRINT "INPUT 1 FOR CYT C, 0 FOR CYT OX"
0160 INPUT A7
0170 PRINT
0180 PRINT "INPUT CHARGE CORRECTION"
0190 INPUT Q6
0200 PRINT
0210 LET Q7=1
0220 LET A2=12000
0230 LET A3=19000
0240 LET E1=1.437
0250 LET E2=.33
0260 LET E3=.35
0270 LET E4=.21
0280 LET E5=.22
0290 LET E6=.25
0300 REM CONC OF FERROCENE
0310 LET C1=.00088
0320 REM CONC OF OXIDASE
0330 LET C2=0
0340 REM CONC OF CYT C
0350 LET C3=1.842E-5
0360 LET C4=C1+C3+C2*4
0370 FOR I=1 TO 100 STEP .2
0380 LET D=.05+.005*I
0390 LET A=EXP(2.30258*(D-E1)/.059)
0400 LET B=EXP(2.30258*(D-E2)/.059)
0410 LET C=EXP(2.30258*(D-E3)/.059)
0420 LET R=EXP(2.30258*(D-E4)/.059)
0430 LET W=EXP(2.30258*(D-E5)/.059)
0440 LET M=EXP(2.30258*(D-E6)/.059)
0450 LET U=C1*A/(1+A)
0460 LET E=C2*B/(1+B)
0470 LET F=C2*C/(1+C)
0480 LET G=C2*R/(1+R)
0490 LET H=C2*W/(1+W)
0500 LET N=C3*M/(1+M)
0510 LET S=U+E+F+G+N
0520 LET J=(C2-E)*A2
0530 LET L=(C2-G)*A2
0540 LET F=(C3-N)*A3
0550 LET T=S*9.6500*(1/(1-06))
0560 LET V=J+L
0570 LET X=INT(38.00*(T-Q1)/(Q2-Q1))+100
0580 IF A7=1 THEN GOTO 0610
0590 LET Y=INT(38.00*(V-A8)/(A9-A8))+100
0600 GOTO 0620
0610 LET Y=INT(38.00*(F-A8)/(A9-A8))+100
0620 IF X<25 THEN GOTO 0710
0630 IF X>3975 THEN GOTO 0710
0640 IF Y<25 THEN GOTO 0710
0650 IF Y>3975 THEN GOTO 0710
0660 CALL 1*X,Y
0670 IF Q7>1 THEN GOTO 0690
0680 GOSUB 0770
0685 CALL 2+1
0690 LET Q7=Q7+2
0700 GOTO 0740
0710 LET E=E+1
0720 GOTO 0740
0740 NEXT I
0750 CALL 2+0
0760 STOP
0770 CALL 1*X,Y
0780 FOR J=1 TO 400
0790 LET K=K
0800 NEXT J
0810 RETURN
0820 END
APPENDIX II

The following is a Digital Simulation program which calculates concentration vs distance profiles and plots a working curve relating normalized absorbance to kinetic parameters for the catalytic regeneration mechanism.

