

AD-A198 747

REPORT DOCUMENTATION PAGE
LECTE

2a. SECURITY CLASSIFICATION AUTHORITY		AUG 25 1988		3. DISTRIBUTION/AVAILABILITY OF REPORT	
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE				Approved for public release; distribution unlimited	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)		H		5. MONITORING ORGANIZATION REPORT NUMBER(S)	
				AFOSR-TR- 88 - 0906	
6a. NAME OF PERFORMING ORGANIZATION Oklahoma State University Department of Zoology		6b. OFFICE SYMBOL (If applicable)		7a. NAME OF MONITORING ORGANIZATION Air Force Office of Scientific Research/NL	
6c. ADDRESS (City, State and ZIP Code) 430 LSW Stillwater, OK 74078				7b. ADDRESS (City, State and ZIP Code) Building 410 Bolling AFB, DC 20332-6448	
8a. NAME OF FUNDING/SPONSORING ORGANIZATION AFOSR		8b. OFFICE SYMBOL (If applicable) NL		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER AFOSR-84-0264	
8c. ADDRESS (City, State and ZIP Code) Building 410 Bolling AFB, DC 20332		10. SOURCE OF FUNDING NOS.			
		PROGRAM ELEMENT NO. 61102F		PROJECT NO. 2312	TASK NO. A5
11. TITLE (Include Security Classification) Hydrophobic Molecules with Heme Proteins		Interaction of			
12. PERSONAL AUTHOR(S) H. James Harmon					
13a. TYPE OF REPORT Final		13b. TIME COVERED FROM 8/1/84 TO 4/30/88		14. DATE OF REPORT (Yr., Mo., Day) August 29, 1988	
15. PAGE COUNT 41					
16. SUPPLEMENTARY NOTATION Publications, Meeting Abstracts					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB. GR.	Local anesthetics, antiarrhythmics, myoglobin, cytochrome c, cytochrome oxidase, spectroscopy, electron paramagnetic resonance, protein conformation, ligand binding		
19. ABSTRACT (Continue on reverse if necessary and identify by block number) The effects of hydrophobic local anesthetics, cardiac antiarrhythmics, and environmental pollutants on the physico-chemical characteristics of myoglobin and cytochrome oxidase have been studied by visible light spectroscopy, electron paramagnetic resonance spectroscopy, and low temperature ligand binding experiments. In this manner, structural and functional changes in these proteins/enzymes can be determined. Three molecules in particular, propranolol, dibucaine, and tetracaine are capable of altering the spin state of the iron of myoglobin and cytochrome <u>a₃</u> of cytochrome oxidase. Tetracaine is a powerful denaturant of myoglobin as well. The changes in the proteins are subtle (except for tetracaine-myoglobin). Small conformations around the heme regions of the proteins are all that are necessary and all that are observed to be responsible for changes in kinetic function. A novel breakthrough in being able to observe cytochrome <u>a</u> separately from cytochrome <u>a₃</u> has been found, as has new information concerning the microenvironment of the heme region of cytochrome oxidase.					
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☒ DTIC USERS ☐			21. ABSTRACT SECURITY CLASSIFICATION Unclassified		
22a. NAME OF RESPONSIBLE INDIVIDUAL Maj. J. Jan Cerveny			22b. TELEPHONE NUMBER (Include Area Code) (202) 767-5021		22c. OFFICE SYMBOL NL

AFOSR-TR. 88-0906

FINAL REPORT

1 Aug. 1984 to 30 April 1988

AFOSR-84-0264

INTERACTION OF HYDROPHOBIC MOLECULES WITH HEME PROTEINS

H. James Harmon
Zoology Department
Oklahoma State University
Stillwater, OK 74078

PERSONNEL INVOLVED IN THIS RESEARCH 1984-1988

H. James Harmon, P.I. Professor, Department of Zoology and Department of Physics

Dr. B. Lukas, Post Doctoral Researcher 1985-1986

Jack Stuart, Technician 1984,1985

J. Swartz, Technician 1985

J.M. Kunkel, Technician (part time) 1985,86,87

J. Eng, Technician (part time) 1987

B. Stringer, Graduate Student 1987-present

A. Beach, Graduate Student 1987-present

Accession For	
NTIS GRA&I	☒
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A-1	

PRESENTATIONS AT SCIENTIFIC MEETINGS

- 1985 Interaction of Local Anesthetics with Mitochondrial Cytochromes and Myoglobin. H. James Harmon. American Society of Biological Chemists, FASEB meeting, Anaheim, April 21-26, 1985.
- 1986 Effect of Cardiac Antiarrhythmics and Anesthetics on Cytochrome Oxidase Activity. H.J. Harmon and J. Swartz. American Society of Biological Chemists. June 9-12, 1986, Washington, D.C.
- 1987 Electron Redistribution in Cytochrome Oxidase at Low Temperatures. H.J. Harmon. American Society of Biological Chemists. June 7-11, 1987, Philadelphia.
- 1988 Effect of Propranolol and Local Anesthetics on Myoglobin and Cytochrome Oxidase. H.J. Harmon and B. Lukas. American Society for Biochemistry and Molecular Biology. May 2-6, 1988, Las Vegas.

Published Articles

1. "Effect of Naphthalene on Cytochrome Oxidase Activity" H. James Harmon. Bull. Environ. Contam. Toxicol. 40, 105-109.

Articles In Review At This Time

1. "Dibucaine Inhibition of the Copper A Center of Cytochrome Oxidase" Brad Stringer and H.J. Harmon. Biochemical and Biophysical Research Communications.
2. "Electron Redistribution in Mixed Valence Cytochrome Oxidase Following Photolysis of Carboxy-Oxidase" H.J. Harmon. J. Biomembranes and Bioenergetics.

Articles in Preparation

1. "Effects of Local Anesthetics on Cytochrome c".
2. "Formation of Low Spin Myoglobin By Local Anesthetics and Propranolol".
3. "Inhibition of Cytochrome Oxidase by Local Anesthetics and Propranolol".
4. "Formation of High Spin Cytochrome Oxidase by Local Anesthetics and Propranolol".
5. "Effect of Benzene and Acenaphthene on Cytochrome Oxidase Activity".

Articles Contemplated/Anticipated

1. "Inhibition of Mitochondrial Respiration by Mixtures of Polyaromatic Hydrocarbons".
2. "Characteristics of Antiferromagnetically Coupled Cytochrome a_3 and Copper".
3. CO Recombination to High Spin Cytochrome a_3 ."

INTRODUCTION

— This research project investigated the effects of hydrophobic molecules on the structure and function of proteins. Proteins have hydrophobic interior regions where interaction with hydrophobic molecules may occur. The amino acid residues concerned with the active site and the binding of prosthetic groups are evolutionarily conserved. In general, the hydrophobic residues in proteins are also conserved, although not highly (ie., a hydrophobic residue, perhaps not the same identical residue, is usually present at a certain position in the protein). This evolutionary conservatism indicates the importance of the hydrophobic interior to the maintenance of the three dimensional protein structure and hence its function. Perturbation of the hydrophobic region can be expected to alter structure/function, particularly in heme proteins, where the heme prosthetic groups lies in a hydrophobic pocket or region.

Proteins that are integral components of membranes have an additional hydrophobic region on the surface of the protein where interaction with the membrane lipids occurs. Integral proteins such as cytochrome oxidase, will have a hydrophobic protein interior as well as an exterior surface where 45-50 "boundary" or interfacial lipids are found. In these integral proteins, conformation and enzymatic activity are dependent on lipid; depletion or exchange of lipid by another type result in decreased activity. Thus, perturbation of the hydrophobic surface of integral proteins may also alter structure/function.

Proteins Studied

The structure and activity of three heme proteins were and are still being studied: cytochrome *c*, a monomeric extrinsic protein that serves as a mitochondrial electron transport component; myoglobin, the water-soluble oxygen binding protein of cells and tissues; and cytochrome *c* oxidase, the multi-subunit intrinsic lipid-requiring protein responsible for the utilization of oxygen (and its reduction to water) and involved in the energy transducing reactions associated with chemiosmotic-driven oxidative phosphorylation of ADP to ATP.

Heme proteins were chosen for several reasons:

1. Their biological and physiological importance.
2. Their ease of commercial availability or ease of isolation.
3. The presence of a large and extensive data base concerning their structure and function.
4. Their similarity to other known and important proteins (eg., myoglobin to the beta chain of hemoglobin, cytochrome oxidase to peroxidases and other oxidases).
5. The presence of a heme iron whose physico-chemical properties are extremely sensitive to the conformation of the protein near the heme. As a result, small changes in protein structure can be detected without resorting to X-ray crystallography.

A brief summary of the characteristics of each protein is included in each pertinent section of the results.

Compounds Used

We have studied two types of compounds:

1. Polyaromatic hydrocarbons such as naphthalene, acenaphthene or benzene that may be frequently encountered in the workplace in fuels, solvents, lubricants, fluids, etc and that have economic industrial importance and are found as environmental pollutants.
2. Local anesthetics and cardiac antiarrhythmic drugs of extreme medical importance used in treatment of cardiac ailments as well as in injury, trauma, and anesthesia.

Much of our research has centered on the latter group since compounds with widely varying hydrophobicities can be used with relatively little change or difference in molecular structure or molecular weight. The anesthetics are chemically similar. These similar compounds are useful in determining the role of hydrophobicity as opposed to the role of molecular structure in effects on protein.

Goals

The goals and aims of the original proposal are stated below. The research with cytochrome c was not heavily pursued since we could obtain only limited results with only one compound tested. Research on cytochrome oxidase was intensified to attempt to more clearly define the molecular basis and mechanism of action of the drugs involved. In addition, the findings have opened a new and unique avenue to study the basic mechanism and characteristics of the oxidase in the absence of drugs; the drugs are a useful tool to study and understand the basic biophysics of the oxidase as well as interaction with hydrophobic molecules. In general, work on the oxidase and myoglobin was pursued because of the possibility of application of the data to drug toxicity reactions as well as mechanism of drug action on physiologically important proteins.

GOALS/AIMS

- A. Study the interaction of hydrophobic compounds with proteins to determine
 1. location of sensitive hydrophobic regions in well-characterized biologically-important proteins
 1. Cytochrome c
 2. Myoglobin
 3. Cytochrome oxidase
 2. the size of the hydrophobic region by using hydrophobic perturbants of different size and molecular weight.
- B. Determine the effect of perturbants on the physico-chemical properties of heme proteins
 1. determine if all regions of the protein equally affect the protein characteristics
 2. determine if the conformational changes can be detected without x-ray crystallographic studies.
 3. determine the effects of perturbants on light absorbance wavelength maxima, EPR resonances, proton NMR resonances, and molar absorptivity of light.
- C. Determine the effects of hydrophobic perturbants on the function of heme proteins:
 1. midpotentials of cytochromes c and oxidase and myoglobin
 2. O₂ and CO binding to cytochrome oxidase and myoglobin
 - a. rate of CO and O₂ binding
 - b. energy of activation
 - c. enthalpy of binding
 - d. entropy of binding
 - e. number of occupancy regions and their capacity
 3. K_m of cytochrome oxidase for cytochrome c
 4. It is possible to measure the formation of oxygen intermediate compounds of cytochrome oxidase to measure alteration, if any, of oxidase function

D. Attempt to correlate the hydrophobicity and molecular size of the perturbant to its protein—altering effectiveness

1. determine a hydrophobicity vs. reactivity series
2. determine structure—perturbation correlates to determine the portion of the perturbant responsible for the effects.

RESULTS

Cytochrome Oxidase

Characteristics of the oxidase

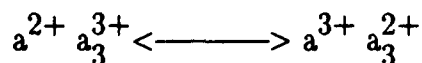
Cytochrome c oxidase is a multi—subunit integral protein of the inner mitochondrial membrane containing two heme proteins and two copper atoms bound to protein via a sulfur bridge (1–3). The four redox centers have markedly different visible light and EPR spectral properties. While cytochromes have three visible light absorbance bands, only the alpha and the gamma (Soret) bands are of interest/utility in the identification of these two cytochromes. In the oxidized form, cytochromes a and a₃ have absorbance peaks at 426

and 412 nm, respectively in the Soret region and slight absorbances in the alpha region at 601 and 604 nm, respectively. In the ferrous form, cytochromes a and a₃ absorb equally strong at 447/442 and 444 nm, respectively (4,5). Differential identification of the two cytochromes is usually made at the alpha band, where at least 80% of the absorbance at 604 nm is due to cytochrome a, the remainder due to ferrous a₃ (4,6).

The two copper centers are each associated with a cytochrome. Cupric CuA, associated with cyt a, is detectable as a shallow trough in reduced minus oxidized spectra centered at 840 nm (7–10); CuA is also responsible for the g=2 EPR signal. The copper associated with cyt a₃, CuB, is not readily detectable in the near-IR and is not readily detected by EPR; it is frequently referred to as the "invisible copper", although its presence can be detected at 720–740 nm in certain intermediate states of the oxidase (3,11).

The basis of the "invisibility" of CuB and its associated cyt a₃ is in the antiferromagnetic coupling (12–14) of these two centers where the S=1/2 of the copper and S=5/2 spin of cyt a₃ give a combined spin of 6/2, which is undetectable by EPR and poorly detected by visible spectroscopy (hence only 20% of the alpha band absorbance). Uncoupling the two centers by mixed valence partial reduction of the oxidase or by addition of a ligand such as sodium azide allows cyt a₃ to be detected as a high spin signal at g=6 (6,15,19). In addition, the absorbance in the Soret region is altered, with the ferric high spin form absorbing at 421 nm (16,20,21) with a second change at 432 nm (22,23).

The midpotentials of the four oxidase centers are very similar in the unliganded state. Numerous investigators have reported the mid potentials of cyt a, CuA, CuB, and cyt a₃ at approximately +220 mV (6,24,25,26). Carbon monoxide shifts the E_m of the ferrous cyt a₃ to +580 mV. In the absence of a ligand, reduction of one of the cytochromes results in an increase of approximately 140 mV in the other. As a result, the single electron is transferred between the two centers; the value of Keq for this transfer between the centers (and the steady—state ratio of the two mixed valence forms



is approximately one. Thus, reduction of one center induces a change in the midpotential of the other; this is a heme—heme interaction. The neo—classical model and subsequent

publications indicate that cyt a is responsible for 80% of the 604 nm absorbance and that ligation of CO merely perturbs the equilibrium between the two centers (6,26,27).

Different Forms of Cytochrome Oxidase

Isolated oxidase exists in several forms. The "resting" or fully oxidized state of the enzyme is commonly isolated and is unperturbed by electron reductants or ligands. This form of the enzyme has $g=3$ Fe and $g=2$ Cu EPR signals and the visible wavelength maxima described above.

Okunuki and co-workers in the late 1950's (28,29) described a different form of oxidase formed by aeration/oxygenation of dithionite-reduced oxidase; this "oxygenated" oxidase exhibits a red-shifted ferric Soret at 428 nm and an increased extinction coefficient at 600nm compared to "resting" oxidase. The oxygenated form can be reduced by four electrons (30,31) and has EPR and MCD spectra (32,33) identical to those of the "resting" enzyme, although differences in CD spectra suggest a conformational change (32). The "oxygenated" enzyme is more easily reduced by dithionite (34) and reacts with HCN (35,36) and azide faster than does the resting oxidase. The oxygenated form reverts to the resting form over time in the presence of cyt c (36).

"Pulsed" oxidase, where the oxidase is allowed to go through at least one catalytic reduction/reoxidation cycle, was once thought to be identical to the original "oxygenated" species but is formed by pulsing reduced oxidase with oxygen (1 mole of O_2 reacts per aa_3) in the presence of cytochrome c (32,38). The pulsed oxidase accepts 4 electrons, but is not identical to the "oxygenated" form". It is likely that the pulsed form is a partially-reduced form with oxygen bound to a_3 . Shaw (39) and co-workers describe

another "oxygenated" type of enzyme derived from reduction of the oxidase by asc+TMPD, dithionite, cyt c , or NADH +PMS followed by oxygenation in the presence of cyt c . This unusual form of the oxidase yielded a ferric high spin a_3 with $g=6$ (axial), 5,

1.78, and 1.69 signals. This is not the same species as the Okunuki "oxygenated" enzyme which is more similar (especially EPR-wise) to the resting enzyme. The pulsed oxidase is kinetically more active with a faster reactivity toward ligands. The Soret absorbance is at 428 nm and EXAFS indicates the lack of a sulfur bridge between CUB and cyt a_3 as in the classical oxygenated form (40). This enzyme also has the 655 nm absorbance band which is attributed to high spin ferric a_3 . The new transient absorbances (particularly $g=5$) are believed due to an iron with spin 5/2 interacting with some other center, but the presence of the axial $g=6$ has not been explained. Wikstrom has provided an excellent review of the oxidase forms (41).

Some workers have suggested that the "pulsed" form is actually a species functional in the normal catalytic cycle (3,27,42). In addition, a ferryl form of the cyt a_3 -Fe is also believed to function in the reduction of oxygen (3,43).

Chance and co-workers (70) describe a different form of pulsed oxidase formed by reduction and reoxidation in the absence of hydrogen peroxide; this is the "420" form that has its Soret at 420 nm like the resting enzyme and shows the enhancement of catalytic activity of the pulsed enzyme. Addition of H_2O_2 to the 420 form results in the appearance of 428 nm Soret, suggesting that the 428nm "pulsed" oxidase is a peroxy intermediate of the fully oxidized resting oxidase (3). They suggest that the Orii and Okunuki form be renamed the "peroxidatic form" of the oxidase.

It is likely that the resting enzyme is in a conformationally "closed" form reflected by its lack of reactivity with oxygen or other ligands. In contrast, the "pulsed" or peroxidatic forms are more "open" and reactive with ligands. This explanation seems to be substantiated by EXAFS findings (3).

Cytochrome Oxidase Oxygen Intermediates

The forms of oxidase just described are stable forms. In the mid 1970's Chance and co-workers trapped and described the characteristics of 3 oxygen intermediates (44-47): the ferrous "oxy" Cmpd A (analogous to the ferrous carboxy form); the partially reduced peroxy form, Cmpd B, where at least 2 electrons are transferred to molecular oxygen; and the mixed valence oxy Cmpd C, derived by internal electron transfer within a mixed valence oxidase following oxygen binding. Subsequent reports define subsets of compound B where the appearance of CuB at 720nm is observed (3,11), apparently uncoupled from cyt a_3 . It is likely that several forms of compound B are to be found due to electron

transfer among the equipotential cyt a , CuA, and CuB centers (44-53). The reader is referred to a recent review for a more detailed treatment of oxygen intermediates (3).

Neither the pulsed/oxygenated oxidase nor the kinetic oxy-compounds of Chance exhibit high spin signals associated with a mixed valence or partially reduced form of the oxidase. Jensen and co-workers (36) have reported the appearance of $g=6$ signals during the oxidation of the enzyme by oxidation in the presence of cyt c (this was unaffected by ligands such as CN^- , however). The appearance of axial vs. rhombic $g=6$ signals has not been adequately explained or understood, although axial $g=6$ is frequently passed off as due to a contaminant minor species (always undefined).

Several investigators (43,6) have substantiated Chance's (44,45) suggestion that a ferryl form of cyt a_3 is involved in the reduction of oxygen. This appears to have been shown in EXAFS studies and also by comparison to cyt c peroxidase ferryl intermediates. A CO-reactive ferryl dioxygen intermediate with an alpha band at 580 nm has been studied; this form oxidizes CO to CO_2 and produces rhombic CuB signal adjacent to a ferrous low-spin CO or oxygen adduct (43).

Inhibition By Local Anesthetics and Antiarrhythmics

Inhibition of cytochrome oxidase activity by local anesthetics such as dibucaine or tetracaine has been described by others (54,55). Singer (56) and Casanovas et al (55) report that dibucaine can interact electrostatically with the oxidase, possibly competing for the charged active site for cyt c and altering the apparent K_m . Because of a strong correlation between the concentration dependence of the inhibition and the octanol/water partition coefficient of the drugs, Singer (56) and Casanovas et al (55) suggest a hydrophobic interaction as well, most likely with the lipid associated with the oxidase. Chazotte and Vanderkooi (57) demonstrated that the concentration of dibucaine needed to inhibit oxidase activity was inversely proportional to temperature, further suggesting a hydrophobic interaction, but they could not determine if the drugs interacted directly with the protein or the "boundary" interface lipid (58). Despite these studies on the mechanism and type of inhibition, the site and mode of action of these drugs has not been identified. Data presented in the next section describes the site and possible mode of action of the local anesthetics and antiarrhythmics in cytochrome oxidase.

Effects of Hydrophobic Molecules

There are at least 2 separate effects of hydrophobics on cytochrome oxidase. One effect involves cytochrome a and its copper and occurs at low concentrations of drug/compound while the other involves cytochrome a_3 and its copper and requires far greater perturbant concentrations.

The effects of hydrophobic local anesthetics and cardiac antiarrhythmics have been studied on lipid-depleted cytochrome oxidase isolated by the procedure of Yu, Yu, and King (59) and intact beef heart mitochondria (60). Spectra were recorded at room temperature and at $-160^\circ C$ using a Johnson Research Foundation DBS-3 scanning dual wavelength spectrophotometer. EPR spectra were recorded using a Bruker ER 2000 series

spectrometer at 7–12 K. All samples were suspended in 0.25 M sucrose–50 mM sodium phosphate buffer (pH 7.4) unless noted otherwise.

The concentration dependence of the inhibition of mitochondrial succinate oxidase activity by local anesthetics and antiarrhythmics is shown in Figure 1. The more water soluble compounds such as procaine or lidocaine are not effective inhibitors until they attain a concentration in excess of 100 mM while compounds such as dibucaine, propranolol, and tetracaine are effective inhibitors at 2 mM or less concentration. The concentration needed to achieve 50% inhibition is proportional to the log of the partition coefficient, as shown in Figure 2. In general, the more hydrophobic the molecule, the more effective an inhibitor and anesthetic it is. The effect of increasing concentrations of dibucaine and propranolol on ascorbate+TMPD–driven cytochrome oxidase activity is shown in Figure 3; the concentration of these drugs needed to inhibit cytochrome oxidase activity is similar to that needed to inhibit succinate oxidase activity.

The effects of three drugs, dibucaine, tetracaine, and propranolol (to be referred to as DTP for convenience) on cytochrome oxidase will be described because other drugs investigated (lidocaine, verapamil, procaine, procainamide, etc) are without additional effect. The effects of these three drugs are identical except for the concentration at which these effects are observed. As a result, the qualitative effects of the drugs will be described using the best data obtained with any of these 3 drugs. They should be considered interchangeable except for the effective concentration needed to elicit the effect.

Addition of DTP to isolated ferric (resting state, fully oxidized) oxidase causes a shift in the Soret band spectrum as shown in Figure 4 where propranolol intensifies and sharpens the absorbance at 426 nm (liq nitrogen temperature). The spectrum in the absence of propranolol shows a broad Soret band centered around 422 nm. Kornblatt and Hui Bon Hoa (61) ascribe such a spectrum to a mixture of high and low spin forms at pH 7.4 with the 426–428 peak being low spin and the shoulder at 419 nm due to high spin oxidase. The EPR spectrum of the non–treated control oxidase (cf. Fig 5) shows that the resting ferric oxidase is all low spin with a prominent $g=3$ signal; a small amount of $g=4.3$ heme due to denatured cytochrome is present and constant in all EPR spectra. The identification of the 428 nm (room temp) peak as low spin (61) is not correct. The addition of propranolol in excess of 10 mM causes the appearance of a high spin $g=6$ signal (cf. Fig. 5) (in addition to the low spin $g=3$ signal) occurring at the same time as the 428 nm band. The 428 nm species is high spin.

Addition of DTP to fully dithionite–reduced ferrous oxidase (cf. Fig 6) results in a 3–4 nm shift in the Soret to approximately 440 nm and the appearance of a prominent 426 nm shoulder due to an altered form of ferrous oxidase.

Addition of the drugs to ferrous oxidase in the presence of CO results in drastic changes in the Soret band spectrum. In the presence of DTP, a sharp absorbance centered at 418 nm appears at the expense of the 427 nm a_3^{2+} CO signal as seen in Figure 7 (in the control spectrum, the presence of the 445 nm peak of ferrous cyt a and the 430 nm peak of ferrous a_3 –CO appear as a broad band at 438 nm. This indicates that the DTP drugs are altering the absorbance characteristics of cytochrome a_3 and not cytochrome a . If cyt a were affected, the absorbance at 445 nm would change in intensity and/or position, but it does not. Presence of the drug alters the intensity of the 430 nm band; the apparent change in the position of the absorbance is due to the presence of a mixture of the 418 and 430 nm forms (61,53). At drug concentrations of 25 mM DTP and higher, however, the only absorbance observed is that at 418 nm; the 427 nm band is not observed (cf. Fig. 8). Analysis of this data allows us to accurately describe the composition of the oxidase in these states. DTP induces a high spin ferric form of the oxidase with an absorbance maximum at 426 nm. Gilmour et al., (5) identified 426 and 412 nm absorbances due to ferric cyt a and a_3 , respectively; the presence of both ferricytochromes gives a broad peak

centered at approximately 420 nm, as we observe in Figure 4. The high spin form of cyt a_3 induced by DTP will absorb at 428 nm and the low spin form of cyt a will absorb at 426 nm (61), thus accounting for the sharpened and intensified absorbance at 426 nm in Figure 4.

Reduction of the ferric high spin a_3 induces the appearance of a 426 nm absorbance that could be due to ferrous high spin a_3 or to non-reduced (and non-reducible) high spin heme. In the control, cyt a and a_3 absorb at 442 and 444 nm respectively (4,5); in the presence of DTP, cyt a will still absorb at 442 nm while a new band, presumably ferrous high spin a_3 , appears at 426 nm. That the 426 nm band is NOT oxidized cytochrome is shown in Figure 7 in the presence of CO. If the 426 nm band were due to non-reduced ferric heme, then the 426 nm band would still be present in the carboxy-reduced spectrum; it is not. This indicates that the 426 nm ferrous band is due to ferrous high spin a_3 ; addition of CO to this form results in a high-spin carboxy form that absorbs at 418 nm; any carboxy-low spin form of cyt a_3 will absorb at 428 nm and accounts for the presence of both 418 and 428 nm absorbances at low (less than 10 mM propranolol or 25 mM dibucaine) drug concentrations (as in Fig. 7).

Thus far, the evidence is quite suggestive that DTP affects only cyt a_3 and not cyt a and that it converts cyt a_3 to a high spin cytochrome.

DTP-treated ferrous oxidase shows a slight decrease in intensity of the 604 nm band, to be expected if cyt a_3 , which contributes 20% of the 604nm absorbance, is converted to high spin. In the presence of CO, the typical 590 nm ferrous a_3 -CO band is not seen and a 575 nm high spin ferrous absorbance is observed. These spectral changes are somewhat similar to those observed by Witt et al., (43) where a ferryl dioxygen intermediate with a 580 nm band was formed; addition of CO shifted the band to 605 nm. While they observed changes in the $g=2$ region, they also observed axial as well as rhombic high spins (while we see only axial signals), indicating that DTP-oxidase may not contain a ferryl iron.

ELECTRON PARAMAGNETIC RESONANCE

Cytochrome oxidase exhibits microwave absorbance only in the oxidized state. Normal untreated oxidase exhibits a pronounced $g=3$ low spin state with accompanying low spin signals at $g=2.2$ and 1.5. The detectable copper has an absorbance at $g=2$ as seen in Figure 5. Our findings thus far have centered only on the heme EPR resonances; we propose to study copper in detail in the future. As shown previously, addition of DTP induces the formation of a high spin signal at $g=6$ and a new resonance at $g=2.8$ or so.

High spin oxidase has been observed before (9-18) and is commonly induced by flash photolysis of mixed valence carboxyoxidase where cyt a and CuA are oxidized and the CuB and cytochrome a_3 are still reduced. The resulting spectrum is shown in Figure 9 and consists of a low spin $g=3$ signal and a high spin cluster of resonances centered around $g=6$ but actually consist of 4 resonances, two that are referred to as "axial" and 2 as "rhombic". Wikstrom et al., (6) and others have suggested that the presence of the axial and rhombic signals is due to two different environments of high spin cytochrome in the mixed valence state (one environment being $a^{2+}a_3^{3+}$ and the other $a^{3+}a_3^{2+}$). The observance of only axial high spin or only rhombic high spin is not common, but has been observed previously in early stages of partial reduction of the oxidase (16,36).

Earlier investigators have observed the appearance of the $g=6$ signal AND a decrease in intensity of the $g=3$ signal. In the presence of DTP, decreases in the intensity of the $g=3$, 2.2, and 1.5 signals are not observed at any concentration of drug, but the intensity of the axial high spin signal increases with increased drug concentration (Figure 10). The intensity of the high spin signal is maximal at those concentrations where the presence of the 418 nm carboxy-ferrous signal and not the 430 nm low spin a_3^{2+} CO absorbance is observed. In other words, the formation of high spin ferric oxidase occurs at the same concentration that the 418 carboxy-form, the 426 nm ferrous form, and the 428 ferric form are seen. This is the basis for our suggestion that the 428 nm ferric is ferric high spin. Reduction of the 428 nm ferric high spin would result in the 426 nm ferrous form; addition of CO to the ferrous high spin results in the 418 nm band which we suggest is carboxy ferrous high spin.

The correlation of the appearance of the $g=6$ signal, the constancy of the $g=3$ and low spin signals, and the identification of the Soret and alpha bands allows us to suggest that DTP induces high spin cyt a_3 but does not affect (low spin) cyt a . Further, the axial high spin signal is due to ferric high spin cyt a_3 in the presence of ferric low spin cyt a ; a mixed valence state where one of the two cytochromes is reduced is not needed.

The identity of the new resonance at $g=2.8$ has not yet been established and warrants further investigation.

Few reports of a purely axial high spin signal are found in the literature. Karlsson et al (62) demonstrated axial $g=6$ signals in partially reduced oxidase in phosphatidylcholine vesicles. Powers and co-workers (2) observed axial $g=6$ in oxidized resting Cu-depleted oxidase where EXAFS had shown the absence of the Fe-S-Cu bridge. Jensen et al., (36) observed an axial $g=6$ (and a decreased $g=3$ intensity compared to fully oxidized enzyme) when either resting oxidase is mixed and rapidly frozen in the presence of cyt c^{2+} or when pulsed oxidase is mixed with cyt c^{2+} and oxygen in the presence or absence of CN $^-$. These axial $g=6$ signals are similar to those observed in the presence of drugs but we do not observe a diminution of $g=3$ intensity. Beinert and Shaw (63) observed an axial $g=6$ following aerobic reduction by dithionite (as in our samples) but without the $g=3$ signal.

SITE OF ACTION OF DTP

From the foregoing discussion it is clear that the drugs do not affect cytochrome a . It is clear that DTP acts at a site(s) in the oxidase to inhibit activity and to induce a high spin signal. Likely candidates for such a site are the copper centers based on the following preliminary evidence.

As shown in Figure 11, addition of 1.5 mM (concentration at which 70% inhibition of activity occurs) prevents the formation of approximately 50% of the 445 nm absorbance signal (relative to the dithionite-reduced absorbance) at room temperature when ascorbate+TMPD and cyt are used as reductants. On the other hand, ascorbate+TMPD and cyt c are capable of eliciting about 80% of maximal formation of the 604 nm absorbance as seen in Figure 12, indicating that all of cyt a (80% of 604 and 50% of 445 nm absorbance; ref 6) is reduced but that none of the cyt a_3 is reduced. This indicates that the site of inhibition is between the two hemes and likely to be a copper. Further, addition of oxygen to the ascorbate+TMPD and cyt c -reduced enzyme does not cause a reduction in either 445 or 604 nm absorbance in the presence of dibucaine (the DTP drug used here); addition of ferricyanide abolishes the 445 and 604 nm absorbances (data not shown).

Ferric cyt a_3 exhibits a weak absorbance at 655 nm that disappears upon reduction. If DTP acts between cyts a and a_3 (at one or more coppers) then we expect to see no

change in the 655 nm absorbance in the presence of drugs. This is what we observed previously. Control oxidase exhibits a decrease in absorbance of the 655 minus 630 nm wavelength pair upon reduction; in the presence of dibucaine, the absorbance decrease due to the loss of 655 nm intensity on reduction of a_3 (630 is near the 626 nm isobestic point) is not observed, (data not shown) confirming the earlier findings at 445nm (50% reduced) and 604nm (80% reduced) that the reduction of a_3 but not a is prevented. In the presence of DTP, electrons from ascorbate+ TMPD cannot reach cyt a_3 (although in the presence of DTP, a_3 can be reduced by dithionite in accord with findings at 445nm).

In the absence of drug, ascorbate +TMPD and cyt c are capable of reducing the IR-detectable CuA center as indicated by the wide trough in the near-IR region of a reduced minus oxidized difference spectrum as shown in Figure 13; note the reduction of cyt a as seen by the 604 nm absorbance. In the presence of 1.5 mM dibucaine, however, the trough in the IR region is not observed when ascorbate + TMPD and cyt c are used as reductant (Fig 13) but is observed if the dibucaine-treated sample is subsequently reduced with dithionite. The drug prevents the reduction of CuA by cyt c and a but not by a strong non-physiological reductant. Dibucaine seems to apparently act between the CuA and cyt a .

A second site of action of DTP is likely the undetectable CuB. The appearance of high spin cyt a_3 can only occur if the antiferromagnetic coupling between cyt a_3 and the CuB center is broken as in the case of reduction of the CuB; in this case the $S=5/2$ heme is visible as $g=6$. We suspect that since CuA is altered by DTP, the CuB may similarly be affected. Extensive investigation of the $g=2$ region of the EPR spectrum is needed to further verify that CuA ($g=2$) is affected and that the antiferromagnetic coupling between CuB and cyt a_3 is broken. We expect that if the centers are uncoupled, CuB should be detectable in the $g=2-2.3$ region as reported by others (43,27,36,64), assuming that DTP does not reduce the copper and render it undetectable ($S=0$). We do not believe reduction of CuB occurs since electron transfer to the equipotential cytochrome a_3 is not detected; further, if the CuA center were reduced by DTP we would not detect it in a reduced minus oxidized difference spectrum (it would already be reduced in the "oxidized" spectrum) and we would not be able to induce the trough by addition of dithionite. The center would also appear upon oxidation with ferricyanide if it were already reduced; this is not observed. Thus, all available evidence supports the notion that DTP does not reduce the coppers and that it prevents CuB reduction.

From the foregoing discussion it now seems clear that the site of action of the drugs is at the coppers such that electron transfer from cyt a to CuA to cyt a_3 is prevented and that the antiferromagnetic coupling between CuB and cyt a_3 is broken. Inducement of this condition by a drug or hydrophobic compound has not been observed before and opens totally new avenues of research approaches to understanding the operation of the oxidase!

REBINDING OF CO FOLLOWING FLASH PHOTOLYSIS

The rebinding of CO to reduced cyt a_3 following flash photolysis follows pseudo-first order single exponential reaction kinetics as described by the applicant and others (6,47,65,66). The binding kinetics are monitored in the Soret band to take advantage of the large extinction coefficients and to optimize signal/noise ratio. The kinetics are measured as a decrease in 445 nm absorbance as ferrous cyt a_3 binds CO and absorbs at 430 nm.

As shown in Figures 14 and 15, the presence of drugs, including DTP at concentrations where we know we have extensive conversion to high spin and where significant quantities of the 418nm high spin ferrous a_3 -CO are present, does not alter the rate or the energy of activation of CO binding as measured at 445 nm. The explanation for this data is quite simple. By measuring the kinetics at 445 nm, we have been measuring the rate of rebinding of CO to ferrous low spin (normal) cyt a_3 . The non-CO bound ferrous high spin cyt a_3 absorbs at 426nm! To measure the binding parameters of the high spin form, we need to measure the kinetics as a disappearance of the 426 nm signal or the appearance of the 418 nm absorbance following flash photolysis.

As shown previously in this section, optical spectra indicate complete conversion to the high spin cyt a_3 above 10 mM drug concentration. We would expect to see a marked decrease in the extent of the 445 nm change with increasing drug concentration; that is exactly what we find. At high concentrations of the drug, changes in 445 nm are not observed following flash photolysis since all ferrous high spin a_3 absorbs at 426nm, not 445 nm. At all concentrations where a fraction of cyt a_3 is still low spin and measurable at 445nm, the energy of activation and rebinding rates are not altered, although the intensity of the post-flash absorbance change decreases with increasing drug concentration. This again suggests that the drug does not alter cytochrome a_3 , other than to convert it to high spin by altering CuB.

If the post-flash kinetics are monitored at 420 nm in the presence of more than 10 mM dibucaine, for example, CO rebinding can be measured, indicating that the 418 nm band is a photolyzable CO-ligated form of cytochrome. We have not yet determined the rate constants at different temperatures for the binding of CO to high spin ferrous cyt a_3 , but preliminary data indicates that rebinding is significantly faster than in the low spin form; in this aspect, the 418 nm CO compound resembles hemoglobin and myoglobin in wavelength and rate of ligand binding.

That the CO binding is faster suggests to us that the binding of molecular oxygen would also be faster as well. We propose to measure the rate and energy of activation of the formation of the "oxy-intermediate" Compound A (44-45,67) in which molecular oxygen binds in place of CO following flash photolysis. Dr. Harmon has considerable experience in measuring the kinetics of the oxygen intermediate compounds of cytochrome oxidase at low temperatures (6,47,67).

RELATION TO OTHER OXIDASE SPECIES

The similarity of the drug-induced forms to the pulsed and oxygenated forms has been shown. The appearance of the 580 nm alpha band in the reduced form is similar to that found in the 3 electron ferryl dioxygen intermediate, but in that form, rhombic as well as axial $g=6$ signals were observed. The ferryl form has a Soret absorbance at 428 nm, also. It is curious that the DTP-induced form has some characteristics of the pulsed, oxygenated, Compound B, and ferryl iron forms. It may be that the DTP-induced forms are more stable than the other forms mentioned and that the other forms represent mixtures or readily interconvertible spin/reduction states. The basis for the similarities and differences is not known at this time and definitely warrants further investigation.

That the 580/428 nm bands we see with DTP are not likely due to ferryl cyt a_3 is seen by the fact that samples that are either 50 or 75% reduced show a ferryl heme and show both axial and rhombic high spin iron signals but not low spin $g=3$ signals (43). Witt et al (43) propose that ferryl a_3 oxidizes CO to yield CO_2 , in accord with the oxidation of

CO observed by Caughey and co-workers (68,69). CO oxidation results in a low spin CuB at $g=2.3$ and a 437 nm absorbance which we do not observe with DTP. Because of these differences, we feel the DTP-induced form is likely NOT a ferryl iron.

PROBLEMS

A potentially serious problem is that some aspects of the data can be clouded by the presence of cyt b or Hb/Mb in the preparation. Cyt b is frequently co-isolated with purified oxidase while Hb/Mb is isolated with mitochondria. The presence of a 560 or 580 nm band could be due to cyt b or Hb/Mb. Binding CO to ferrous isolated (denatured) cyt b would result in a decrease/loss of 560 nm absorbance; binding to Hb/MB would mean a loss of 580 and an increase in 419 nm! Presence of cyt b could explain the 560 nm absorbance in isolated DTP-treated oxidase and the loss of that absorbance when CO is added. The presence of 5% cyt b or Hb/Mb (calculated contamination levels) does not explain the loss in 445 nm absorbance in ferrous, the presence of the 418 nm and not the 430 band in ferrous-CO, the loss of the 590 ferrous-CO alpha band, the appearance of the 426 ferrous band, or the appearance of the 428 nm ferric band in treated enzyme.

The fact that treated, pulsed, oxygenated, and compound B oxidase forms have Soret absorbances similar to each other and cyt b is unfortunate. Cyt b contamination can be minimized by isolating the purest oxidase possible. The use of ascorbate + TMPD (+80 and +225 mV, respectively) as reductant instead of -400 mV dithionite will allow reduction of the oxidase but not cyt b (+/- 30 mV; ref 24).

Attributing the 560 nm band to the beta band of the oxidase is questionable since the intensity of this band relative to the alpha band is very variable and the intensity of the 560 band can be substantially decreased by retreatment of the oxidase with ammonium sulfate (unpublished data); this procedure solubilizes oxidase and leaves a red cyt b pellet following centrifugation.

The presence of deoxy Hb/Mb in the mito prep can alter the shape and intensity of the 840 CuA band since they also absorb in this region (860 nm). The presence of Hb/Mb can be checked by looking for the decrease in 430 minus 465 nm absorbance in the presence of ascorbate; a decrease in signal corresponds to the binding of oxygen to the protein, which would also decrease 860 nm absorbance. In our preps where the mito are KCl-washed, Hb/Mb contamination (measured in the Soret) is less than 5% of the cytochrome content of the preparation. We are confident that Hb or Mb are not involved in our results based on the effects of DTP on Mb (and Hb by analogy). DTP induces the conversion of high spin $g=6$ Mb to $g=2.9$ low spin with shifts in Soret spectra. In addition, DTP induces the loss of heme from oxyMb. As a result, the data we have described are due to oxidase, not Hb/Mb contaminants.

Chance and co-workers have proposed a mechanism of oxygen reduction in which cytochrome a_3 assumes a structure and properties similar to those of hemoglobin (3). The similarities between our drug-treated cyt a_3 and Hb/Mb have not been ignored. Treated oxidase becomes high spin with an axial $g=6$ signal like Hb/Mb. The ferrous treated oxidase assumes a 428 nm Soret and a 580 nm alpha band, again like Hb/Mb. In the presence of CO, the treated oxidase has a 418 nm band compared to 419 nm for CO-Hb. In general, treated cyt a_3 resembles a protoporphyrin IX heme protein!

While the optical properties of ferryl heme are similar to those of Hb/Mb, the EPR properties are not. Axial as well as rhombic $g=6$ signals are seen with ferryl iron while only axial signals are seen in treated oxidase or Hb/Mb. This leads us to suggest that the treated oxidase may be more like myoglobin than a ferryl heme.

Final comment: Effects of hydrophobic molecules are often attributed to non-specific "detergent" effects of the compound. This is not the case here; the effects are very specific

and not mimicked by addition of SDS, Tween, or deoxycholate detergents. Further, the effects are reversible; control characteristics return following dialysis of DTP-treated enzyme.

Classical kinetics

Table 1 summarizes the effects of 8 compounds on cytochrome oxidase activity. Acenaphthene and procainamide do not alter K_m or V_{max} values; naphthalene, lidocaine, and verapamil effectively double these parameters. Benzene lowers these values while tetracaine and procaine cause a 5-fold increase in K_m . We can classify the inhibitors as follows: procainamide and acenaphthene are without apparent effect; tetracaine and procaine are competitive inhibitors; naphthalene, lidocaine, benzene, and verapamil are mixed inhibitors.

Location of Inhibitory Sites in Cytochrome Oxidase

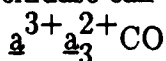
Minimally, one can envision at least 5 separate steps in electron transport in cytochrome oxidase:

1. transfer from cyt c to cytochrome a (or copper).
2. transfer between cyt a and its copper (EPR and visible light detectable).
3. transfer from copper to copper.
4. transfer from copper to cytochrome a_3 .
5. transfer from cyt a_3 to oxygen.

Measurement of the reduction of oxygen (oxygen consumption) or the overall rate of cyt c or cyt a oxidation provides a measurement of the total enzyme function. If a molecule such as naphthalene increases the value of V_{max} , where does it act? What electron transfer step is affected? We know that the binding of cytochrome c is affected since K_m changes. We know that the rate of CO (and thus probably oxygen) binding is unaltered. This suggests that an electron transfer step(s) is altered by naphthalene or other drugs/compounds.

In a previous report we stated that naphthalene caused an increase in the rate of CO recombination. This has been shown subsequently to be untrue. Naphthalene does not alter CO binding. What we measured initially was intra-oxidase electron transport, i.e. electron transfer between the redox centers in the oxidase. We undoubtedly measured faster kinetics using partially reduced cytochrome oxidase.

In partially oxidized cytochrome oxidase in the presence of CO, the reduction state of the oxidase can be written as



Following flash photolysis, the oxidase is in its half-reduced or mixed valence form. The midpotential of cytochrome a in this case is approximately 380 mV while cyt a_3 is reduced with a midpotential of +240 mV (6); cytochrome c and the copper associated with cytochrome a also have midpotentials at approximately 223–240 mV (24). Thus, following photolysis of the CO compound, electrons will transfer from the lower potential cyt a_3 to the higher potential centers. Thus by observing the rate of absorbance change at wavelengths corresponding to changes in cyt c , copper, cyt a and cyt a_3 , the electron transfer steps can be measured and rates calculated.

Mixed valence oxidase is easily produced at room temperature in the absence of CO; small quantities of ascorbate are added to cytochrome oxidase in the presence of cytochrome c and TMPD such that only partial reduction of cyt c and cyt a (observed at 550 and 604 nm) are observed as seen in Figure 16. This cytochrome oxidase suspension is then bubbled with CO to yield the mixed valency carboxy-oxidase where the reduction

state of cyt *a* and cyt *c* is again calculated. From the data in Figure 16, approximately 75% of the molecules of cyt oxidase are in the mixed valence state with cyt *c*, cyt *a*, and the copper oxidized.

The kinetics of the 445 nm absorbance after photolysis (Fig. 17) show biphasic kinetics. The temperature-dependence (Arrhenius plot) of the reaction shows two distinct processes (Figure 18); one (the slower phase), is identical to CO recombination to cyt *a*₃.

The faster phase has a similar energy of activation, but represents a different process that results in a change in 445 nm absorbance. A decrease in 445 nm absorbance can occur when either/or cyt *a* or *a*₃ is oxidized or when cyt *a*₃ binds CO or oxygen (at which time

absorbance at 430 nm due to carboxy-*a*₃²⁺ increases). Since previous studies (66) have shown that the kinetics of CO binding are identical in fully reduced or partially-reduced oxidase, the faster phase of 445 nm decrease cannot be CO binding to mixed valence oxidase.

Following flash photolysis, a transient increase in the absorbance at 550nm and 604 nm occurs (cf. Figs 19,20), suggesting that these cytochromes are being reduced and then re-oxidized. Observation of changes at 830nm (Fig 21) indicates that the copper is also undergoing oxidation and reduction.

These observations are consistent with the following explanation. Following photolysis, electrons from +240 mV cyt *a*₃ move to the lower potential copper or cyt *a*. The copper and cyts *a* and *c* are essentially equi-potential; thus, electrons could flow from cyt *a*₃ to either copper or cyt *a* depending on the sequence of carriers. Once cyt *a*₃ becomes oxidized and cyt *a* becomes reduced, the midpotential of cyt *a* is lower than *a*₃, and the electron can be re-transferred to the +380 mV cyt *a*₃ or can be equipotentially redistributed to copper or cyt *c*.

The fast phase kinetics show that cyt *a*₃ becomes oxidized. The increase in reduction state of cyts *c* and *a* and copper indicate that the electron is indeed reducing these centers. Electron redistribution occurs so that all centers are eventually reduced. As soon as a center is reduced, it acts as reductant for an oxidized center, hence each center undergoes a cycle of reduction followed by oxidation. Observing the sequence of the reduction/oxidation cycles will allow the sequence of electron transfer steps in the oxidase to be determined in reverse order!

Once oxidized, cyt *a*₃ is at +380 mV. Thus electrons will eventually return to cyt *a*₃ where CO recombination can again occur.

Measuring the time sequence of these changes will allow the sequence of electron transfer to be determined. If a perturbant acts between two centers, then a change in the reduction/oxidation cycles will be apparent. If, for example, a compound were to interfere with the electron transfer step between cyt *c* and cyt *a*, then after flash photolysis in mixed valence state, cyt *a* and copper would be observed to undergo reduction/oxidation, but changes at 550 would not be seen. Measurement of the temperature-dependence of the transfer steps may also reveal the site of action of a compound.

We now have a method of determining the site(s) of action of the drugs and compound in cytochrome oxidase by measuring the transient reduction and re-oxidation of CuA, cyt *c*, and cyt *a*. We intend to utilize this procedure to determine the specific sites of naphthalene and local anesthetics.

In a previous annual report (1986) we stated that naphthalene caused an increase in the rate of CO recombination. Subsequent research has shown this to be incorrect;

naphthalene does not alter CO binding. What we were measuring was intra-oxidase electron transport, i.e., the redistribution of electrons between redox centers in partially-reduced enzyme. This redistribution occurs in the absence of CO and in the absence of hydrophobic perturbant.

These data imply that a portion of the procaine and tetracaine molecule, likely the long "-caine" tails, can compete with cytochrome c for the active site. It is likely that the mixed inhibitors cause an allosteric change in the oxidase to alter the active site and another region of the protein. We cannot explain why procaine but not procainamide acts as an inhibitor.

MYOGLOBIN

Background

Myoglobin is a monomeric single heme protein that physiologically exists in the ferrous state and binds/stores oxygen in tissues. It is a single polypeptide chain whose EPR, NMR, and X-ray crystallographic characteristics are well-known. The molecular weight is approximately 16 kilodaltons. Myoglobin can bind oxygen or CO and has been used to measure low temperature ligand-binding kinetics and the size/nature of the thermodynamic barriers to ligand binding (71).

Myoglobin is a 5-coordinate heme protein with a ferric Soret band at 411nm; in the ferrous state, a Soret band at 428nm is observed as is a weaker alpha band at 560nm. In the presence of CO, a prominent CO-ferrous band at 418 nm appears.

Myoglobin is a high-spin heme protein under normal physiological conditions but can be made low-spin at pH 12 or so. The high-spin resonances appear at $g=6$ and $g=2$ while the low spin resonances appear at approximately $g=3$, 2.2, and 1.5 (typical for a low spin heme). The intensity of the $g=6$ resonance is approximately 10% that of the $g=3$ low-spin resonance.

Results

We have observed several effects of drugs on the visible spectra of myoglobin. Several compounds were observed to cause a blue shift in the absorbance maximum in the Soret region of Mb and a red shift in oxy Mb. While even more hydrophilic compound like procainamide ($\log P = 1.77$) can alter the spectrum of Mb at 411nm, only the more hydrophobic molecules are effective in affecting oxyMb, suggesting that the oxyMb conformation is more "closed" and does not allow access to the hydrophobic interior as readily as the deoxy form. Tetracaine concentration-dependent shift in Soret spectra are illustrated in Fig. 22. It is extremely important to note that tetracaine (as well as dibucaine) induces the loss/removal of heme from oxyMb rendering it inactive.

In the alpha-band (500-600nm) of the spectrum, addition of dibucaine, tetracaine, and propranolol cause the formation of a new absorbance at 536 nm, indicative of the formation of a low-spin form of myoglobin (Fig. 23).

EPR spectroscopy indicates that our initial indication of the formation of a low-spin form of Mb in the presence of tetracaine is indeed correct. Figure 24 shows the EPR spectrum of myoglobin, with the $g=6$ and $g=2$ resonances. Figure 25 shows that in the presence of low concentrations of dibucaine, as may be encountered in anesthetic or cardiac therapy situations, the intensity of $g=6$ decreases and new resonances at $g=2.95$, 2.28, and 2.05, indicative of low spin compounds, appear. As shown in Figure 26, the decrease in $g=6$ intensity (and $g=3$ intensity increase) are dose-dependent and that the same spin state conversion is observed with other compounds, even though changes in Soret absorbance may not have been observed, as in the case of dibucaine. The data suggests that the spin state change is not a function of hydrophobicity of the molecule.

This suggests (means) that the iron atom of the hexacoordinate high-spin metmyoglobin which sits above the plane of the porphyrin ring is now located in the

porphyrin plane with the water molecule as the sixth ligand being replaced by a different stronger ligand. This stronger axial ligand may be a hydroxyl group or possibly a molecule of the drug itself. The latter possibility seems rather unlikely since it is known that a low-spin heme complex is formed when the fifth and sixth axial ligands of the iron atom are strong field ligands. The exception occurs for CO-Fe(II) mesotetraphenyl porphyrin where only one CO molecule is sufficient to cause the iron to be in the low spin state. The iron in metmyoglobin has an imidazole group (strong field ligand) of proximal histidine and a water molecule (a weak field ligand) as its fifth and sixth axial ligands. To convert this high spin met myoglobin to a low spin complex, another strong field ligand is needed to replace the water molecule. Each drug used in these experiments is a very large molecule which contains a hindered nitrogen group (unhindered nitrogen groups such as imidazole, pyridine, or primary amines are the only nitrogen ligands which can convert the high spin metmyoglobin into a low-spin species). This indicates that the drugs are not likely capable of acting as a strong field ligand. Further evidence that the drugs do not act as ligands is shown by the EPR spectra of naked hemin in the presence and absence of these drugs under the same conditions used in myoglobin experiments. These spectra show that the spin state of iron in hemin remains high spin in the presence of these drugs (data not shown).

The other possibility, namely that the strong field ligand in this low spin myoglobin compound is a hydroxyl group, is also unlikely even though the visible spectrum of myoglobin in the presence of 50 mM dibucaine-HCl (with dithionite added to insure reduction) is similar to that obtained for reduced myoglobin at pH 12. That OH⁻ is the sixth axial ligand is unlikely because the EPR *g*-values of Mb(III)OH done in this and other laboratories are *g*=2.60, 2.15, and 1.83 as shown in Figure 25, and not the *g*-values shown in Figure 24.

This leaves an attractive alternative explanation that an imidazole group of a distal histidine is the sixth axial ligand to the iron in the presence of these drugs. Since the closest imidazole group to the heme is that of the distal histidine E7, this suggests that the binding of the drug to the protein has perturbed either the heme itself or the E-helix from its original position such that this distal imidazole group, which could not normally bind to the iron can do so in the presence of the drugs, is now the sixth axial ligand of the iron. Though the binding of the distal imidazole group to the heme in myoglobin has not yet been reported, this explanation is not unreasonable since other workers (72) have reported that under various conditions the optical and EPR characteristics of ferric hemoglobin A and of isolated alpha and beta chains (similar to myoglobin) are consistent with the imidazole group of the distal histidine E7 can bind to the heme as its sixth axial ligand (73).

From *g*-values and the maxima of the optical absorbance bands it is clear that hemichromes (low spin species) are formed when dibucaine-HCl, tetracaine-HCl, or propranolol-HCl are added to low spin myoglobin. Unlike the low spin species produced from ferric hemoglobin A and its isolated alpha and beta chains which could not be converted into high spin ferric hemoglobin, the hemichrome formed from met Mb can be reversibly converted into the high spin deoxyMb by removal of the added reagent following aerobic dialysis at 4 C for 3 hours followed by addition of sodium dithionite as reductant. The reversibility of this formation of this low spin species may suggest that the perturbation of the myoglobin structure which causes either the E-helix or the prosthetic heme group itself to move from its original position is not as extensive as was reported for ferric hemoglobin A (72).

Although X-band EPR cannot be used to differentiate high spin (*S*=2), intermediate spin (*S*=1), and low spin (*S*=0) Fe(III) porphyrins, it can be safely assumed that the reduced myoglobin in the presence of 50 mM dibucaine, tetracaine, or propranolol-HCl at liquid nitrogen temperature exists mainly (predominately) as a low spin species (hemochrome) based on the data obtained from the low temperature visible spectra.

SIGNIFICANCE

Our findings indicate that hydrophobic molecules can function as powerful perturbants of protein conformation and alter the physical and chemical characteristics as well as the function of the proteins. The research did not explore the physiological aspects of these functional alterations, although we can speculate on the ramifications.

Alteration of the E7 helix of Mb and alteration of the heme ligation may have far-reaching effects, particularly since the individual chains of Hb are similar in structure; by analogy we can extend the effect to Hb (this would be an excellent research project!) The binding of O_2 to the altered Mb/Hb should be greatly affected. Application of the DTP drug may result in localized loss of oxy Mb/Hb and altered O_2

association/dissociation reactions. Our evidence indicates loss of heme from the oxy Mb/Hb and loss of functional protein! Because of the high concentrations of drug needed and the circulation of blood, we would not expect to see loss of O_2 transport to tissues where substantial circulation is present to dilute the drug and to transport unaltered Hb to the area. In situations of poor circulation or sustained application of the compound, however, O_2 transport may be affected.

The effect on Mb in cells/tissues is different. Since altered Mb cannot be "flushed out" and the drugs will not be rapidly diluted, storage of O_2 in cells and tissues may decrease, a form of drug-induced temporary ischemia. The effects of the ischemia might not become noticeable except under at least a few situations.

1. Hypoxia
2. High stress or metabolic activity
3. Immediately following a large exposure to the compound
4. Chronic long-term exposure
5. Any combination of the above

Because of the similarity of tetracaine and dibucaine to cocaine and its derivatives, we wonder if local ischemia and tissue damage would not result from cocaine usage or that even occasional usage would cause O_2 transport/storage and utilization in those tissues/organs where O_2 supply is critical (heart and brain).

Could our spectroscopic results be used as a non-invasive rapid test for drug usage for damage as a result of exposure to noxious compounds.

In 1982 we (Crider et al Bull. Env. Contam. Toxicol. 28, 52-57) demonstrated that naphthalene decreases the Hb content in *Daphnia* and decreases O_2 uptake as well. Thus, PAH may alter the levels of functional Mb/Hb in organisms and also decrease the metabolism and energy production in the mitochondria.

Problems associated with O_2 transport/storage by the globins will only be exacerbated by the alteration of the site of oxygen utilization, cytochrome oxidase.

The effect of the anesthetics /drugs on the oxidase is two-fold

1. Inhibition of O_2 reduction and /or
2. Acceleration (orders of magnitudes) of O_2 /CO binding. Rapid binding of CO will only exacerbate #1 above.

The effect of hydrophobic PAH such as benzene or naphthalene on the oxidase is to accelerate O_2 uptake and may alter the K_m for O_2 (we didn't check it) as well as cytochrome c. These effects would cause a decrease in ATP and energy production in the cells/tissues and the inducement of an anaerobic or ischemic-like condition. Even if O_2 can get to the mitochondria, it can't use it! Again these effect my manifest themselves in hypoxic or high stress/metabolic activity following exposure of the compound.

An extremely detrimental situation may occur to personnel exposed to the PAH and/or detrimental under medication/local anesthetic (or using drugs) in a high stress/activity situation where O_2 is two and/or CO may be present at high than normal levels as in pilots or flight line personnel. We are investigating studies to determine if physiological effects will indeed occur.

In a related view, we have, under a different research program, studied the effects of aging on brain mitochondria in rats (we believe the rat to be a good human model). With increasing age, the concentration of cytochrome oxidase in neuronal brain tissue decreases with the net result that oxidative phosphorylation and O_2 utilization also decrease! (see Harmon et al., Mechanisms of Aging and Development 38, 167-177, 1987; enclosed as a reprint). We do not yet know is O_2 binding or the K_m for O_2 is altered. Thus, the effects of exposure to hydrophobics may vary with the age of the individual as well as the conditions described earlier.

The role of these effects on personnel are undetermined but may alter performance. Further research on the basis of our results may be warranted.

References

1. Powers, L., Chance, B., Ching, Y., and Angiolillo, P. (1981) *Biophys. J.* 34, 465-498.
2. Powers, L., Chance, B., Ching, Y., Muhoberac, B., Weintraub, S.T., and Wharton, D.C. (1982) *FEBS Lett.* 138, 245-248.
3. Naqui, A., Chance, B., and Cadenas, E. (1986) *Ann. Rev. Biochem.* 55, 137-166.
4. Vanneste, W.H. (1966) *Biochemistry* 5, 838-848.
5. Gilmour, M.V., Wilson, D.F., and Lemberg, R. (1967) *Biochim. Biophys. Acta* 143, 487-499.
6. Wikstrom, M.K.F., Harmon, H.J., Ingledew, W.J., and Chance, B. (1976) *FEBS Lett.* 65, 259-277.
7. Wharton, D.C. and Tzagoloff, A. (1964) *J. Biol. Chem.* 239, 2036-2041.
8. Harmon, H.J. and Crane, F.L. (1974) *Biochim. Biophys. Acta* 368, 125-129.
9. Beinert, H., Shaw, R.W., Hansen, R.E., and Hartzell, C.R. (1980) *Biochim. Biophys. Acta* 591, 458-470.
10. Griffiths, D.E. and Wharton, D.C. (1961) *J. Biol. Chem.* 236, 1850-1856.
- 11a. Chance, B. and Leigh, J.S., Jr. (1977) *Proc. Nat. Acad. Sci.* 74, 4777-4780;
- 11b. Chance, B., Saronio, C., Leigh, J.S., Jr., Ingledew, W.J. and King, T.E. (1978) *Biochem. J.* 171, 787-798.
12. Palmer, G., Babcock, G.T., and Vickery, L.E. (1976) *Proc. Nat. Acad. Sci.* 73, 2206-2210.
13. Tweedle, M.F., Wilson, L.J., Garcia-Iniguez, L., Babcock, G.T., and Palmer, G. (1978) *J. Biol. Chem.* 253, 8065-8071.
14. Van Gelder, B.F. and Beinert, H. (1969) *Biochim. Biophys. Acta* 189, 1-24.
15. Wilson, D.F. and Leigh, J.S., Jr. (1972) *Arch. Biochem. Biophys.* 150, 154-163.
16. Leigh, J.S., Jr., Wilson, D.F., and King, T.E. (1974) *Arch. Biochem. Biophys.* 160, 476-486.
17. Aasa, R., Albracht, S.P.J., Falk, K.-E., Lanne, B., and Vanngard, T. (1976) *Biochim. Biophys. Acta* 422, 260-272.
18. Wever, R. and Van Gelder, B.F. (1974) *Biochim. Biophys. Acta* 368, 311-317.
19. Hartzell, C.R. and Beinert, H. (1974) *Biochim. Biophys. