SITE OF INHIBITION OF CYTOCHROME OXIDASE BY DIBUCAINE, TETRACAINE AND THE CARDIAC ANTIARRYTHMIC PROPRANOLOL

By

BRADLEY KENT STRINGER

Bachelor of Science

in Arts and Sciences

Oklahoma State University

Stillwater, Oklahoma

1987

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 1989

SITE OF INHIBITION OF CYTOCHROME OXIDASE

BY DIBUCAINE, TETRACAINE AND

THE CARDIAC ANTIARRYTHMIC

PROPRANOLOL

Thesis Approved:

Thesis Adviser

Dean of the Graduate College

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to my advisor, Dr. H. James Harmon. His guidance, insight, and persistence during the course of this work has proved invaluable to my training as a scientist. I would also like to thank Dr. C. A. Yu and Dr. J. T. Blankemeyer for their assistance by serving as committee members.

Thanks are also given to Andy Beach, John Berry, and Carol Hefler for their friendship and moral support during this work.

I would like to especially thank my mother, Carolyn Ricks for her continued dedication and support during not only this work, but also throughout my entire college career. It is through her that the importance of education has been stressed in my life.

Finally, I would like to thank Jana Lynch for her support and making my life more enjoyable.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

In every living system, there must exist a means to convert energy taken in from food consumption to a form of energy that can be used by the organism. Some form of energy currency must be available in order for chemical reactions to be driven and in order for life processes to be carried out. Nature has provided a way for this enormous task to be accomplished by supplying this "currency" in the form of adenosine triphosphate (ATP). In order to make ATP, a highly complex phosphorylation mechanism has evolved to convert adenosine diphosphate (ADP) into ATP.

A highly specialized organelle called the mitochondrion is now known to be responsible for the phosphorylation mechanism. Peter Mitchell's chemiosmotic hypothesis of ATP synthesis is now widely recognized as the mechanism by which phosphorylation takes place. The nature of this organelle is one of a highly membranous structure rich in proteins. The oxidative phosphorylation system is contained within the inner membrane of the mitochondria which is composed of approximately 70% protein and 30% lipid (Hatefi, 1985).

There is an abundance of literature on all aspects of oxidative phosphorylation and the nature of the organelle itself. As a result, there are many aspects of the mitochondria that have been elucidated. But at the same time, this large amount of research has generated controversy between researchers and has opened many new topics of discussion and research. One such area of research has been the use of inhibitors. Various compounds have been discovered that will block the flow of electrons in the respiratory chain (see Hatefi 1985 for review). These inhibitors not only slow mitochondrial respiration but also act as a probe to allow researchers to functionally seperate the respiratory complexes and study their function without interference from a neighboring complex.

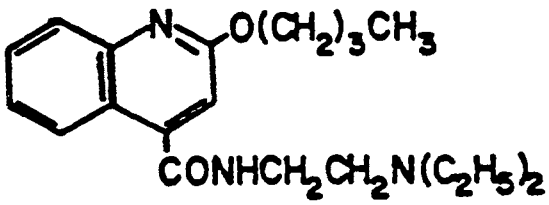
One class of compounds that has been shown to interfere with mitochondrial respiration are the tertiary amine local anesthetics and cardiac beta blockers. It is the purpose of this research to characterize the effects of these types of compounds on cytochrome oxidase. This research has four goals:

1. To determine the inhibition of respiration in complex IV;
2. To determine a site of action within the cytochrome oxidase;
3. To determine the effect on the individual redox centers;

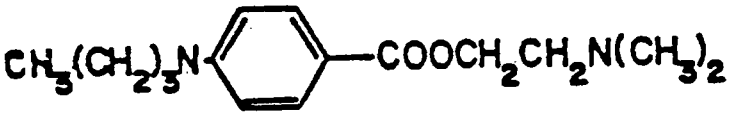
4. To determine the basis of the inhibition by observing changes in the following:
   a. Visible Spectra
      1. Cytochrome a
      2. Cytochrome a_3
      3. Copper associated with cytochrome a
   b. Enzyme Kinetics
      1. K_m
      2. V_{max}
   c. Oxidation/Reduction Potentials

Since there are numerous drugs of these types, only three will be used in this study, dibucaine, tetracaine, and propranolol. Their chemical structures are shown in Figure 1. These drugs were chosen because they each represent some structural similarities and structural differences; this is evident in that they each have ring structures and a long hydrocarbon chain containing a nitrogen atom. Tetracaine has a single ring whereas dibucaine and propranolol have two. Also, they vary in the degree of hydrophobicity, which, as will be pointed out later, is an important consideration in determining the degree to which they inhibit mitochondrial respiration.

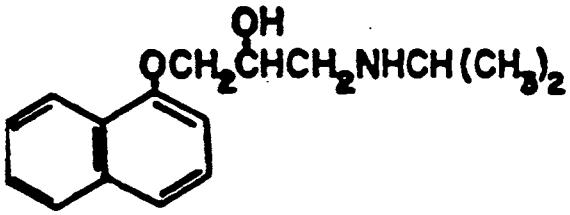
Figure 1. Chemical structures of the local anesthetics dibucaine, tetracaine, and the cardiac beta-blocker propranolol.



DIBUCAINE



TETRACAINE



PROPRANOLOL

CHAPTER II

REVIEW OF LITERATURE

The phosphorylation system in mitochondria is divided into five protein-lipid enzyme complexes. They are the following: 1. NADH: ubiquinone oxidoreductase (complex I); 2. succinate: ubiquinone oxidoreductase (complex II); 3. ubiquinol: ferricytochrome c oxidoreductase (complex III); 4. ferricytochrome c: oxygen oxidoreductase (complex IV) 5. ATP synthase (complex V). Functionally, complex V is not a part of the electron transport chain, but interaction between the ATP synthase and the electron carriers is essential for the phosphorylation system to function properly. While all of the enzyme complexes are important and an intergal part of the phosphorylation process, it is complex IV that catalyzes the reduction of molecular oxygen to water and completes the electron transport process.

Complex IV, or more readily known as cytochrome oxidase, E.C. 1.9.3.1., catalyzes the 4-electron reduction of molecular oxygen by the donation of electrons from ferricytochrome c. Cytochrome oxidase is composed of essentially two oxidation-reduction centers,

two heme centers known as cytochromes a and a_3, and their associated coppers, CuA and CuB (Hatefi 1985). The enzyme complex spans the mitochondrial membrane completely (Tzagoloff, 1982), allowing the enzyme to accept electrons from cytochrome c on the cytosol side and to reduce oxygen on the matrix side.

Cytochrome c oxidase is an enzyme complex that has been the focus of numerous studies for over one hundred years. Although much has been learned, there is still much more to be elucidated about its structure and function. Cytochrome oxidase has a two fold function: first, the enzyme complex reduces molecular oxygen to water; second, in the reduction of molecular oxygen to water, protons are translocated across the inner membrane to create an electropotential gradient to provide free energy for the production of ATP. Many methods of study have been used to elucidate the function, structure, and mechanism of this complex enzyme system. I will summarize some of these methods and the information being obtained from them.

The sequence of electron transfer in the mechanism of reduction of molecular oxygen to water has been the subject of much experimentation. Potentiometric as well as spectrophotometric techniques have been used to elucidate the mechanism (Tzagoloff, 1982). Oxygen intermediates have also been studied at low temperatures by Chance and others (Chance et. al, 1975a,b,c; Lemberg

and Mansley, 1966; Wharton and Gibson, 1968). By using low temperature trapping techniques, the oxidase can be "trapped" into various intermediate stages of oxidation. Use of multiple beam spectrophotometry permits the study of all the redox centers at the same time, allowing the sequence of electron transfer to be postulated by observing the time sequence of oxidation and reduction of the centers in question. These studies suggest that a sequential mechanism is operating such that electrons flow sequentially from cytochrome a > CuA > a_3 (Chance and Williams, 1955, 1956). Even with all of the work that has been done, the mechanism is poorly understood.

A second important aspect of the function of cytochrome c oxidase is the translocation of protons across the membrane to form an electrochemical gradient used in the formation of ATP. The foundation of the chemiosmotic hypothesis proposed by Mitchell requires protons to be translocated across the mitochondrial membrane and is linked to the oxidation-reduction of the cytochromes that compose the electron transport chain (Mitchell, 1961). Three sites have been identified as being involved in the translocation of protons, one of which is cytochrome oxidase (Wikstrom et. al, 1981a,b). Potentiometric evidence suggests the location of these points. Electrons pass down the respiratory chain via an increased affinity for the redox componants of the chain for the electrons (due to an increasing electromotive

mid potential). The midpotential (relative to the hydrogen couple) is used to measure the relative ease that a compound can either accept electrons or donate electrons. Compounds with a negative midpotential tend to donate electrons, therefore acting as reductants, while compounds with a positive midpotential accept electrons, therefore acting as an oxidant. By measuring the midpotentials of the cytochromes as described by Dutton and Wilson (1974), the ability of a cytochrome to reduce or oxidize a neighboring cytochrome can be determined. Potentiometric studies of electron transfer components can be used to elucidate aspects of electron transport mechanisms such as the determination of the sites of ADP phosphorylation in the chain. By measuring the potential difference between two neighboring cytochromes, one can then estimate if there is enough potential difference between the two centers for phosphorylation to take place. 7.3 kcal of standard free energy is needed to esterify the terminal phosphate bond of ATP. Using the Nernst equation, this translates to roughly a 158 mV change in potential between redox components needed to produce this amount of free energy. Cytochrome oxidase (between cytochrome c and oxygen) has a potential difference of 520 mV; this potential drop translates to a free energy change of -23.9 kcal, more than enough to phosphorylate ADP (Tzagoloff, 1982).

A tremendous amount of investigation has focused on the mechanism of proton pumping associated with cytochrome oxidase (see Wikstrom and Krab, 1979, Wikstrom and Krab, 1980, for reviews). Current views suggest that cytochrome a and its associated copper play an important role in the tranlocation of protons within this complex (Wikstrom et. al, 1981a,b; Wikstrom and Krab, 1979; Wikstrom and Saari, 1977). It is suggested that cytochrome a and CuA act as a "gate" by which protons are shunted across the membrane, and the mechanism is most likely not a redox linked cycle as is the proton motive Q cycle in complex III (Boyer and Trumpower, 1981). Li et al., (1989), as well as Scholes and Malmstrom, (1986), suggest that a conformational change in the protein is responsible for this "gating" effect. This change is postulated to be redox linked, thereby altering acid-base groups to account for the change in structure for proton translocation to occur. It is indicated that both cytochrome a and its associated copper (CuA) must be in the reduced form for the transition from the closed to open state to take place (Scholes amd Malmstrom, 1986), therefore resulting in a translocation of protons across the mitochondrial membrane.

One of the greatest contributions to the understanding of the structure and function of cytochrome oxidase was the development of the dual-

wavelength spectrophotometer by Dr. Britton Chance as described previously (Chance, 1952c, 1951). Through a series of elegant spectrophotometric assays using the dual wavelength instrument, absorption bands were assigned to the many cytochromes in the electron transport chain including cytochrome a and cytochrome a_3. Keilin and Hartree (1939), suggested that each cytochrome had a characteristic absorption band in the electromagnetic spectrum. An alpha band and a shorter wavelength Soret band were assigned by Keilin and Hartree as early as 1934 using a hand held spectroscope. They recognized that a cytochrome could be studied using the absorption bands for that particular heme. Dr. Chance's instrument, however, made it possible to measure a cytochrome even in a turbid sample (Chance, 1952a). Much of what is known about the structure and function of cytochrome oxidase has been elucidated by Chance's methods.

One important concept that was brought about by the use of dual wavelength spectroscopy was the study of crossover points in the respiratory chain. Chance and Williams, (1955 and 1956), defined the crossover point to be the site where a large difference in the reduction levels of neighboring carriers is observed. More succinctly, for a single phosphorylation step, the component involved will become oxidized. The net result being that the component on the oxygen side will become

more reduced while those on the substrate side more oxidized. If the reduction levels of the cytochromes are followed during steady state respiration (after the addition of ADP), the point at which the reduction changes to an oxidation is the crossover point (Chance et al., 1955). Experiments were performed to determine the reduction levels of the various electron transport chain components including those of cytochrome oxidase. Not only was this method useful in determining crossover points, but also in determining the sequence of electron carriers. By measuring the absorbance change at the appropriate wavelengths in the presence of a reductant, which cytochromes were reduced at the same time another was oxidized could be made. Through these experiments, a good indication of the relative positions of the electron carriers was determined (Chance and Hollunger, 1961a,b,c,d,e,f).

The use of compounds that inhibit electron transport at various sites has been applied using spectrophotometric methods to functionally isolate the various redox complexes. Use of the inhibitors not only defines the sequence of electron carriers, but also defines the location of the carrier in the membrane. Each complex of the electron transport chain has been found to be sensitive to one or more inhibitor compounds. Complex I is sensitive to rotenone, piericidin, and some barbiturates (Palmer, et. al, 1968).

Complex II is sensitive to 2-thenoyltriflouroacetone (TTFA). Complex III is inhibited by a number of compounds with one of the most potent being Antimycin A, an antibiotic from Streptomyces (Slater, 1973). Along with Antimycin A, a number of quinone analogs have been shown to inhibit this complex as well (Yu et al. 1988). 5-n-undecyl-6-hydoxy-4,7-dioxobenzothiazole (UHDBT) (Trumpower, 1981), 2-hydroxy-3-undecyl-1,4 napthoquinone (HUNQ) (Harmon and Struble, 1983), and BAL (2,3-dimercaptopropanol, (British anti-Lewsite), (Grigolova and Konstantinov, 1977), have been shown to inhibit complex III. In addition, myxothiazole has been shown by Trumpower, (1981), to inhibit complex III as well.

Cytochrome c oxidase is inhibited by a number of compounds as well. Carbon monoxide and cyanide have been shown to interact with cytochrome a_3, while azide has been shown to bind to cytochrome a, thereby inhibiting the reduction of oxygen to water (Tzagoloff, 1982). Of these inhibitors, carbon monoxide is of particular intrest in this research. Carbon monoxide will react with reduced cytochrome a_3 to form a carboxy-oxidase complex; cytochrome a can be subsequently oxidized while cytochrome a_3 remains reduced (Tzaoloff and Wharton, 1965). Spectral evidence for the carboxy-oxidase formation is seen by a 590 nm shoulder that appears adjacent to the 604 nm cytochrome a band. Also, a 430 nm band appears as well as a decrease in the 445 nm Soret

band (Chance, 1953a, Tzagoloff and Wharton, 1965). These spectral properties of carboxy-oxidase have been used extensively to determine the contributions of cytochrome a and a_3 to the alpha and Soret bands (see Wikstrom et. al, 1976).

The spectral properties of CuA are of interest to the current study in order to determine the oxidation-reduction state of the copper atom. The reduced MINUS oxidized spectrum of CuA exhibits a trough instead of the characteristic peak that is seen in a reduced MINUS oxidized spectrum of an iron atom. The valence state of oxidized copper is +2. The paired electron valence state of the copper will absorb light in the near infra-red region of the electromagnetic spectrum. The absolute oxidized spectrum of CuA, therefore,has a peak at 830 nm. The reduced form of CuA has a valence state of +1 and is undetectable because of an unpaired electron spin, and the absolute reduced spectrum is indicated by a flat line. A reduced MINUS oxidized spectrum for CuA, then, is a trough, because a flat line subtracted from a peak produces a trough.

Recent research has led to the discovery of a new class of inhibitors of cytochrome c oxidase. Local anesthetics and cardiac beta-blockers have been found to inhibit electron transport in cytochrome oxidase as well as alter some of the ion exchange mechanisms associated with the outer membrane.

Local anesthetics act reversibly to block the transmission of nerve impulses by decreasing the sodium conductance through the nerve membrane (Casonovas, 1983). However, there is much debate as to the mechanism by which the local anesthetics block the sodium channel; some investigators have postulated that the molecule interacts with the lipid membrane that surrounds the protein (Seeman, 1972; Cerbon, 1972), while others have postulated that the protein itself is being altered (Richards et al., 1978). However, as pointed out by Gutierrez-Merino et al., (1989), Singer, (1980), and Chazotte and Vanderkooi, (1981), there is a strong correlation between anesthetic potency and the solubility of the anesthetic in the lipid bilayer (which is related to the value of log P). Despite the studies that indicate that the protein is directly affected, the vast majority of the literature indicates that perturbation of the lipid membrane confers the change upon the protein instead of the protein being acted on directly.

Dibucaine and tetracaine inhibit Ca^{2+} + Mg^{2+}-ATPase from skeletal mucsle sarcoplasmic reticulum. Calorimetric studies as well as flouresence measurments of sarcoplasmic reticulum membranes and determined that dibucaine and tetracaine shifted the ATPase to a more unstable conformation at concentrations close to those that inhibit the Ca^{2+}-Mg^{2+}-ATPase activity (4.0 mM

tetracaine and 2.0 mM dibucaine) (Gutierrez-Merino et al., 1989). Bradford and Marinetti, (1981) have proposed that specific lipid-protein interactions play a role in sodium channel function and that local anesthetics can disrupt these interactions. According to Casonovas et al., (1983), the lipid solubility of local anesthetics determines the amount of molecules that are able to cross the membrane; once inside the cell, the protonated hydrocarbon "tail" of the drug acts to block nerve conduction. The lipid solubilty of the drugs has also been correlated to their ability to inhibit the the protein calmodulin. Dibucaine, tetracaine, and lidocaine showed an affinity for this protein with a correlation to the octanol/water partion coefficient; namely, the more hydrophobic the molecule, the higher the affinity for the protein (Tanaka and Hidaka, 1981).

Inhibition of electron transport in mitochondria by local anesthetics has been reported previously (Chazotte and Vanderkooi, 1981). Chazotte and Vanderkooi reported that anesthetics of the alcohol and tertiary amine type inhibited electron transport in succinate oxidase, NADH oxidase, NADH dehydrogenase, succinate dehydrogenase, and cytochrome c oxidase. A concentration dependence of the inhibition by the anesthetic was noted (Singer, 1980; Vanderkooi and Chazotte, 1982; Chazotte and Vanderkooi, 1981). It was noted that the relative hydrophobicity of the molecule was related to the

potency of electron transport inhibition (Singer, 1980, 1982; Casanovas, et al,. 1983), just as the hydrophobicity of the molecule contributes to their potency as anesthetics. These investigators suggested an interaction most likely with the lipid associated with the oxidase as described by Jost et al., (1973).

Several investigators have used cytochrome oxidase as a model membrane protein to test the effects of various local anesthetics. Since much is known about the structure and function of cytochrome oxidase (and that the oxidase requires lipids to make it functional (Yu et al., 1975; Crane, 1968), this protein, is an excellent model for the study of anesthetic interaction. Singer, (1980, 1982), and Casonovas et al., (1983), report that the molecule can interact electrostatically with the oxidase, possibly competing for the charged cytochrome c binding site. Casonovas et al., (1983), characterized eight local anesthetics as being competitive, non-competitive, or a mixed type inhibitor of cytochrome oxidase activity. Using isolated oxidase using 1% Tween 80 to reconstitute lipid depleted cytochrome oxidase, Chazotte and Vanderkooi (1981) and Vanderkooi and Chazotte (1982) demonstrated that the concentration of dibucaine needed to inhibit cytochrome oxidase in rat liver submitochondrial membranes was inversely related to temperature, further suggesting a hydrophobic interaction. They suggested a reversible perturbation of

protein conformation but could not determine if the anesthetics react with the protein directly (as is the case of lipid-free isolated ATPase (Adade et al., 1984, 1987; Chazotte et al., 1982; Vanderkooi and Adade, 1986) or with its "boundary lipid" (Jost et al., 1973).

In addition to these studies, Singer (1983) has studied the effect of dibucaine, tetracaine, procaine, and benzocaine on the rate of electron transport of cytochrome oxidase incorporated into proteoliposomes. In addition, the drugs effect upon hydrogen ion uptake in ionophore stimulated liposomes was investigated. Liposomes that are treated with trifluoromethoxycarbonylcyanide-phenylhydrozone (FCCP) and valinomycin plus K^+ cause the membrane to be permeable to hydrogen ions and thereby dissipate the pH gradient/electrical gradient resulting from the respiratory activity (Hinkle et al., 1972). Singer found that in the presence of anesthetics, liposomes treated with an ionophore showed a much greater decrease in the turnover number as opposed to liposomes that were untreated with an ionophore. Dibucaine, at a concentration of 2.5 mM, inhibited the enzyme turnover by 25%; but when the liposomes were treated with an ionophore, the inhibition of enzyme activity by the drug increased to 75%. Singer postulates that a lipid site of action by the drugs could account for the decrease in turnover number. Studies by Singer using azide, a known

heme ligand (Nicholls and Chance, 1974), does not change the degree of oxidase inhibition either in the presence or absence of an ionophore. Singer suggests that an interaction with the lipid boundry could account for these effects; also the relative potencies of the anesthetics studied follow their degree of hydrophobicity in the order dibucaine > tetracaine > benzocaine > procaine. Therefore, it could be inferred that the site of action by the drugs is not directly affecting the heme, but the membrane associated with the protein.

Propranolol is used to block beta-andergenic receptors; this results in a lowered heart rate, cardiac output, stroke volume, and oxygen uptake (Ledingham and Lees, 1981). Propranolol inhibits respiration dependent ADP and Ca^{2+} uptake (Komai and Berkoff, 1979); Sobel et al., (1982), reported that it did not inhibit oxidative phosphorylation. Komai and Berkoff (1979) found that 0.4 mM propranolol inhibited uncoupler-stimulated respiration as well as calcium stimulated respiration in intact beef heart mitochondria. They also found that the affinity for propranolol was greatly lowered by adding 50 mM KCl. At 0.5 mM propranolol, using malate-glutamate as substrate, state 4 respiration was measured to be 0.12 uatoms O_2/min/mg and in the presence of 50 mM KCl 0.5 mM propranolol, state 4 respiration increased to 0.20 uatomsO_2/min/mg. They suggested that the charge

interaction between the KCL and propranolol prevented the hydrophobic interaction needed between the drug and the membrane. Herdette, et al., (1983) reports a non-specific interaction of propranolol with dimyristoyl phosphatidyl choline (DMPC) bilayers. They also reported that this drug inhibits the calcium pump in sarcoplasmic reticulum by partioning into the bulk lipid matrix. Further, propranolol will bind to the mitochondrial membrane with a high affinity (Huunan-Seppala, 1972).

Despite these extensive studies on the mechanism and type of inhibition that is seen by these drugs, the site of action of these compounds in cytochrome oxidase has not been identified.

CHAPTER III

MATERIALS AND METHODS

Mitochondrial Preperation

All experiments utilized intact, coupled mitochondria that were isolated according to the method of Harmon and Crane, 1974. Fresh beef hearts were obtained from a local slaughter house. All subsequent steps were performed on ice or in a cold room. Hearts were trimmed of all fat, valves, and pericardial membranes and ground in an industrial meat grinder. After grinding the heart, 1.5 times the volume of isolation medium containing 0.35 M sucrose, 0.5 M Na_2PO_3, 7.5mM $MgCl_2$, and 1.0 mM succinate was added to the ground meat. The meat mixture was then separated into 400 ml aliquots and homogenized with a Tekmar Tissuemizer blender on setting 9 for 45 seconds. The homogenate was centrifuged in 1000 ml bottles at 500 x g in an International swinging bucket centrifuge for 20 minutes. The supernatant was strained through 4 layers of cheese cloth (the pellet was discarded), and centrifuged at 26,500 x g for 15 minutes in a Sorvall RC5-B using a SA-600 rotor. The supernatant from this

step was discarded. The mitochondrial pellet was resuspended in the initial isolation medium and homogenized using a size C Potter-Evelhjem homogenizer. The resuspended pellet was again centrifuged at 26,500 x g for 15 minutes. The supernatant was discarded and the pellet resuspended in a solution of 0.35 M sucrose, 7.5 mM $MgCl_2$, and 0.15 M KCl. The mitochondria were washed twice with this solution and resuspended in a medium of 0.35 M sucrose and 7.5 mM $MgCl_2$. The mitochondria were suspended at a protein concentration of approximately 40-50 mg/ml and stored frozen at -40° C in 1 ml aliquots. The protein concentration was determined by the Biuret method described by Yontani (1961).

Oxygen Consumption Assays

Measurement of oxygen consumption by the polarographic method described previously by Harmon and Crane, 1976, was used to determine cytochrome oxidase activity as well as K_m and V_{max} (Harmon, 1988). Oxygen consumption was measured with a Clark oxygen electrode in a glass, water-jacketed chamber kept at a constant 25° C. The following assay mixture was added to the 1.7 ml chamber: 1.2 ml water, 0.3 ml sodium phosphate buffer (pH 7.4, 80mM final concentration), 100 ug mitochondria, 100 uM cytochrome c (Sigma, Type VI). Ascorbate (5.0 mM final concentration)and 12 ug N,N,N',N'-tetramethylphenylenediamine (TMPD) were added as

reductant. Protamine sulfate was used to inhibit the oxidase so an autoxidation rate could be measured. This value was then subtracted from the original value in order for a true measurement of cytochrome oxidase activity.

Inhibition curves were generated by adding various amounts (0-10 mM final concentration) of either dibucaine, tetracaine, or propranolol to the assay mixture. The values of K_m and V_{max} for cytochrome c were obtained by varying the amount of cytochrome c and adding the drugs at a concentration needed for 70% inhibition. The data was plotted using Eadie-Hofstee plots as describe by Ferguson-Miller et. al. (1976), and Segal, (1976) to determine the high and low affinity binding sites for cytochrome c.

Log P values for the drugs were obtained from Cruickshank, (1980), as determined using an octanol/water solvent system. The values reported for dibucaine, tetracaine, and propranolol are 4.4, 3.7, and 3.9 respectively.

Spectrophotometric Assays

Dual wavelength kinetics using the 445 minus 465 nm or 604 minus 630 nm wavelength pair were recorded with a Johnson Research Foundation (University of Pennsylvania) DBS-3 scanning dual wavelength spectrophotometer. Spectra in the near infra-red region were recorded with

the same insturment with a Kodak Wratten #15 filter used as a second order filter. Spectra of carboxy-oxidase were recorded using a Varian DMS-100S spectrophotometer modified to function in a dual wavelength mode.

Mitochondria were suspended at 2 mg protein/ml in 0.25 M sucrose-50 mM sodium phosphate buffer (pH 7.4). 3 ml of this suspension was added to a 3 ml cuvette and 100 ug of cytochrome c was added. Ascorbate (5 mM final concentration) plus 12 ug TMPD was added as reductant. The absorbance change indicative of a reduction of cytochrome aa_3 was followed at either 445 nm minus 465 nm or 604 nm minus 630 nm and measured on a strip chart recorder. Sodium dithionite was then added to ensure complete reduction of the sample. Where appropriate, the drugs were added before the reductant.

Spectra of CuA were measured using the DBS-3 instrument scanning from 700 nm to 900 nm using a 900 nm reference wavelength. Mitochondria were suspended at 6 mg/ml protein in 0.25 M sucrose-50 mM sodium phosphate buffer (pH 7.4). Exogenous cytochrome c (100 ug) was added to a 3 ml sample and an oxidized spectrum was recorded. Ascorbate (5.0 mM) plus 12 ug TMPD was added as reductant. A reduced minus oxidized spectrum was generated by subtracting the oxidized spectrum from the reduced spectrum.

Carboxy-oxidase spectra were recorded using a Varian DMS-100S spectrophotometer modified to function in dual wavelength mode. The mitochondrial suspension was scanned from 400 nm to 650 nm using a 630 nm reference wavelength. Mitochondria were suspended at a 2 mg/ml protein concentration in 0.25 M sucrose-50 mM sodium phosphate buffer (pH 7.4); the 3 ml sample was reduced with 180 mM ascorbate, 5 ug TMPD, and 100 ug cytochrome c. Carboxy-oxidase was generated by bubbling 100% carbon monoxide (CO) into the sample for 5 minutes. Where appropriate, the drugs were added before reduction with ascorbate, TMPD, and cytochrome c. Drugs were present at concentrations needed for 70% inhibition of mitochondrial respiration as determined polarographically in all assays where drugs were used.

The oxidation-reduction potentials of cytochrome oxidase were measured with a platinum-calomel electrode pair as described by Dutton and Wilson (1974) using the DBS-3 dual wavelength instrument as described previously (Harmon and Basile, 1984). Mitochondria were suspended at a concentration of 2 mg/ml in 0.25 M sucrose- 50 mM Na_2PO_4 buffer pH 7.4 that had been bubbled with nitrogen to exclude oxygen. The mitochondrial suspension (10ml) was introduced into a "Dutton" cuvette. The cuvette is designed to allow the electrodes to be immersed into the solution to facilitate potential measurment. Side arms

present on the cuvette allow addition of reductant or oxidant.

Cytochrome c (100 ug) was added to the 10 ml of mitochondrial sample. 2,3,5,6-tetramethyl-p-phenylene diamine (DAD, 60 uM), 156 uM phenazine methosulfate (PMS), and 3 uM duroquinone (final concentrations) were used as voltage (potential) mediators (Dutton and Wilson, 1974). Where used, drugs were present at concentrations needed for 70% inhibition. The potential of the solution was varied by addition of ferricyanide and/or dithionite solutions. The potential was set at -300 mV with dithionite, and a reduced spectra was recorded from 530 nm to 630 nm using a 630 nm reference wavelength. The sample was subsequently oxidized by step-wise addition of ferricyanide. A spectrum was recorded for approximately every 5 mV change in potential resulting in an oxidized minus reduced spectra. Midpotentials were calculated by measuring the extent of oxidation of the sample as compared to a final oxidation by setting the potential to +400 mV. Potentials for each subsequent oxidation were plotted against the log of the ratio of oxidized and reduced portion of the sample.

CHAPTER IV

RESULTS

The inhibition of ascorbate plus TMPD driven cytochrome oxidase activity in intact beef heart mitochondria is dependent on the concentration of drug present (Figure 2). In the presence of dibucaine, 50% inhibition is observed at a concentration of 1.1 mM with over 90% inhibition obtained at a concentration of 2 mM dibucaine. Propranolol is slightly less toxic in that the concentration needed for 50% inhibition is 2.0 mM. Tetracaine is the least toxic to ascorbate plus TMPD driven cytochrome oxidase respiration requiring a concentration of 5 mM for 50% inhibition. Figure 3 demonstrates that the concentration dependence is directly related to the log of the octanol/water partition coefficient (log P). If the concentration of drug is plotted versus log P, a linear relationship is evidenced as was reported by Chazotte, et. al., (1981).

The enzyme kinetics for cytochrome oxidase activity are altered by these drugs. The K_m and V_{max} using cytochrome c as a substrate for the oxidase were measured. Table I displays the values for K_m and V_{max} for cytochrome c for the control as well as in the

Figure 2. Inhibition of mitochondrial respiration by dibucaine, tetracaine, and propranolol plotting percent inhibition vs. concentration. A 50% inhibition occurs at a concentration of 1.1 mM for dibucaine, 1.2 mM for propranolol, and 5.0 mM for tetracaine.

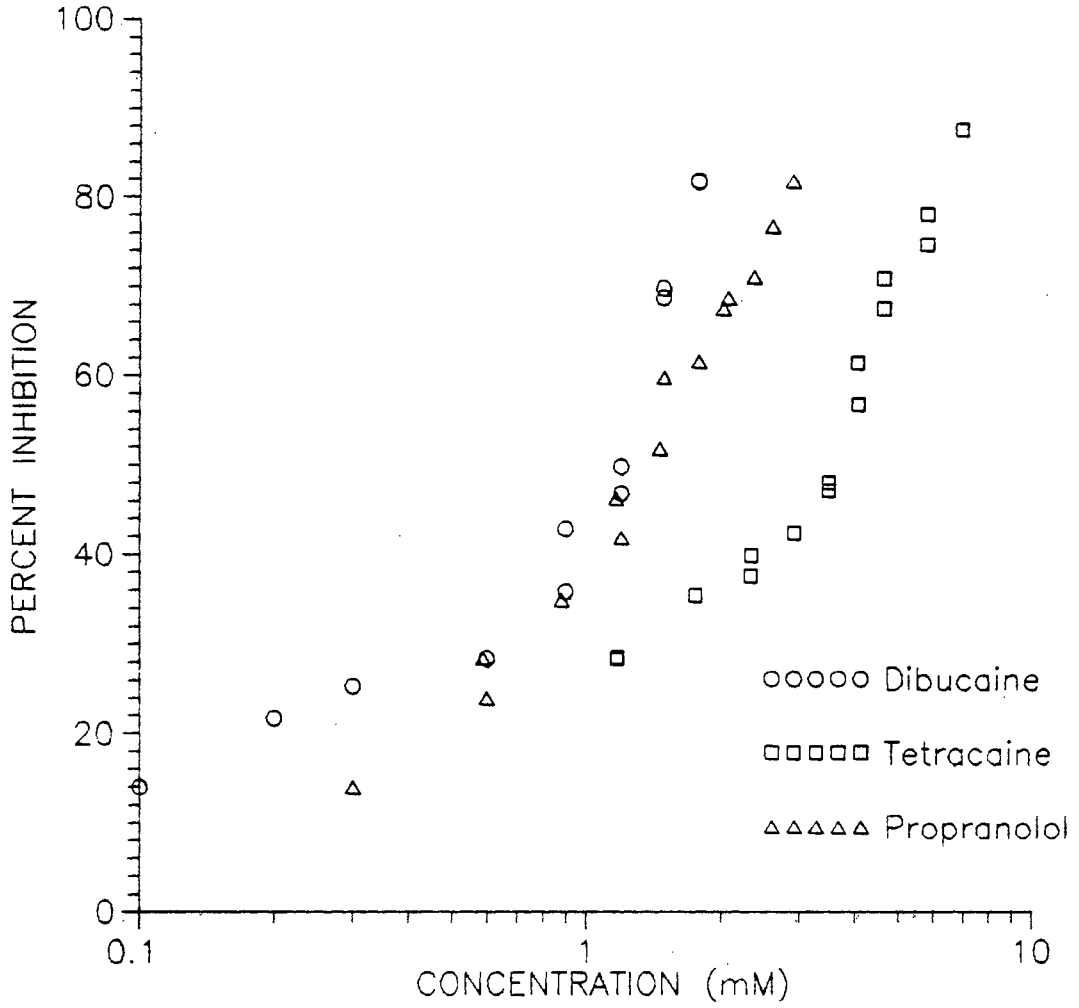
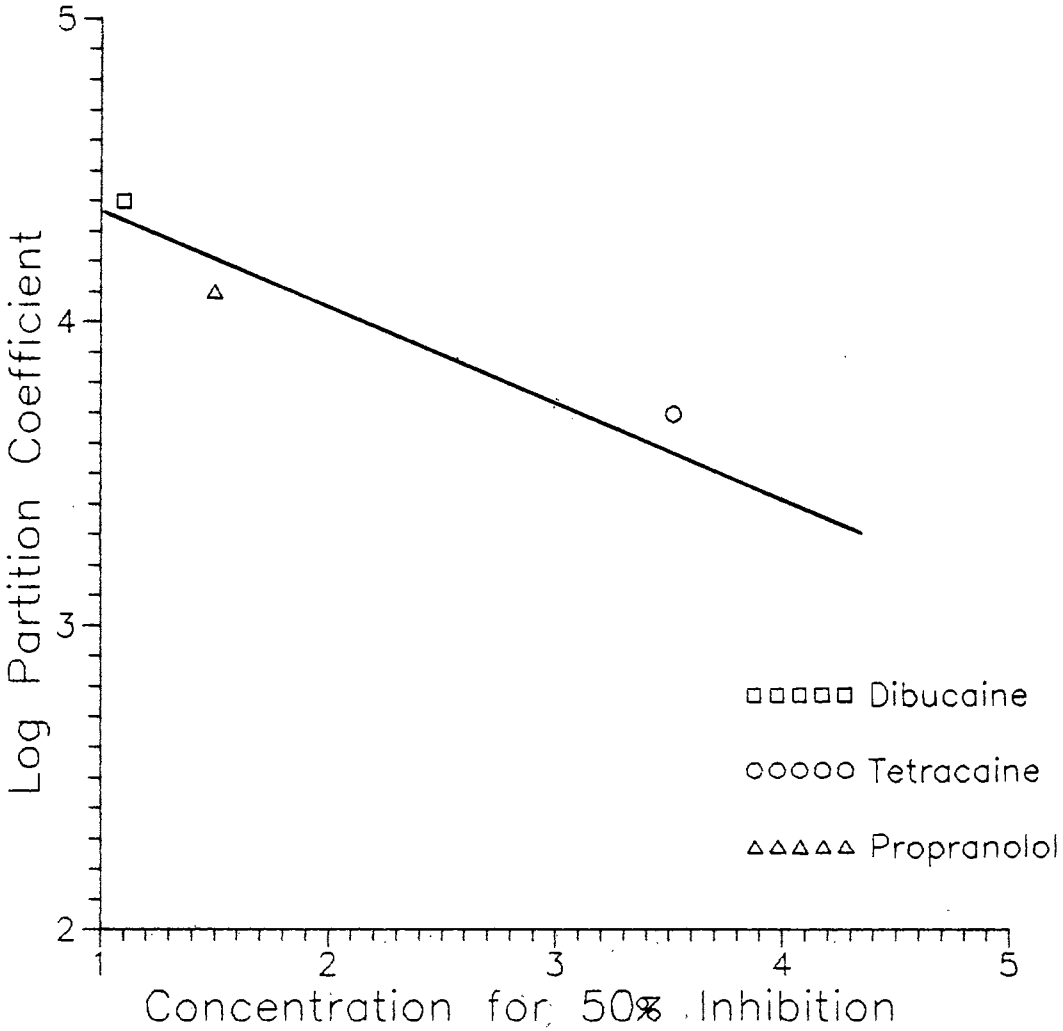

100
80
60
40
20
0
PERCENT INHIBITION
0.1
1
10
CONCENTRATION (mM)
Dibucaine
Tetracaine
Propranolol

Figure 3. Log partition coefficient vs. concentration needed for 50% inhibition of cytochrome oxidase respiration.


Log Partition Coefficient
5
4
3
2
1
2
3
4
5
Concentration for 50% Inhibition
□□□□□ Dibucaine
○○○○○ Tetracaine
△△△△△ Propranolol

presence of dibucaine, tetracaine, and propranolol. When drugs were used, concentrations of 1.5 mM dibucaine, 2.0 mM propranolol, and 5.0 mM tetracaine were added to the assay, the concentrations needed for 70% inhibition of cytochrome oxidase respiration. Figures 4, 5, 6, and 7 are the Eadie-Hoffstee plots for the control and the experiments with the drugs present. The high and low affinity binding sites are clearly visible as two distinct lines with separate K_m's and V_{max}'s.

Addition of ascorbate plus TMPD to mitochondria in the presence or absence of dibucaine, tetracaine, or propranolol results in approximately 90% reduction of cytochrome c oxidase (compared to dithionite reduced sample) as measured by the absorbance increase at 604-630 nm as seen in Figure 8. In contrast, only 50% reduction of the oxidase is observed at 445-465 nm when ascorbate plus TMPD are used as a reductant when the drugs are present at a concentration needed for 70% inhibition (Figure 9).

As shown in Figure 10, reduced cytochrome oxidase exhibits the characteristic alpha band at 604 nm; addition of CO results in an increase in 590 nm absorbance as well as the appearance of a 430 nm band due to the formation of ferrous carboxy-cytochrome a_3. In the presence of 1.5 mM dibucaine, the alpha band at 604 nm is still observed using ascorbate plus TMPD as

TABLE I

SUMMARY FOR Km AND Vmax FOR CYTOCHROME OXIDASE

	Drug	High Affinity		Low Affinity	
		Km (um)	Vmax (nm O2/min/mg)	Km (um)	Vmax (nm O2/min/mg)
	None	0.6	95.56	23.4	389.6
1.5 mM	Dibucaine	29.4	289.73	204.0	882.34
5.0 mM	Tetracaine	8.71	138.02	26.28	911.94
2.0 mM	Propranolol	21.5	245.09	42.19	489.12

Figure 4. Eadie-Hoffstee plot of cytochrome oxidase activity using cytochrome c as substrate. Assay contained the following: 1.2 ml water, 0.3 ml Na_2PO_4 buffer (pH 7.4), 100 ug mitochondria (2mg/ml protein), 100 ug ascorbate plus 5 ug TMPD as reductant. Cytochrome c (Sigma type VI) was varied from 0.5 uM to 100 uM.

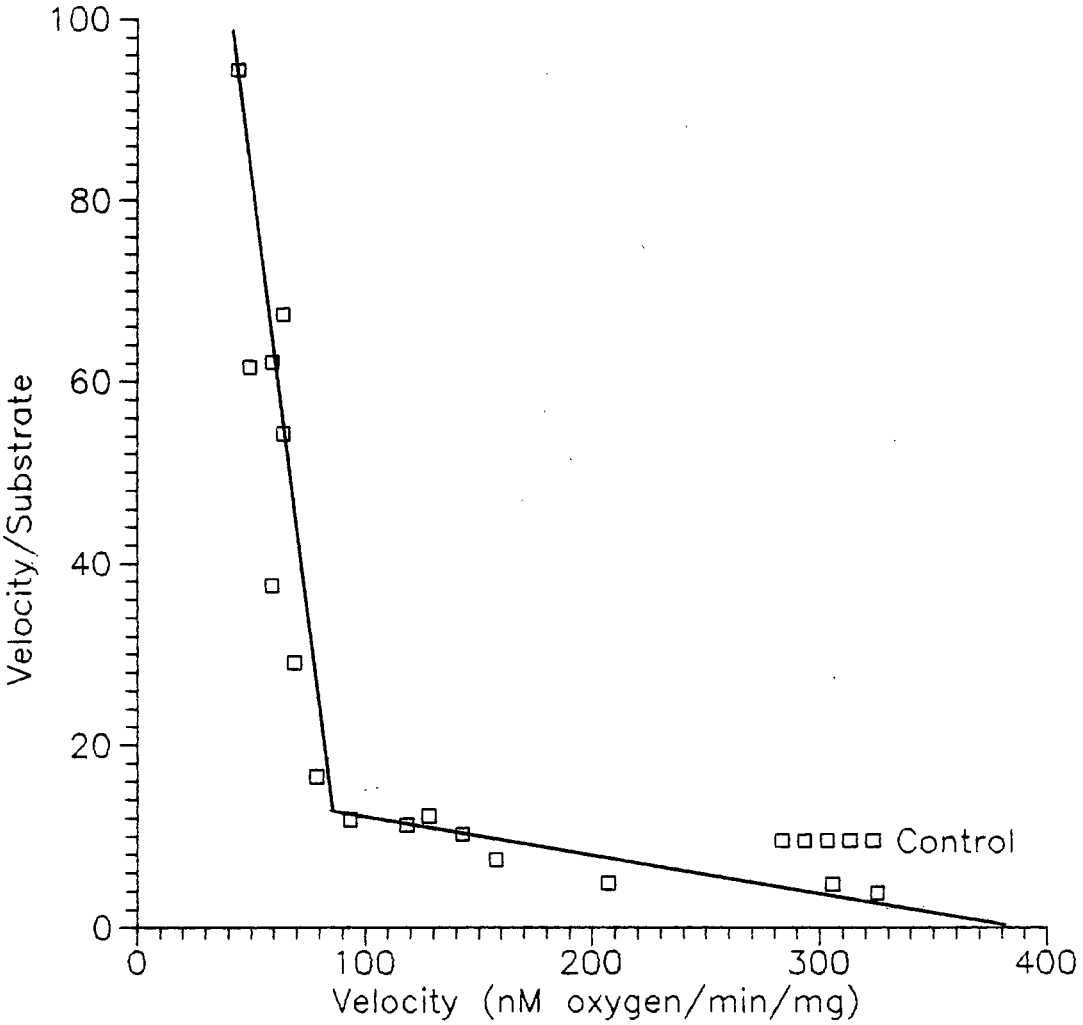

100
80
60
40
20
0
Velocity/Substrate
0
100
200
300
400
Velocity (nM oxygen/min/mg)
Control

Figure 5. Eadie-Hoffstee plot of cytochrome oxidase kinetics using cytochrome c as substrate in the presence of 1.5 mM dibucaine. Conditions are as described in Figure 4.

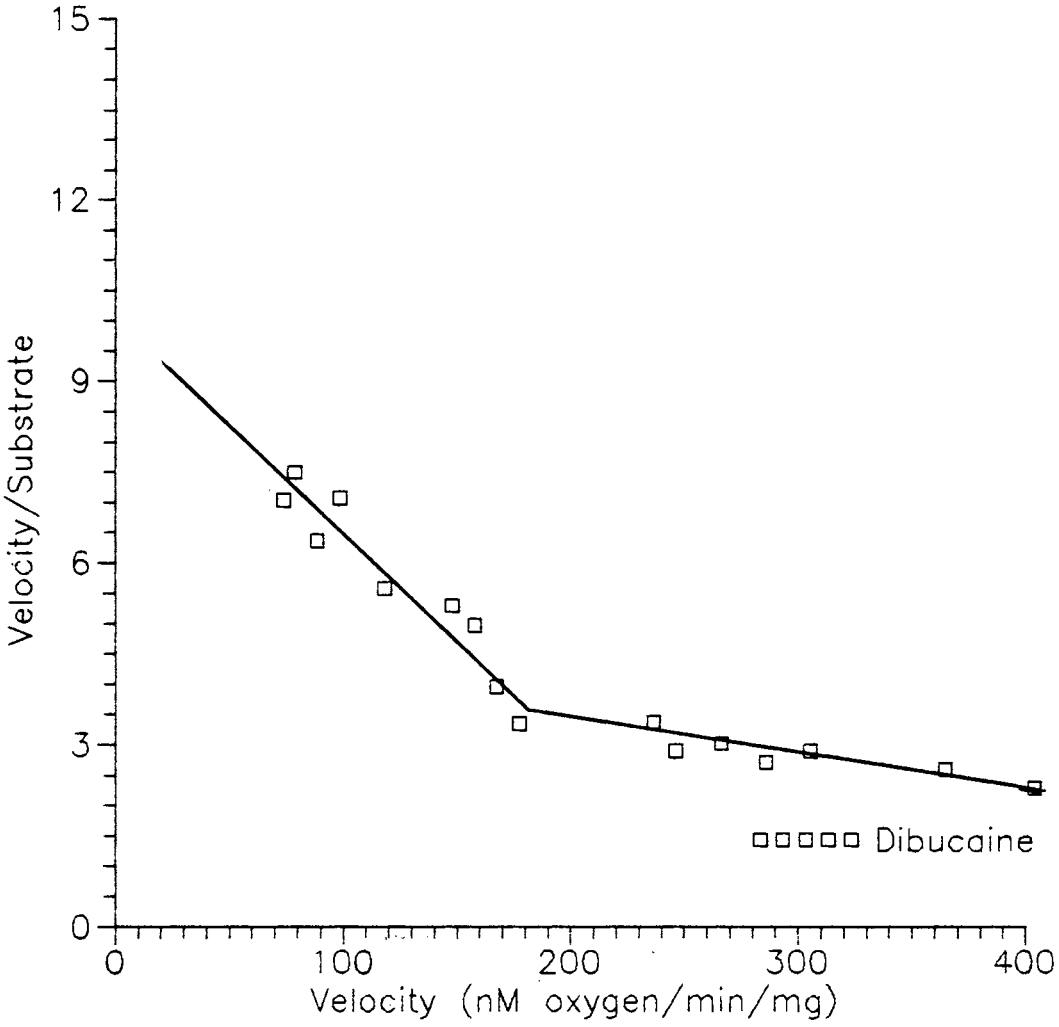

15
12
9
6
3
0
Velocity/Substrate
0
100
200
300
400
Velocity (nM oxygen/min/mg)
Dibucaine

Figure 6. Eadie-Hoffstee plot of cytochrome oxidase kinetics using cytochrome c as substrate in the presence of 5.0 mM tetracaine. Conditions are as described in Figure 4.

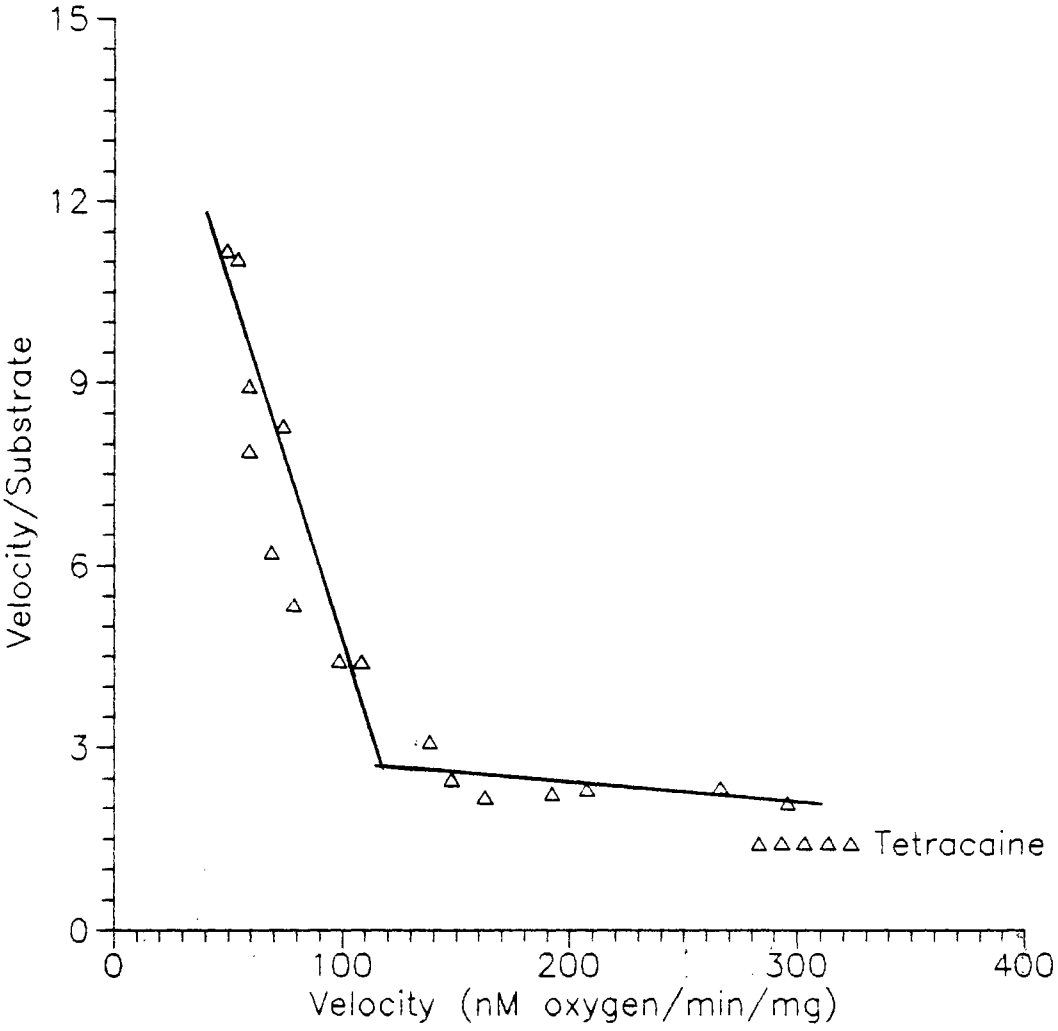

15
12
9
6
3
0
Velocity/Substrate
0
100
200
300
400
Velocity (nM oxygen/min/mg)
Tetracaine

Figure 7. Eadie-Hoffstee plots of cytochrome oxidase kinetics using cytochrome c as substrate in the presence of 2.0 mM propranolol. Conditions are as described in Figure 4.

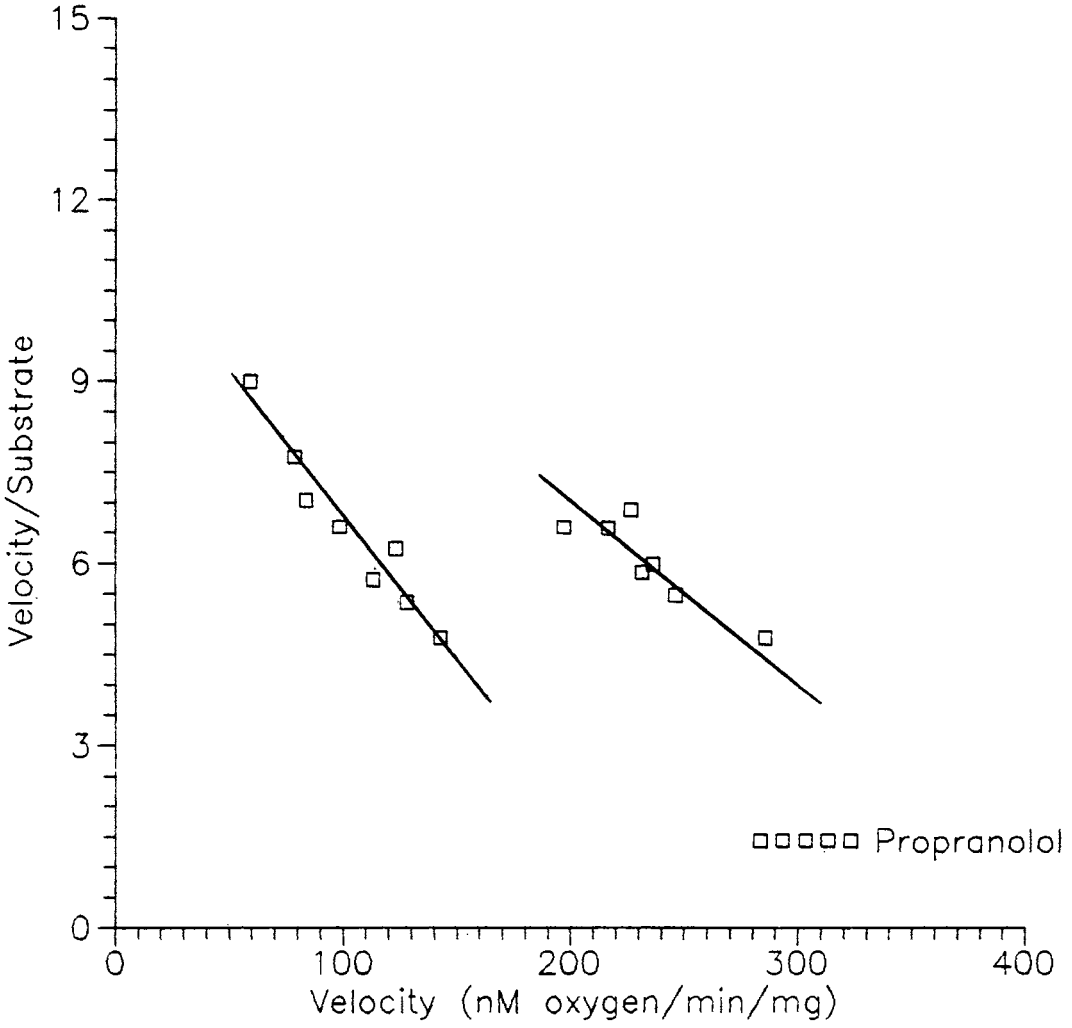

15
12
9
6
3
0
Velocity/Substrate
0
100
200
300
400
Velocity (nM oxygen/min/mg)
Propranolol

Figure 8. Reduction of cytochrome oxidase as measured using the 604 minus 630 nm wavelength pair. Cytochrome c depleted mitochondria were suspended at 2 mg/ml in sucrose-50 mM sodium phosphate buffer (pH 7.4). 5 mM ascorbate and 12 ug TMPD with 200 ug cyt. c were used as reductants.
Drugs were present where indicated at 1.5 mM dibucaine, 5.0 mM tetracaine, and 2.0 mM propranolol.

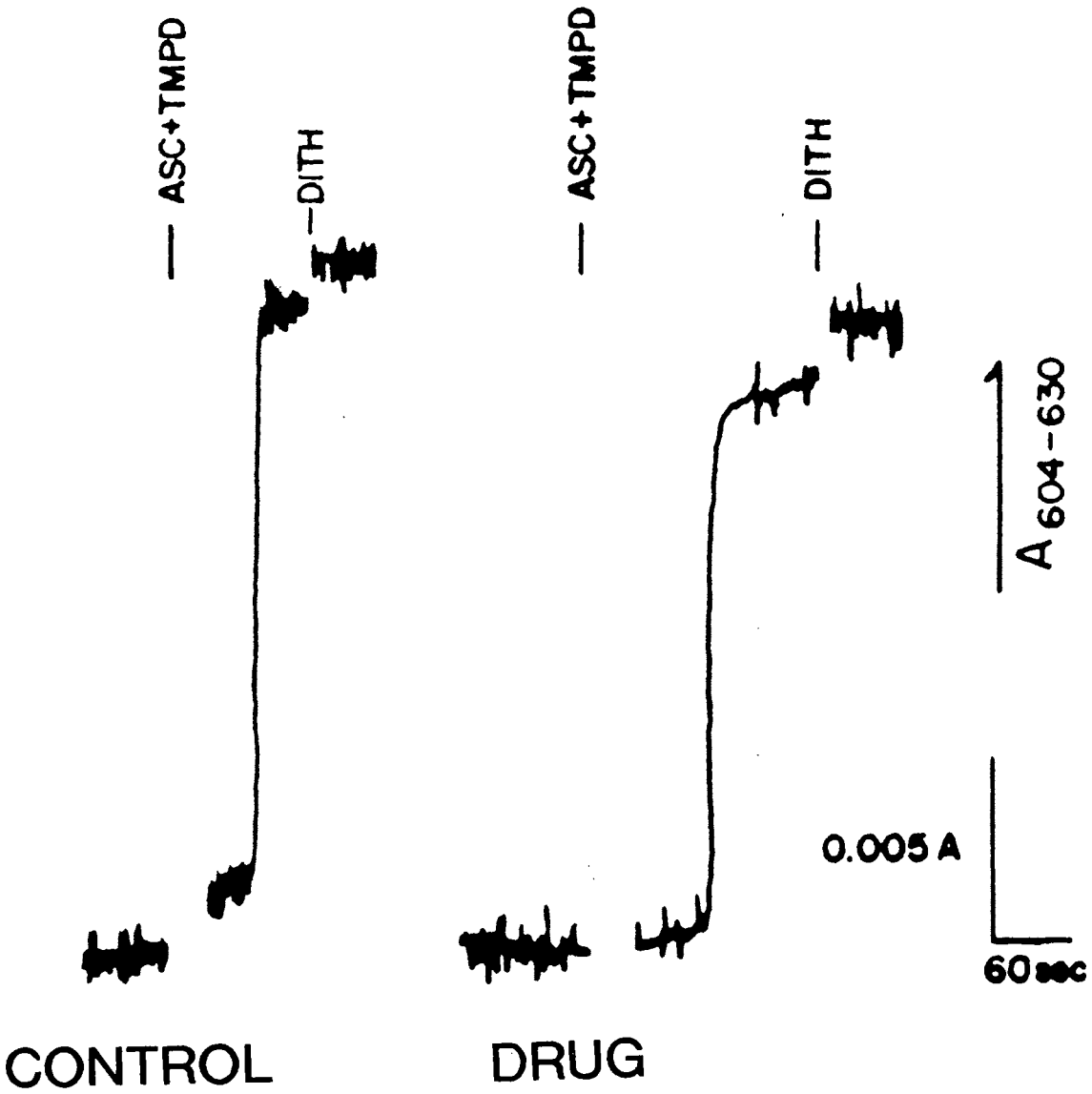
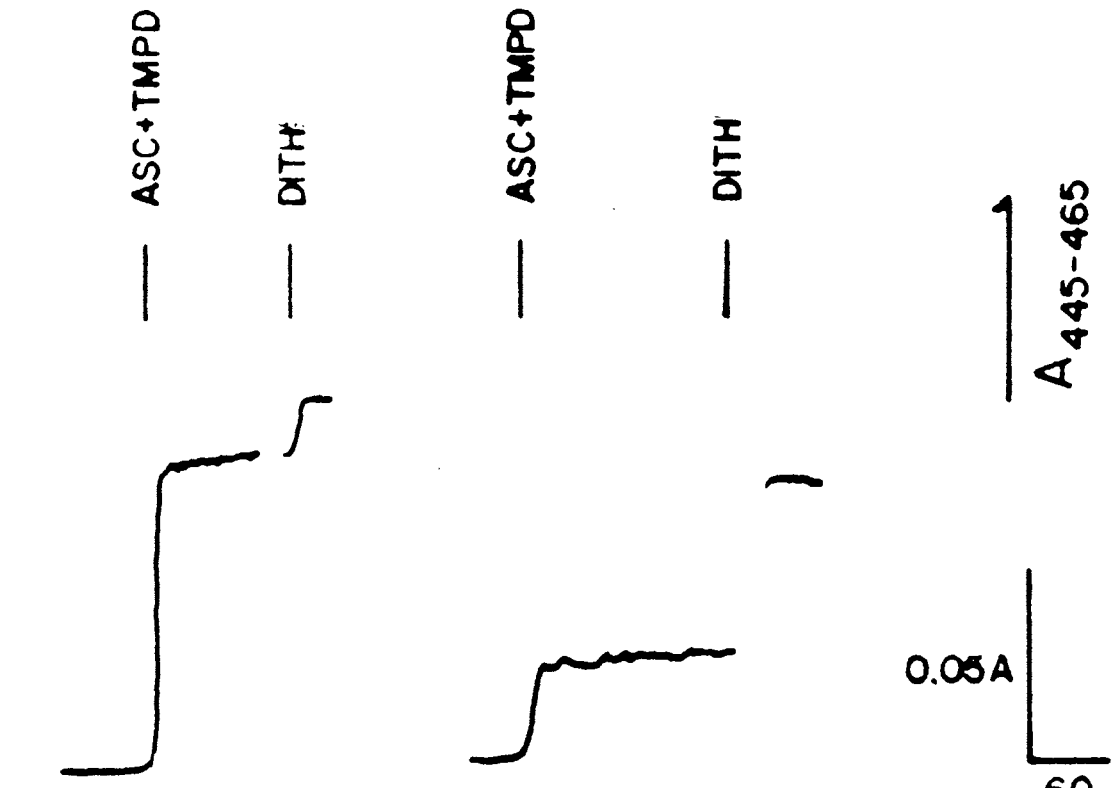

ASC+TMPD
DITH
ASC+TMPD
DITH
A 604-630
0.005 A
60 sec
CONTROL
DRUG

Figure 9. Reduction of cytochrome oxidase as measured using the 445 minus 465 nm wavelength pair in the presence of either 1.5 mM dibucaine, 2.0 mM propranolol, or 5.0 mM tetracaine. Conditions as described in Figure 8.



CONTROL DRUG

Figure 13. Effect of 2.0 mM propranolol on the formation of carboxy-oxidase. Trace 1, reduced MINUS oxidized in the presence of propranolol. Trac 2, same as trace 1 except 100% CO was bubbled into the sample. Conditions are as described in Figure 8.

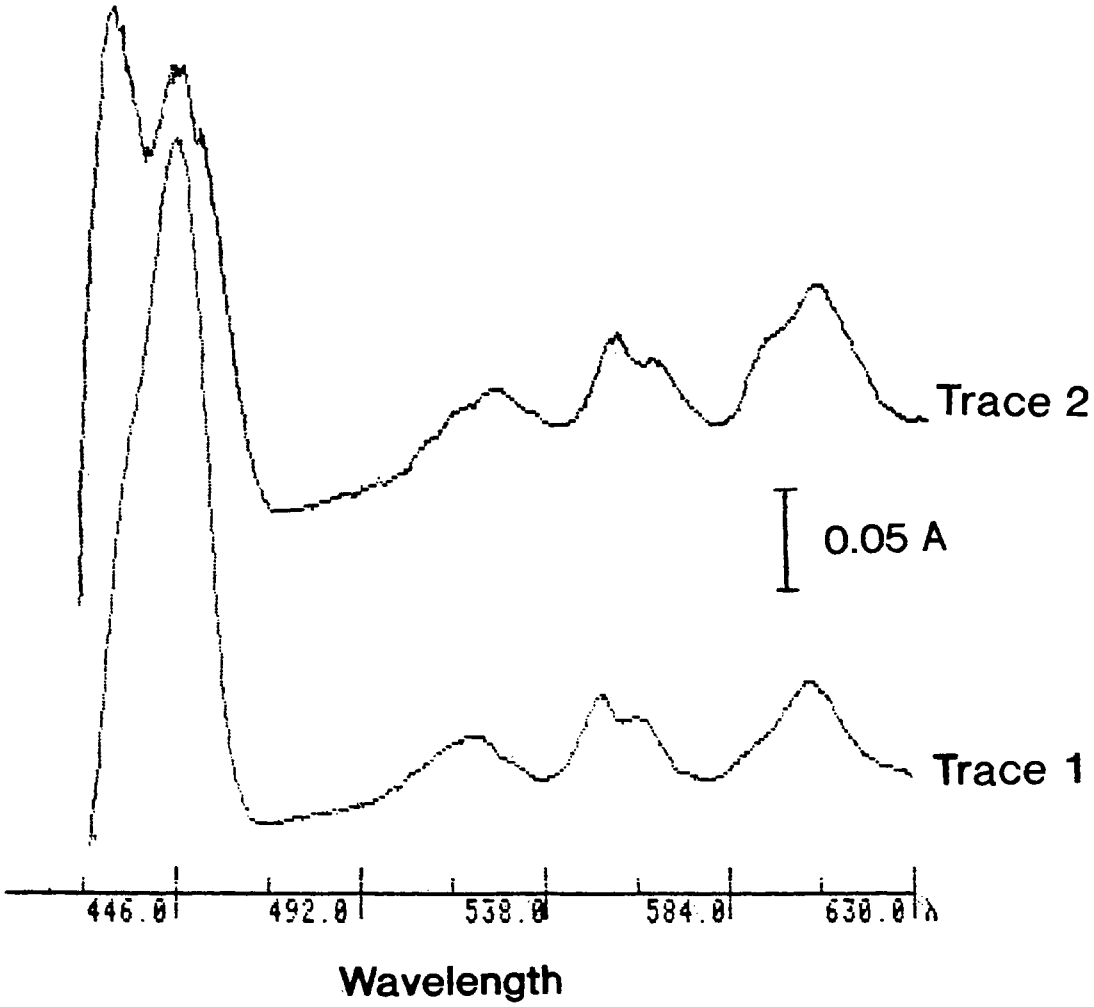

Trace 2
0.05 A
Trace 1
446.0
492.0
538.0
584.0
630.0
λ
Wavelength

Figure 11. Effect of 1.5 mM dibucaine on spectrum of carboxy-ferrous cytochrome oxidase. Samples prepared as desribed in figure 8. Trace A: reduced minus oxidized difference spectrum in the presence of dibucaine. Trace 2: same as trace 1 except 100% CO was bubbled into the sample for 5 minutes. Conditions are as described in Figure 8.

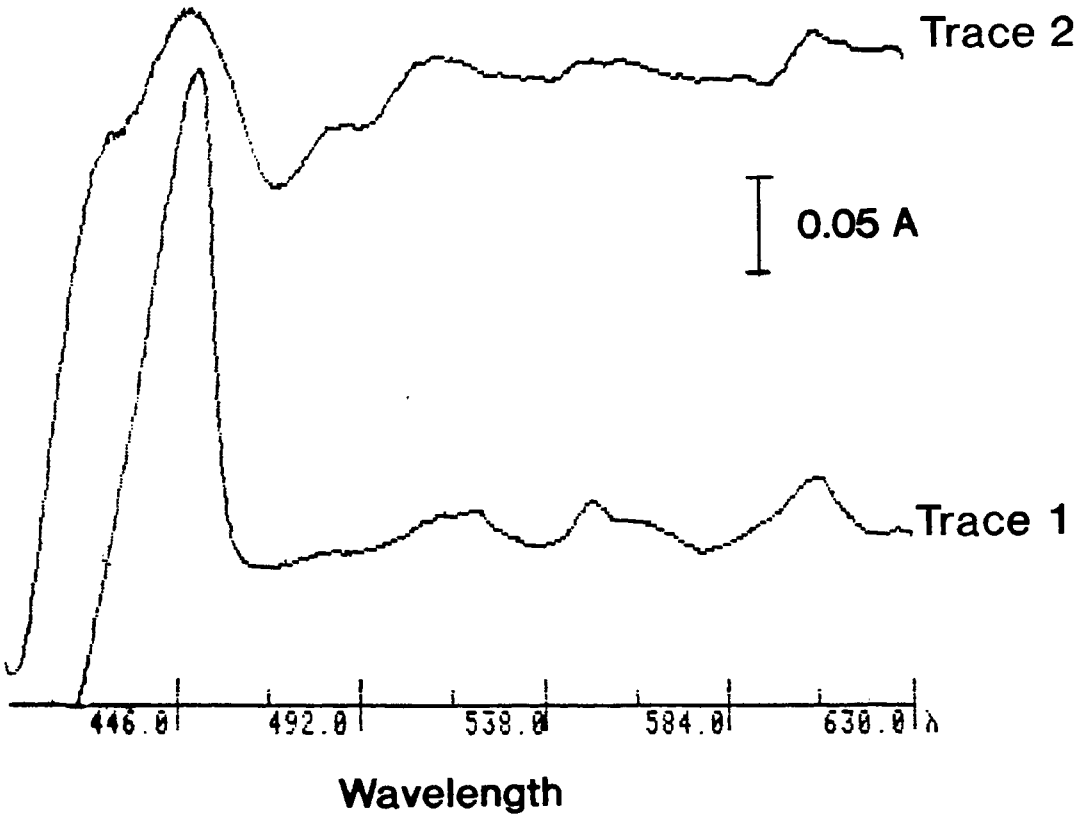

Trace 2
0.05 A
Trace 1
446.0
492.0
538.0
584.0
630.0
λ
Wavelength

Figure 12. Effect of 5.0 mM tetracaine on the formation of carboxy-oxidase. Trace 1:reduced MINUS oxidized spectra in the presence of tetracaine. Trace 2, same as trace 1 except 100% CO was bubbled into the sample. Sample prepared as in Figure 8.

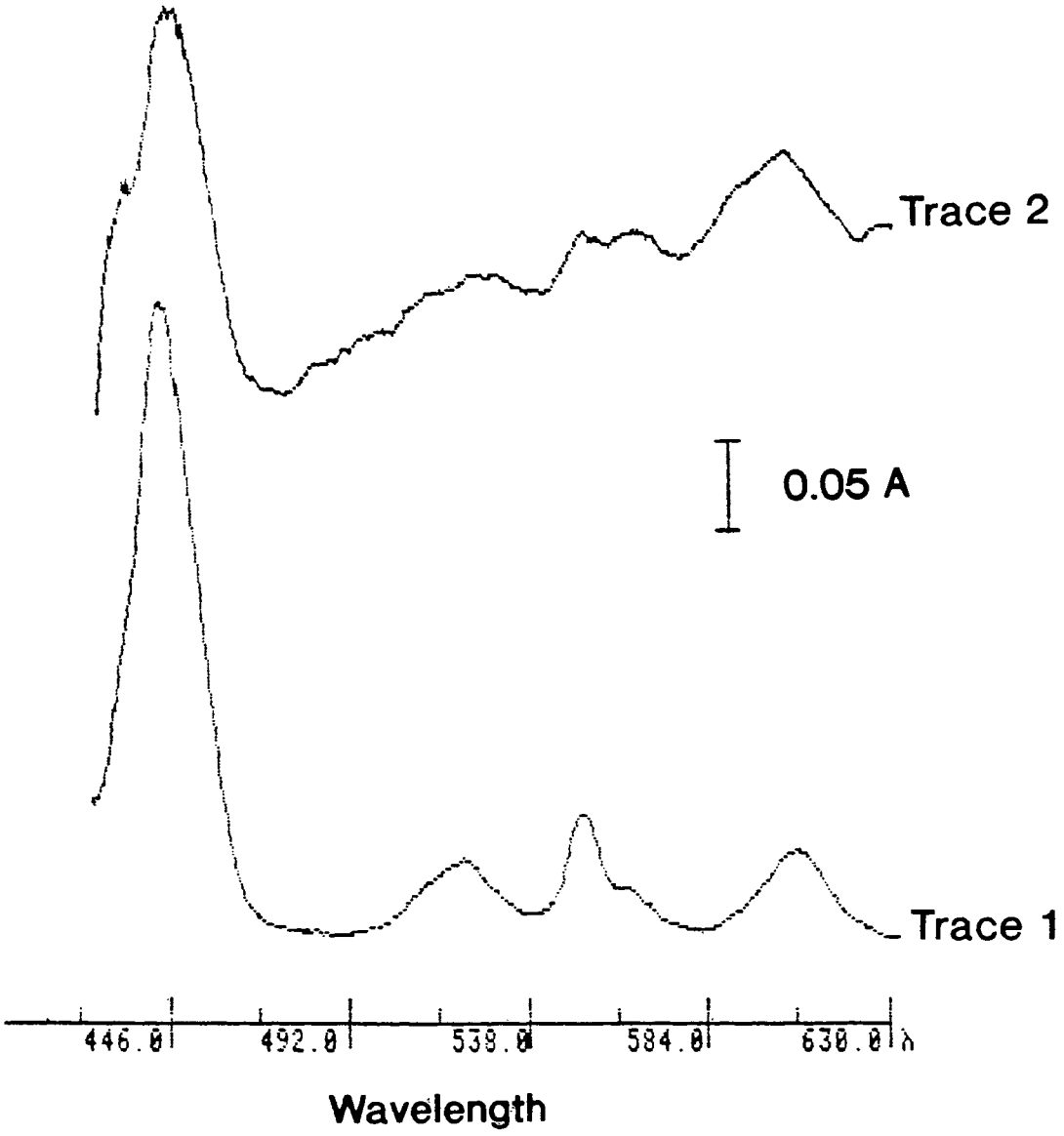
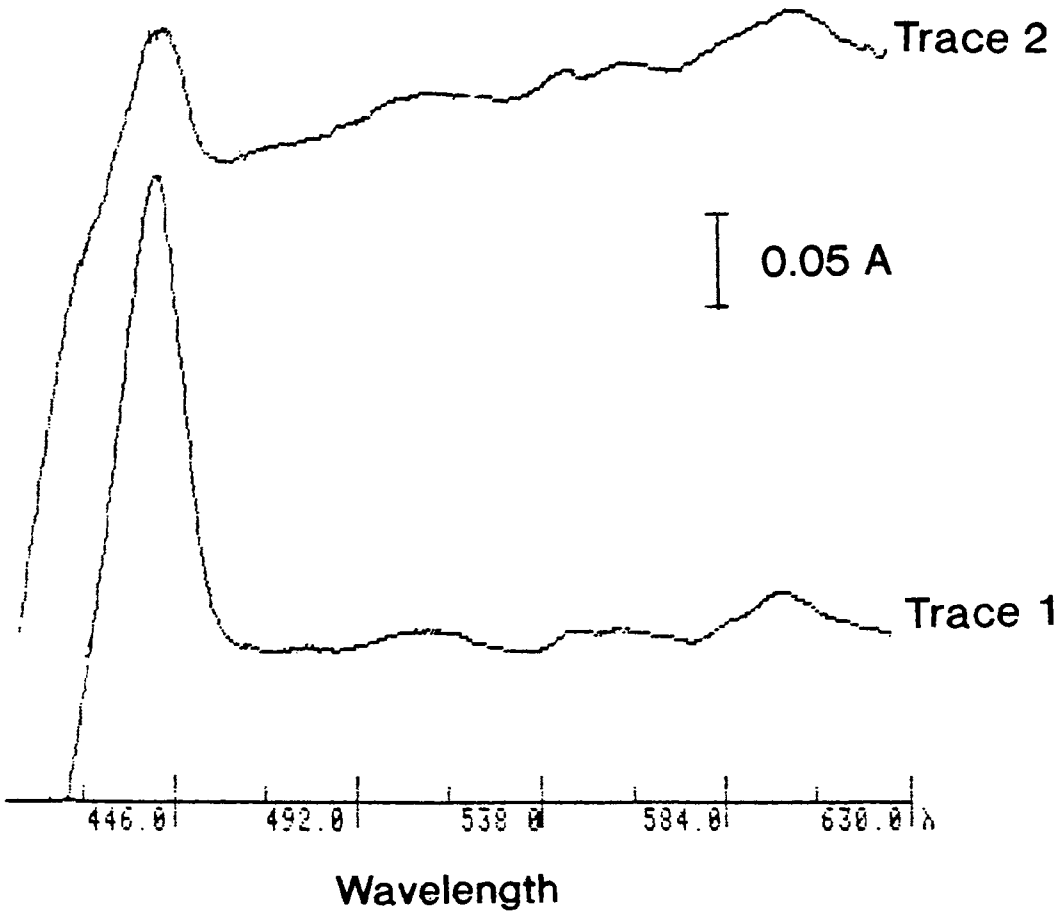

Trace 2
0.05 A
Trace 1
446.0
492.0
538.0
584.0
630.0
λ
Wavelength

Figure 10. Spectra of carboxy-ferrous cytochrome oxidase in intact mitochondria. Samples were prepared as described in Figure 8. Trace 1: reduced minus oxidized. Trace 2: same as trace 1 with 100% CO bubbled into the sample for 5 minutes. Conditions are as described in Figure 8.


Trace 2
0.05 A
Trace 1
446.0
492.0
538.0
584.0
630.0
λ
Wavelength

reductant, but the absorbance increase at 590 nm is not observed (Figure 11). This is to be expected since the kinetic date 445-465 nm shows the dibucaine prevents the reduction of cytochrome $\underline{a}_3$, which is necessary for the formation of carboxy-oxidase. Tetracaine at a concentration of 5.0 mM and propranolol at a concentration of 2.0 mM also prevents the formation of carboxy-oxidase as shown in Figures 12 and 13. In all cases, the drugs inhibit the reduction of cytochrome a_3. Not only is the 590 nm band not present, but there is the absence of the 430 nm band that is also due to ferrous carboxy-oxidase (Chance, 1953a).

The presence of dibucaine, tetracaine, and propranolol alters the midpotential of cytochrome $\underline{c}$ oxidase. Figure 14 displays a typical oxidation-reduction titration of cytochrome oxidase as measured at 604-630 nm. Figures 15, 16, and 17 show the redox titration for dibucaine, tetracaine, and propranolol. The midpotential is the potential at which the log of the ratio of oxidized-reduced is a value of 0, or when exactly half of the oxidase sample is reduced and half is oxidized. The midpotentials for the control and oxidase treated with 1.5 mM dibucaine, 5.0 mM tetracaine, and 2.0 mM propranolol are shown in Table II. Tetracaine alters the shape of the redox titration curve much more than does either dibucaine or propranolol. There appears to be two different mid-

TABLE II

SUMMARY FOR MID POTENTIALS FOR CYTOCHROME OXIDASE

Drug	Mid Potential
None	243 mV
Dibucaine (1.5 mM)	255 mV
Tetracaine (5.0 mM)	350 mV 270 mV
Propranolol (2.0 mM)	265 mV

Figure 14. Oxidation-reduction titration for cytochrome oxidase as measured using the 604 nm band. Mitochondria were suspended at 2 mg/ml protein in 0.25 M sucrose-50 mM Na_2PO_4 buffer (pH 7.4). Cytochrome c was present at a concentration of 100 ug. Redox mediators used were the following: 60 um 2,3,5,6-tetramethyl-p-phenylene diamine (DAD), 156 uM phenazine methosulfate (PMS), and 3 uM duroqinone. Mitochondria were kept under an atmosphere of argon.

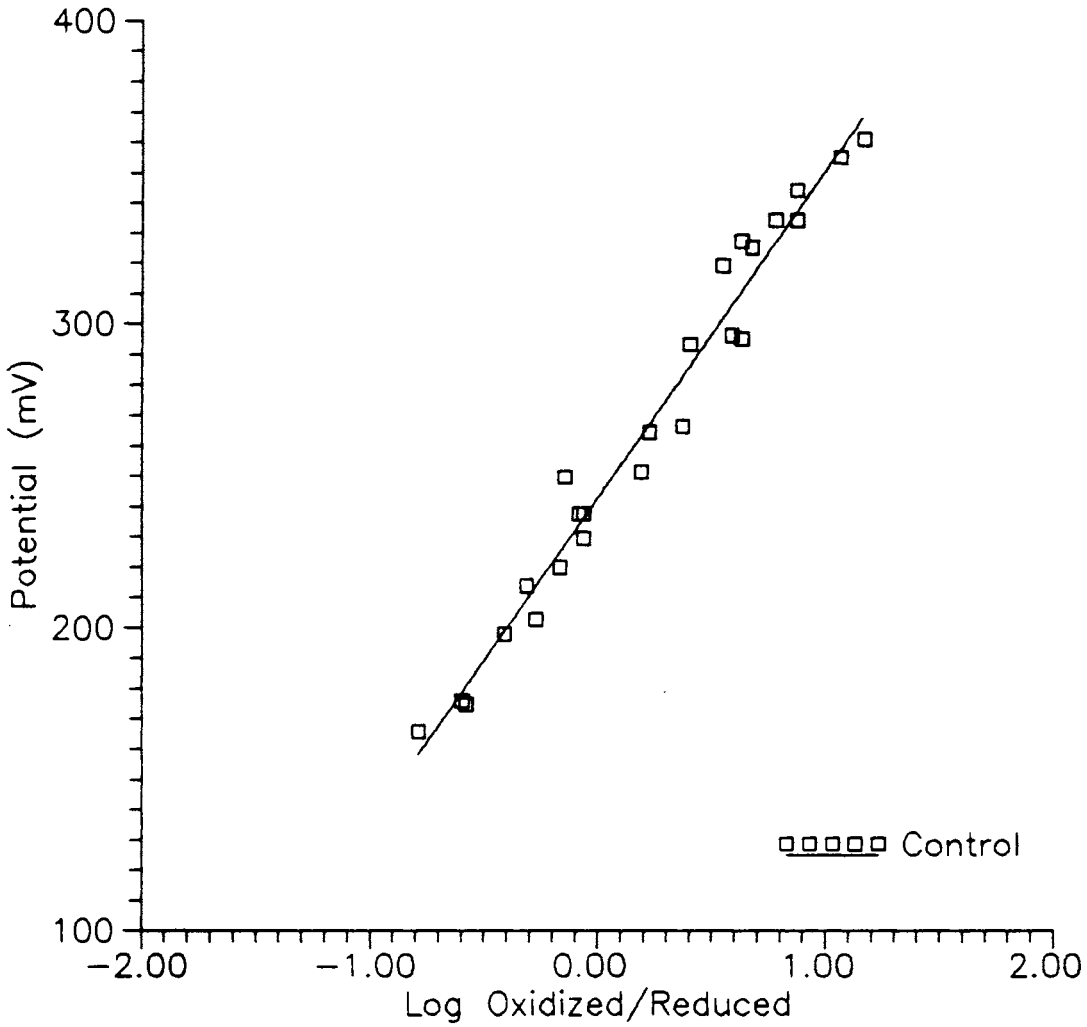

400
300
200
100
Potential (mV)
−2.00
−1.00
0.00
1.00
2.00
Log Oxidized/Reduced
Control

Figure 15. Oxidation-reduction titration for cytochrome oxidase in the presence of 1.5 mM dibucaine. Conditions are as described in Figure 14.

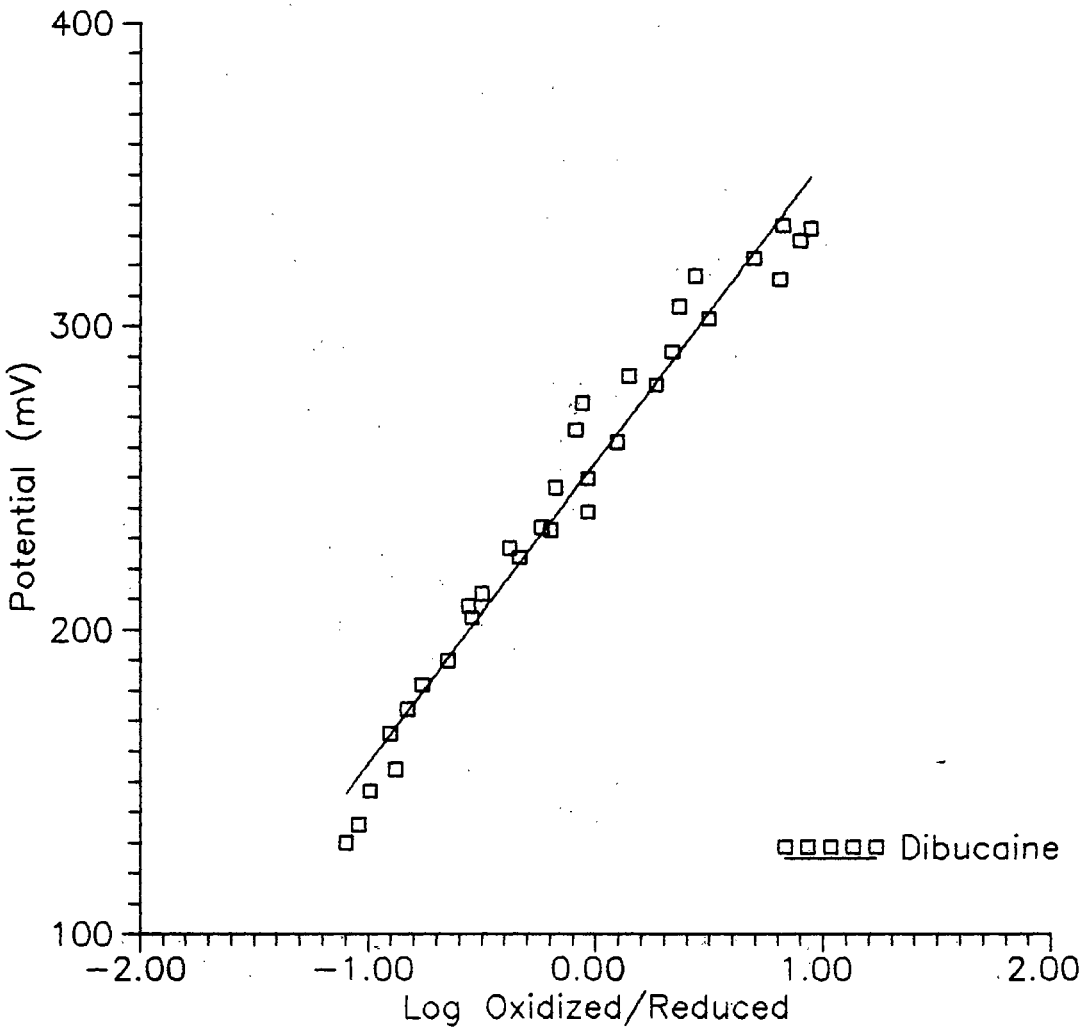

400
300
200
100
Potential (mV)
−2.00
−1.00
0.00
1.00
2.00
Log Oxidized/Reduced
Dibucaine

Figure 16. Oxidation-reduction titration for cytochrome oxidase in the presence of 5.0 mM tetracaine. Conditions are as described in Figure 14.

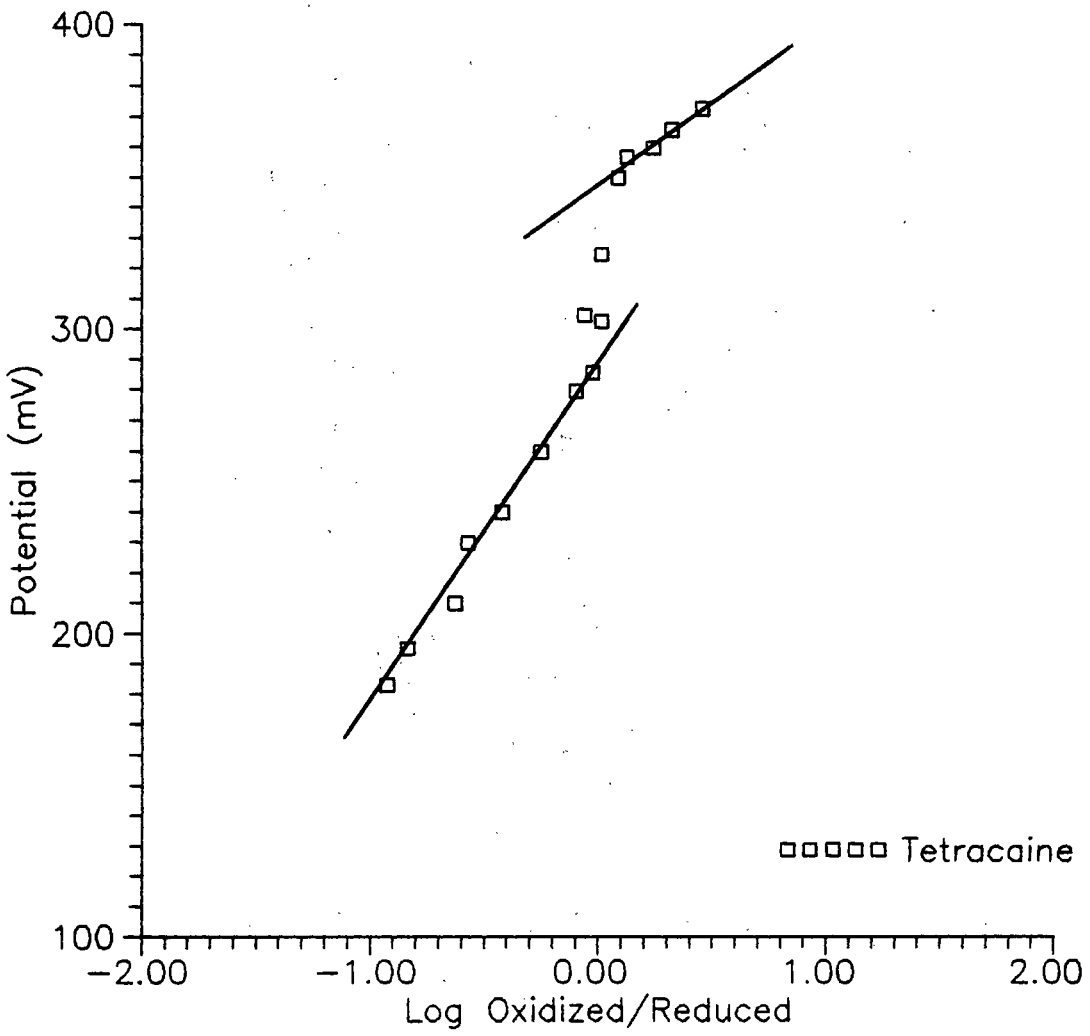
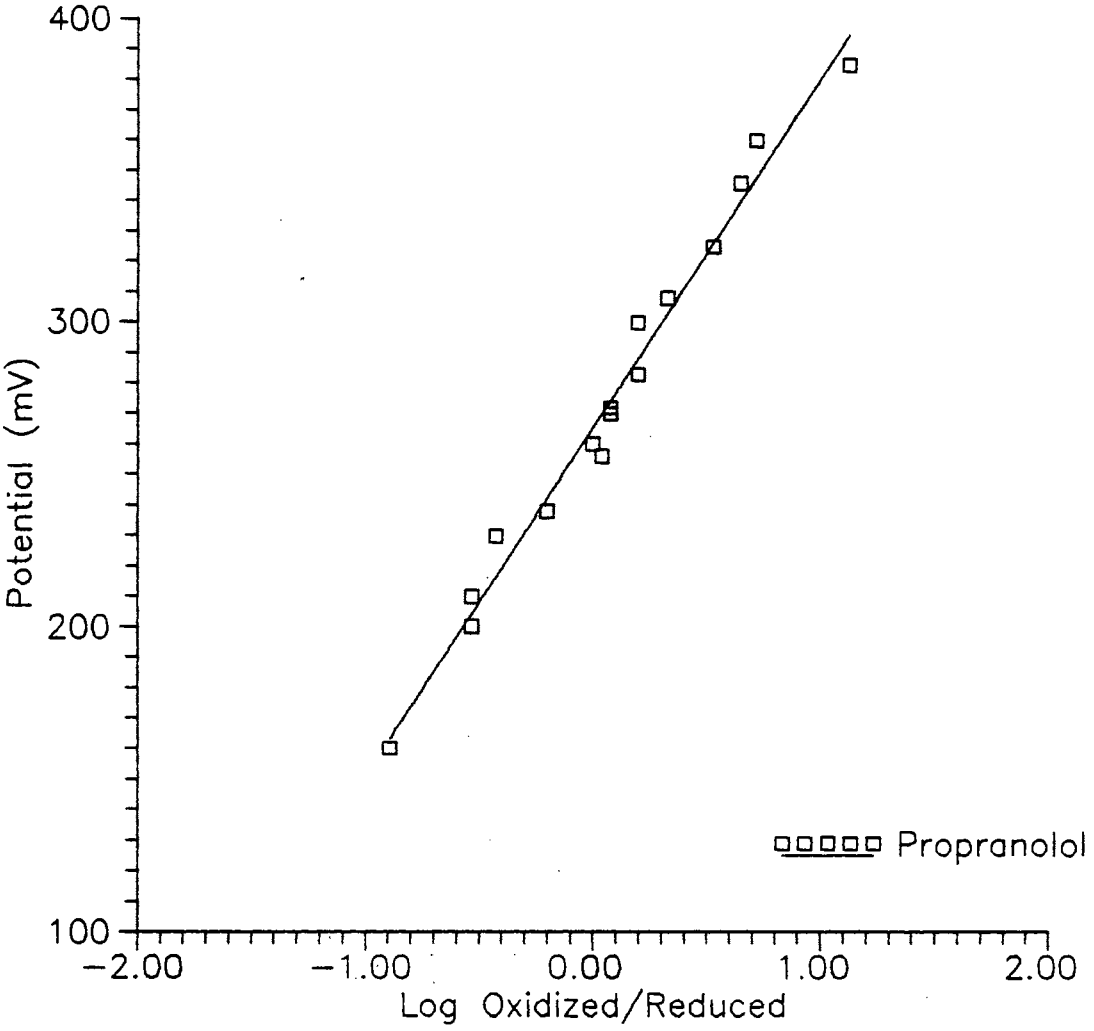
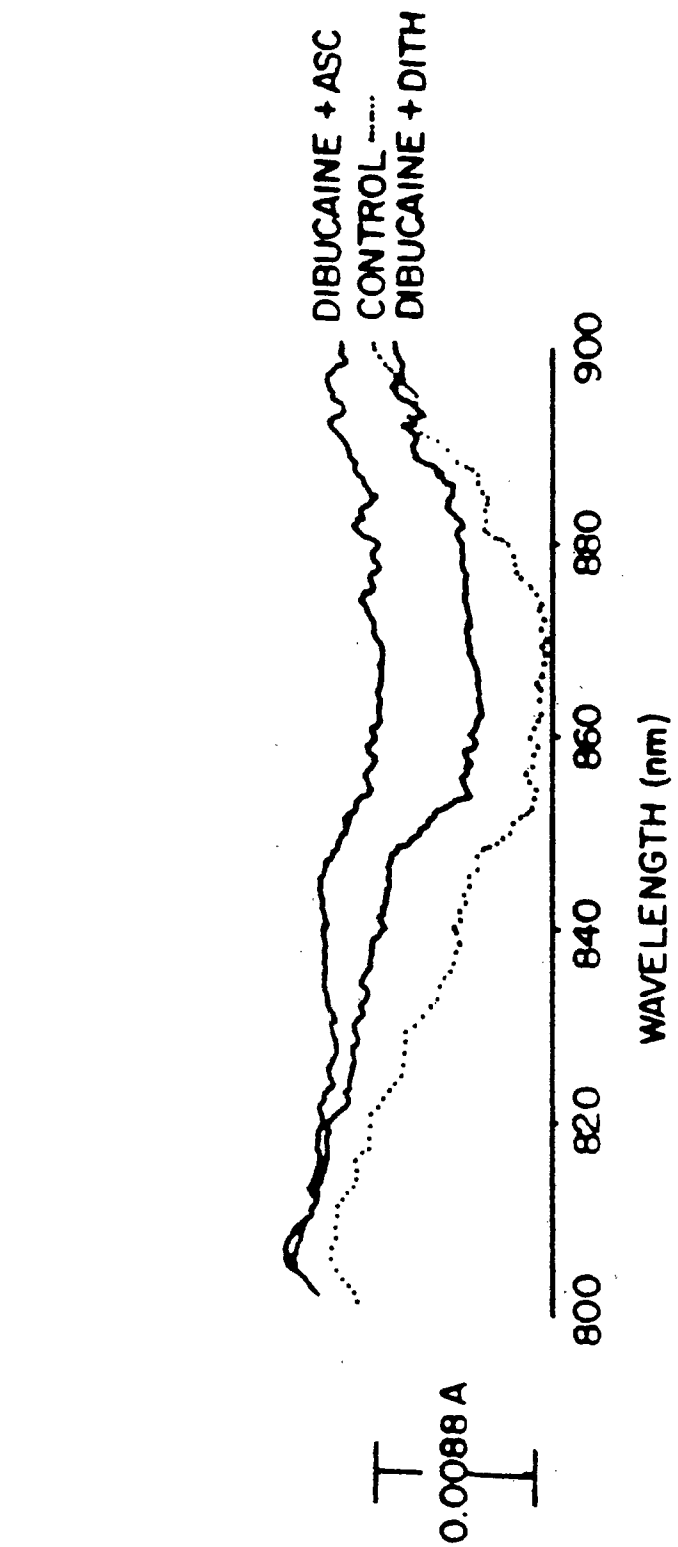

400
300
200
100
Potential (mV)
−2.00
−1.00
0.00
1.00
2.00
Log Oxidized/Reduced
Tetracaine

Figure 17. Oxidation-reduction titration for cytochrome oxidase in the presence of 2.0 mM propranolol. Conditions are as described in Figure 14.


400
300
200
100
Potential (mV)
−2.00
−1.00
0.00
1.00
2.00
Log Oxidized/Reduced
Propranolol

potentials, one at 245 mV and one at 385 mV. These values correspond to the midpotentials for cytochrome a and cytochrome a_3, respectively, as reported by Wikstrom, et al., (1976). This observation will be examined further in the Discussion section. As demonstrated in Figure 18, ascorbate plus TMPD is capable of reducing the "visible" CuA moiety in the absence but not in the presence of 1.5 mM dibucaine. Reduction of the CuA center is shown by a decrease in the absorbance in the 840-860 nm region of a reduced minus oxidized spectrum (Griffith and Wharton, 1961; Wharton and Tzagoloff, 1964). In the presence of dibucaine, addition of dithionite causes a decrease in the near infra-red absorbance indicating that the CuA center is capable of being reduced by a strong reductant. Similar results are obtained using mitochondria washed with bathophenanthroline sulfate to remove adventitious copper (Griffith and Wharton, 1961; Beinert, et. al., 1970; Harmon and Crane, 1974b). Therefore, the copper signal indicated in Figure 18 is not due to adventitious copper, but the copper associated with cytochrome a (CuA).

Figure 18. Spectra of CuA in intact beef heart mitochondria. Mitochondria were suspended at 6 mg/ml protein in 0.25 M sucrose-50 mM Na_2PO_4 buffer (pH 7.4). A Kodak # 15 filter was used as a second order filter. The sample was reduced using 100 ug cytochrome c, 5.0 mM ascorbate and 12 ug TMPD. Where appropriate, dibucaine was present at a concentration of 1.5 mM.


DIBUCAINE + ASC
CONTROL
DIBUCAINE + DITH
800
820
840
860
880
900
WAVELENGTH (nm)
0.0088 A

CHAPTER V

DISCUSSION

This study has examined the effects of three local anesthetics and a cardiac beta-blocker, propranolol, on cytochrome c oxidase respiration as well as the effects on the individual redox centers cytochrome a, CuA, and cytochrome a_3. Unlike previous studies (Chazotte and Vanderkooi, 1981; Singer, 1983; & Singer, 1980) that only characterized the effects of the drugs on electron transport activity this investigation sought to elucidate an exact site of inhibition by these compounds within the oxidase. Chazotte and Vanderkooi (1981) investigated the effects of local anesthetics on the entire mitochondrial electron transport chain utilizing many of the partial reactions to isolate each complex (Hatefi, 1985). These investigators reported LD-50 values for dibucaine of 9 mM and tetracaine of 25 mM for cytochrome oxidase activities. According to Harmon (unpublished results), 50% inhibition of succinate oxidase activity is observed in the presence of 1.25 mM dibucaine in intact mitochondria and at 9 mM dibucaine concentration in 94% inverted submitochondrial particles. The concentration dependence of the

inhibition by the local anesthetics is dependent upon the orientation of the membrane vesicles used and indicates that the inhibitory site is present on the C-side of the membrane and not on the M-side. That the the sites of action of the anesthetics are on the C-side of the membrane would explain why Chazzotte and Vanderkooi's 50% inhibition values were an order of magnitude higher than the ones reported in this current study (Chazotte and Vanderkooi used submitochondrial particles).

Kinetic parameters of cytochrome c oxidase were also investigated; dibucaine, tetracaine, and propranolol all exhibited a mixed-type inhibition. That is, both K_m and V_{max} are altered by the drugs. Singer (1980) reported the same results using the local anesthetics dibucaine, tetracaine, and procaine. He postulates that the charged end of the anesthetics competes with substrate for binding while the non-polar end interacts with oxidase-associated phospholipids to produce non-productive complexes. This conclusion seems logical in light of supporting the assumption that these drugs interact with the lipid bilayer (Casanovas, et. al, 1983; Komai & Berkoff, 1983); Singer, 1983; Gutierrez-Merino, et. al, 1989). However, the data in the current study as well as data of Singer (1980)

indicates that the more hydrophobic the drug molecule, the lower the affinity of cytochrome oxidase for cytochrome c, thus resulting in an elevated K_m and V_{max}.

Each of the molecules studied, dibucaine, teteracaine, and propranolol, have structural similarities in that they each have a hydrophobic head group and a long hydrocarbon tail containing a tertiary amine. It is feasible that the head group dictates the relative hydrophobicity of the drug and determines to a great extent the log P of the molecule. Therefore, the head group could dictate the ease by which the molecule may cross the mitochondrial membrane. Once penetrating the membrane, the amine group, which would bear a positive charge at physiological pH, could be responsible for the competition for the charged cytochrome c binding site. The disruption of the lipid bilayer by the hydrophobic end of the molecule could be responsible for a conformational change to the oxidase resulting in the mixed-type inhibition. If the drugs competed only for the charged cytochrome c binding site, then a strictly competitive inhibition would be displayed.

An aspect of the inhibition of cytochrome oxidase by these compounds not readily apparent to previous investigators are the effects upon the oxidation and reduction levels between the redox centers, cytochrome a, CuA, and cytochrome a_3. Using the dual wavelength

spectrophotometric methods developed by Chance (1952c, 1951, 1961; Chance & Hollinger, 1961a, 1961b, 1961c), oxidation-reduction levels between the redox centers could be resolved. The "neoclassical" model for cytochrome oxidase holds that approximately 80-90% of the absorbance of reduced oxidase at 604 nm is due to ferrous cytochrome a, the remainder due to ferrous cytochrome a_3; both cytochromes absorb light equally at 445 nm (Wikstrom, et. al., 1976). The data in Figures 8 and 9 are consistent with this model. In the presence of dibucaine, tetracaine, or propranolol, over 80% of the 604 nm and approximately 50% of the 445 nm absorbance is recorded when reduced with ascorbate plus TMPD. Thus, these three drugs inhibit electron transport between cytochrome a and a_3, allowing the reduction of cytochrome a (80% of 604 nm at 50% of 445 nm absorbance) but not cytochrome a_3.

The neoclassical 50/50 hypothesis, as reviewed by Wikstrom et al., (1976), relies upon the formation of a mixed valence cytochrome oxidase consisting of a ferric cytochrome a and a ferrous cytochrome a_3 (a^{3+} a_3^{2+}). "Mixed valence" oxidase can be made using several methods (Greenwood, et. al., 1974) the simplest method being to reduce the oxidase with a reductant such as dithionite, bubbling the sample with carbon monoxide, and subsequently oxidizing cytochrome a with ferricyanide. The carbon monoxide binds cytochrome a_3

"trapping" it in the reduced form, while cytochrome a is free to be oxidized. By using this form of the oxidase, absorbance bands may be assigned to cytochrome a or a_3 and also the relative contributions to the size of the band in question (Wikstrom, et al.,1976). Ferrous carboxy-oxidase has a very distinct spectrum; not only is the alpha band at 604 nm present due to reduced cytochrome a, but also a 590 nm shoulder appears. This shoulder at 590 nm is due the 20% of the 604 nm that is due to cytochrome a_3 caused by the ligand of carbon monoxide bound to this cytochrome. In addition, the appearance of a 430 nm band due to the formation of carboxy oxidase is seen (Chance, 1953a). In the current study, the bands were used extensively to provide information concerning the site of inhibition of the drugs in the oxidase. The data obtained from absorbance measurments at the 604-630 nm and 445-465 nm wavelength pairs suggested that cytochrome a is reduced while cytochrome a_3 is oxidized in the presence of the drugs. The carboxy-oxidase spectra validated this finding.

By using the anesthetics or the propranolol, a "reverse" mixed-valence oxidase can be made. Spectra in Figures 10 and 11 demonstrate that cytochrome a is reduced due to the fact the 604 nm peak is present, but the 590 nm and 430 nm peaks are much smaller or not seen at all. That there is a small 430 nm band and a small 590 nm band when the drugs are present needs to be

addressed. First, the drug concentrations used were only at a concentration needed to inhibit the oxidase 70%, therefore, a small amount of the oxidase is not inhibited and $\underline{a}_3$ is able to bind carbon monoxide because it is reduced. A second possibility is that other cytochromes, namely cytochrome b, could be sufficiently perturbed by the drugs as to bind CO and cause a small 430 nm peak to be seen. As can be clearly seen in the figure, the spectrum for cytochrome b is altered to a great extent. The implications of this data are twofold; first, the data demonstrates that the drugs inhibit the flow of electrons between cytochrome a and $\underline{a}_3$, second, it is possible to form a "reverse" mixed-valence oxidase using an inhibitor not currently known to be a ligand of either cytochrome.

Oxidation-reduction titrations were carried out using the band at 604 nm that has been assigned to cytochrome a (Kielin and Hartree, 1939; Wikstrom et al., 1976). The data shows that dibucaine and propranolol do not alter the midpotential of the oxidase to any great degree. However, the Nernst plot in the presence of tetracaine indicates two distint mid potentials, one midpotential is apparent at a value of 270 mV and a second with a midpotential at a value of 365 mV. These numbers correspond well with the potential reported to be that of cytochrome a and $\underline{a}_3$ respectively (Wikstrom et al., 1976). A possible explanation of the split that is

observed in the presence of tetracaine is that the molecule inserts itself into the bilayer (or perhaps into the protein itself) thereby interfering with the heme-heme interaction as described by Leigh, et al., (1974). Separation of the midpotentials could be related to the structure of the tetracaine in that this molecule is the smallest of the drugs studied. The tetracaine might have the ability to insert itself around the heme and cause a steric effect not possible with a larger molecule such as dibucaine or propranolol.

The potentials for cytochrome oxidase are based on the state of the enzyme, that is, energized oxidase using ATP, aerobic oxidase, and anaerobic oxidase have been demonstrated to have different mid potentials. Malmstrom (1973) has demonstrated that heme-heme interaction could cause the midpotentials to shift. This heme-heme interaction is not of the type in which there is a direct dipole-dipole interaction between the iron atoms, but a much more subtle interaction (Wilson and Leigh, 1972; Leigh et al., 1974). It is suggested by Leigh and coworkers (1972, 1974) that both hemes are in electropotential equillibrium with each other and that when one cytochrome is reduced, the potential of the other shifts to a higher potential via a heme-heme interaction. This interaction is postulated to be the reason why two distinct high and low potentials can be resolved using the 604 nm band that is due to 80%

cytochrome a, the low potential heme (Wikstrom et al., 1976; Malmstrom, 1973).

The data obtained for the copper associated with cytochrome a indicates that CuA is not reduced in the presence of the drug dibucaine. This, along with the other data in this study, indicates that dibucaine blocks electron transport between cytochrome a and CuA.

The data that has been collected in this study indicate that these drugs do in fact inhibit cytochrome oxidase respiration in intact mitochondria; moreover, the data suggests that the site of inhibition is between cytochrome a and CuA. Harmon (unpublished data) has shown that carbon monoxide recombination is not effected in the presence of these compounds. Flash photolysis experiments as described previously (Harmon and Sharrock, 1982) have shown that CO recombination is not affected with respect to either the energy of activation or the occupancy of the CO molecules. While these drugs do prevent cytochrome a_3 reduction, once reduced, cytochrome a_3 behaves in a normal manner with respect to its ability to bind carbon monoxide. This data suggests that the drugs do not alter cytochrome a_3.

CHAPTER VI

CONCLUSIONS

This study did not try to elucidate the mechanism of anesthesia and their action as antiarrythmic drugs. The site of inhibition was found to be between cytochrome a and CuA, and all three drugs, tetracaine, dibucaine, and propranolol, have the same apparent site of action within the oxidase differing only in the concentration needed to inhibit respiration.

The ramifications of this research are two fold; one, the sites of inhibition by the drugs were dtermined. Two, the data indicates that the drugs could be a useful tool to study the structure and function of cytochrome oxidase more fully. Three areas of research could benifit by the use of these compounds. The first area is in the study of the midpotentials of cytochrome a and a_3 in the presence of these drugs. This research only looked at the effect of the drugs on the mid-potential as measured at 604-630 nm using the same mediators and the same oxidants and reductants. The potentiometric data indicates that these drugs (especially tetracaine) could be used to further study the interaction of midpotentials between cytochrome a

and cytochrome a_3. Secondly, proton pumping experiments could be very productive in so far as information that could be gained about the mechanism in cytochrome oxidase. The drugs allow cytochrome a to be reduced while CuA is oxidized. The proposal of Wikstrom and Sarri (1977) and Li et al. (1988) (that in order for the oxidase to pump protons cytochrome a and CuA must both be reduced) could be further investigated.

Thirdly, the use of the drugs in the study of oxygen intermediates at low temperatures might also prove interesting. The reverse mixed-valence oxidase that is formed in the presence of the drugs could used to further understand the mechanism of oxygen reduction and the sequence of electron transfer between the centers and to molecular oxygen.

By further experimentation, these drugs, which have the ability to functionally seperate cytochrome a and cytochrome a_3, could prove to be instrumental in our understanding of cytochrome oxidase.

LITERATURE CITED

Adade, A.B., Chignell, D., and Vanderkooi, G. (1984). J. Bioenerg. Biomemb. 16, 353-363.

Adade, A. B., O'Brien, K. L., and Vanderkooi, G. (1987). Biochemistry 26, 7297-7303.

Beinert, H., Hartzell, C. R., VanGelder, B. F., Ganapathy, K., Mason, H. S., and Wharton, D. C. (1970). J. Biol. Chem. 245, 225-229.

Bowyer, J. R., and Trumpower, B. (1981). J. Biol. Chem. 256, 2245-2251.

Bradford, P., and Marinetti, G. V. (1981). J. Membrane Biol. 61, 193-198.

Casanovas, A. M., Nebot, M. F. M., Courriere, P., and Oustrin, J. (1983). Biochem. Pharmacol. 32, 2715-2719.

Casanovas, A. M., Labat, C., Courriere, P., and Oustrin, J. (1985). Biochem. Pharmacol. 34, 3187-3150.

Cerbon, J. (1972). Biochim. Biophys. Acta 290, 51-57.

Chance, B. (1951). Rev. Sci. Instr. 22, 619-624.

Chance, B. (1952a). J. Biol. Chem. 197, 557-566.

Chance, B. (1952b). J. Biol. Chem. 197, 567-576.

Chance, B. (1952c). Nature 169, 215-221.

Chance, B. (1953a). J. Biol. Chem. 202, 383-396.

Chance, B. (1953b). J. Biol. Chem. 202, 397-406.

Chance, B. (1953c). J. Biol. Chem. 202, 407-440.

Chance, B., Harmon, H. J., and Saronio, C. (1975a). Proceedings Tenth FEBS Meeting, pp. 187-194.

Chance, B., and Hollunger, G. (1961a). J. Biol. Chem. 236, 1534--1543.

Chance, B., and Hollunger, G. (1961b). J. Biol. Chem. 236, 1544-1554.

Chance, B., and Hollunger, G. (1961c). J. Biol. Chem. 236, 1555-1561.

Chance, B., and Hollunger, G. (1961d). J. Biol. Chem. 236, 1562-1566.

Chance, B., and Hollunger, G. (1961e). J. Biol. Chem. 236, 1567-1576.

Chance, B., and Hollunger, G. (1961f). J. Biol. Chem. 236, 1577-1586.

Chance, B., Saronio, C., and Leigh, J. S., Jr. (1975b). Proc. Nat. Acad. Sci. 72, 1635-1640.

Chance, B., Saronio, C., and Leigh, J. S., Jr. (1975c). J. Biol. Chem. 250 (24), 9226-9237.

Chance, B., and Williams, G. (1955). Nature 135, 1120-1129.

Chance, B., and Williams, G. (1956). Adv. Enzymol. 17, 65-72.

Chazotte, B., and Vanderkooi, G. (1981). Biochim. Biophys. Acta, 636, 153-161.

Chazotte, B., Vanderkooi, G., and Chignell, D. (1982). Biochim. Biophys. Acta 680, 310-316.

Dutton, P. L., and Wilson, D. F. (1974). Biochim. Biophys. Acta 346, 165-212.

Ferguson-Miller, S., Brautigan, D. L., and Margoliash, E. (1976). J. Biol. Chem. 251, 1104-1115.

Greenwood, C., Wilson, M. T., and Brunori, M. (1974). Biochem. J., 137, 205-215.

Griffiths, D. E., and Wharton, D. C. (1961a). J. Biol. Chem. 236, 1850-1856.

Griffiths, D. E., and Wharton, D. C. (1961b). J. Biol. Chem. 236, 1856-1862.

Grigolova, I. V., and Konstantinov, A. (1977). FEBS Lett. 78, 36-40.

Gutierezz-Merino, C., Molina, A., Escudero, B., Diez, A., and Laynez, J. (1989). Biochemistry 28, 3398-3406.

Hanson, F. B., Miller, M., and Nicholls, P. Biochim. Biophys. Acta 502, 385-399.

Harmon, H. J. (1982). J. Bioenerg. Biomemb. 14, 377-386.

Harmon, H. J. (1988a). Bull. Environ. Contam. Toxicol. 40, 105-109.

Harmon, H. J. (1988b). J. Bionerg. Biomemb. 14, 735-748.

Harmon, H. J., and P. F. Basile (1982). J. Bioenerg. Biomemb. 14, 23-43.

Harmon, H. J. ,and Crane, F. L. (1974a). Biochim. Biophys. Res. Commun. 59. 326-333.

Harmon, H. J., and Crane, F. L. (1974b). Biochim. Biophys. Acta 368, 125-129.

Harmon, H. J., and Crane, F. L. (1976). Biochim. Biophys. Acta 440, 45-48.

Harmon, H. J., and Sharrock, M. (1978). Biochim. Biophys. Acta 503, 56-66.

Harmon, H. J., and Struble, V. G. (1983). Biochem. 22, 4394-4400.

Hatefi, Y., (1985). Ann. Rev. Biochem. 54, 1015-1069.

Herbette, L., Katz, A.M., and Sturtevent, J.M. (1983). Mol. Pharmacol. 24, 259-269.

Hinkle, P. C., Kim, J. J., and Racker, E. (1972). J. Biol. Chem. 246, 1338-1339.

Huunan-Seppala, A., (1972). Acta Scand. Chem. 26, 2713-2733.

Jost, P., Griffith, O. H., Capaldi, R. A., and Vanderkooi, G. (1973). Proc. Nat. Acad. Sci. 70, 480-484.

Keilin, D., and Hartree, E. F. (1939). Proc. Roy. Soc. London, Series B, 127, 167-191.

Komai, H., and Berkoff, H. A. (1979). Biochem. Pharm. 28, 1501-1504.

Krab, K., and Wikstrom, M. (1978). Biochim. Biophys. Acta 504, 200-214.

Krab, K., and Wikstrom, M. (1979). Biochim. Biophys. Acta 548 (1), 1-15.

Ledingham, J. M., and Lees, D. (1981). Br. J. Pharmacol. 74, 635-641.

Leigh, J., and Wilson, W., and Owen, C. (1973). Archives Biochem. Biophys. 150, 154-163.

Lemberg, R., and Mansley, G. E. (1966). Biochim. Biophys. Acta 118, 19-35.

Li, P. M., Morgan, J., Nilsson, M., Ma, M., and Chan, S. (1988). Biochemistry 27, 7538-7546.

Malmstrom, S. (1973). Quart. Rev. Biophys. 6, 389-431.

Mitchell, P. (1961). Nature 191, 144-148.

Nicholls, P., and Chance, B. (1974). pp. 479-534. Academic Press. New York.

Nicholls, P., and Hildebrandt, V. (1978), Biochem. J. 173, 65-72.

Nilsson, T., Copeland, R. A., Smith, P. A., and Chan, S. I. (1988). Amer. Chem. Soc. 27, 8254-8260.

Ohnishi, T., Harmon, H. J., and Waring, A. J. (1985). Biochem. Soc. Trans. 13, 607-611.

Palmer, G., Horgan, D., Tisdale, H., Singer, T. P., and Beinert, H. (1968). J. Biol. Chem. 243, 844-865.

Person, P., and Zipper, H. (1964) Biochim. Biophys. Acta. 92, 607-609.

Richards, C. D., Martin, K., Gregory, S., Keightley C. A., Hesketh, T. R., Smith, G. A., Warren, G. B., and Metcalfe, J. C. Nature, Lond. 276, 775-779.

Scholes, C. P., and Malmstrom, B. G. (1986). FEBS 3504. 198, 125-129.

Seeman, P. (1972). Pharmac. Rev. 24, 583-590.

Segal, I. (1976). Biochemical Calculations, John Wiley & Sons, USA.

Singer, M. A. (1980). Biochem. Pharmacol. 29, 2651-2655.

Singer, M. A. (1982). Biochem. Pharmacol. 31, 527-534.

Singer, M. A. (1983). Biochem. Pharmacol. 32, 1619-1625.

Slater, E. C. (1973). Biochim. Biophys. Acta 301, 129-154.

Sobel, B., Jequier, A., Sjoerdsma, A., and Lovenberg, W. (1966). 19, 1050-1060.

Tanaka, T., and Hidaka, H. (1981). Biochem. Biophys. Res. Commun. 101, 447-453.

Tang, H., and Trumpower, B. L. (1985). J. Biol. Chem. 261, 6209-6215.

Tiesjema, R. H., Muijsers, A. O., and Van Gelder, B. F. (1973). Biochim. Biophys. Acta 305, 19-28.

Trumpower, B. L. (1981). Biochim. Biophys. Acta 639, 129-155.

Tzagoloff, A., and Wharton, D. C. (1965). J. Biol. Chem. 240, 2628-2633.

Tzagoloff, A., (1983). Mitochondria. New York: Plenum.

Vanderkooi, G., and Chazotte, B. (1982). Proc. Natl. Acad. Sci. 79, 3749-3753.

Vanderkooi, G., and Adade, A. B. (1986). Biochemistry 25, 7118-7124.

Wharton, D. C., and Gibson, Q. H. (1964). J. Biol. Chem. 243, 702-706.

Wharton, D. C., and Tzagoloff, A. (1964a). J. Biol. Chem. 239, 2036-2041.

Wharton, D. C. (1964b). Biochim. Biophys. Acta 92, 607-609.

Wikstrom, M. K. F., Harmon, H. J., Ingledew, W. J., and Chance, B. (1976). FEBS Lett. 65, 259-275.

Wikstrom, M. K. F., and Krab, K. (1979). Biochim. Biophys. Acta 549, 177-222.

Wikstrom, M. K. F., and Saari, H. (1977). Biochim. Biophys. Acta 462, 347-361.

Wikstrom, M. K. F., Krab, K., and Saraste, M. (1981). Annu. Rev. Biochem. 50, 623-655.

Wikstrom, M. K. F., Krab, K., and Saraste, M. (1981). Cytochrome Oxidase - A Synthesis, Academic Press, London.

Wilson, D., and Leigh, J. (1974). Archives Biochem. Biophys. 160, 476-486.

Wilson, D. F., Lindsay, J. G., and Brocklehurst, E. S. (1972). Biochim. Biophys. Acta 256, 277-286.

Yonetani, T., (1961). J. Biol. Chem. 236, 1680-1688.

Yu, C.-A., Yu, L., and King, T. E. (1975). J. Biol. Chem. 250, 1383-1392.

Yu, C.-A., Yu, L., and King, T. E. in Developments in Biochemistry (Eds. T. E. King, Y. Orii, B. Chance, and K. Okunuki), p. 219. Elsevier/North Holland, Amsterdam (1979).

VITA

Bradley Kent Stringer

Candidate for the Degree of

Master of Science

Thesis: SITE OF INHIBITION OF CYTOCHROME OXIDASE BY DIBUCAINE, TETRACAINE AND THE CARDIAC ANTIARRYTHMIC PROPRANOLOL

Major Field: Zoology

Biographical:

Personal Data: Born in Sayre, Oklahoma, September 30, 1964, the son of Lynn and Carolyn Stringer.

Education: Graduated from Edmond High School, Edmond, Oklahoma, in May 1983; received Bachelor of Science Degree in Biology from Oklahoma State University in May, 1987; completed requirements for the Master of Science degree at Oklahoma State University in July, 1989.

Professional Experience: Teaching Assistant, Department of Zoology, Oklahoma State University, August, 1987, to May, 1989. Research Assistant, Department of Zoology, Oklahoma State University, May, 1987, to August, 1987, and May, 1988, to August, 1988.