REAL K1, K2, K3
INTEGER 0
DIMENSION X(150), Y(150), R(150), S(150),
Z(150), V(150), W(150)
TYPE "DIGISIM DOUBLE POTENTIAL STEP"
TYPE "X + E = Y \& Y + Z = X + U MONITOR Y\&U<15>"
D= -0.45
ACCEPT "MIN \& MAX X-COORDS " , XMIN
ACCEPT XMAX
ACCEPT "MIN \& MAX Y-COORDS " , YMIN
ACCEPT YMAX
ACCEPT "DIFF COEFF RATIO " , E
C=1000
ACCEPT "CE/C RATIO " , Q4
ACCEPT "U/Y EXTINCTION RATIO " , ERAT
ACCEPT "U/Y EXTINCTION RATIO 1 " , FRAT
ACCEPT "EQUILIBRIUM CONSTANT = " , RKEQ
ACCEPT "TMAX = " , T3, "<15>"
WRITE (10, 20)
C1=Q4*C
B=1-2*D
CALL PEN(0, 20)
T2=9
T1=0
1 T1=T1+.5
IF(T1.LT.10) GO TO 2
T2=T2-1
T1=1
2 K1=T1/10.**T2
DO 3 I=1, 150
X(I)=C
Z(I)=C1
3 162
\[ Y(I) = 0 \]
\[ U(I) = 0 \]
\[ T = 0 \]
\[ T = T + 1 \]
\[ Q = \text{INT} \left(6 \times \text{SQRT}(D \times T) + 3\right) \]
\[ D \ 5 \ I = 2 \times Q \]
\[ C3 = K1 \times Y(I) \times E(I) - (K1 / RKEQ) \times U(I) \times X(I) \]
\[ R(I) = B \times X(I) + D \times (X(I+1) + X(I-1)) + C3 \]
\[ V(I) = (1 - 2 \times E \times D) \times E(I) + E \times D \times (E(I+1) + E(I-1)) - C3 \]
\[ S(I) = B \times Y(I) + D \times (Y(I+1) + Y(I-1)) - C3 \]
\[ W(I) = (1 - 2 \times E \times D) \times U(I) + E \times D \times (U(I+1) + U(I-1)) + C3 \]
\[ C3 = K1 \times Y(I) \times E(I) - (K1 / RKEQ) \times U(I) \times X(I) \]
\[ R(I) = B \times X(I) + D \times (X(I+1) + X(I-1)) + C3 \]
\[ V(I) = (1 - 2 \times E \times D) \times E(I) + E \times D \times (E(I+1) + E(I-1)) - C3 \]
\[ S(I) = B \times Y(I) + D \times (Y(I+1) + Y(I-1)) - C3 \]
\[ W(I) = (1 - 2 \times E \times D) \times U(I) + E \times D \times (U(I+1) + U(I-1)) + C3 \]
\[ D \ 6 \ I = 1 \times Q \]
\[ X(I) = R(I) \]
\[ Y(I) = S(I) \]
\[ E(V) = V(I) \]
\[ U(I) = W(I) \]
\[ \text{IF}(T \times LT \times T3) \ G3 \ T0 \ 4 \]
\[ A1 = 0 \]
\[ B1 = 0 \]
\[ F7 = 1.72745 / (2 \times C \times \text{SQRT}(D \times T)) \]
\[ D \ 7 \ I = 1 \times Q \]
\[ A1 = A1 + Y(I) \]
\[ B1 = B1 + U(I) \]
\[ A3 = (A1 + \text{ERAT} \times B1) \times F7 \]
\[ A4 = (A1 + \text{FRAT} \times B1) \times F7 \]
\[ T = 0 \]
\[ T4 = 0 \]
\[ T4 = T4 + 1 \]
\[ T = T + 1 \]
\[ Q = \text{INT} \left(6 \times \text{SQRT}(D \times (T3 + T)) + 3\right) \]
\[ D \ 9 \ I = 2 \times Q \]
\[ C3 = K1 \times Y(I) \times E(I) - (K1 / RKEQ) \times U(I) \times X(I) \]
\[ R(I) = B \times X(I) + D \times (X(I+1) + X(I-1)) + C3 \]
\[ V(I) = (1 - 2 \times E \times D) \times E(I) + E \times D \times (E(I+1) + E(I-1)) - C3 \]
\[ W(I) = (1 - 2 \times E \times D) \times U(I) + E \times D \times (U(I+1) + U(I-1)) + C3 \]
\[ C3 = K1 \times Y(I) \times E(I) - (K1 / RKEQ) \times U(I) \times X(I) \]
\[ R(I) = B \times X(I) + D \times (X(2) - X(1)) + C3 \]
\[ S(I) = Y(I) + D \times (Y(2) - Y(1)) - C3 \]
\[ V(1) = 2(1) + E \cdot D \cdot (z(2) - z(1)) - C3 \]
\[ w(1) = U(1) + E \cdot D \cdot (U(2) - U(1)) + C3 \]
\[ D \ 10 \ I = 1, Q \]
\[ X(I) = R(I) \]
\[ Y(I) = S(I) \]
\[ z(I) = v(I) - v(I) \]
\[ U(I) = w(I) \]

10

\[ I F (T \cdot L T \cdot 2 \cdot T 4 \cdot T 3) G O T O 8 \]
\[ A2 = 0 \]
\[ B2 = 0 \]
\[ F9 = 1.0 / (S O R T (T + T 3) - S O R T (T)) \]
\[ F8 = (1.77245 / (2 \cdot C \cdot S O R T (D))) \cdot F9 \]
\[ D O 11 I = 1, Q \]
\[ A2 = A2 + Y(I) \]

11

\[ B2 = B2 + U(I) \]
\[ K3 = A L O G 1 0 (K 1 \cdot T 3 \cdot C 1) \]
\[ A5 = (A2 + E R A T \cdot B2) / (A1 + E R A T \cdot B1) \]
\[ A6 = (A2 + F R A T \cdot B2) / (A1 + F R A T \cdot B1) \]
\[ A7 = B2 / B1 \]

\[ W R I T E (10, 30) A3, A4, A5, A6, A7, K3 \]
\[ I F (T 4 \cdot L T \cdot 5) G O T O 12 \]
\[ A7 = (3800 \cdot (A7 - Y M I N) / (Y M A X - Y M I N)) + 100 \]
\[ K2 = (3800 \cdot (K3 - X M I N) / (X M A X - X M I N)) + 100 \]
\[ I F (K2 \cdot L T \cdot 0) G O T O 14 \]
\[ I F (K2 \cdot G T \cdot 4095) G O T O 14 \]
\[ I F (A7 \cdot L T \cdot 0) G O T O 14 \]
\[ I F (A7 \cdot G T \cdot 4095) G O T O 14 \]
\[ N D E L = S 0 0 \]
\[ C A L L P L O T (I N T (A7), I N T (K2), N D E L) \]
\[ C A L L P E N (1, N D E L) \]
\[ C A L L P E N (0, N D E L) \]

14

\[ I F (K3 \cdot L T \cdot 0) G O T O 1 \]
\[ 2 0 \]
\[ F O R M A T (6 X \cdot "Y / Y 0", 9 X \cdot "Y / Y 0 1", 7 X \cdot "A / A T A U", 1 X \cdot "A / A T A U 1", 6 X \cdot "L O G (K T C 2)") \]

30

\[ F O R M A T (6 F 1 2. 5) \]
\[ E N D \]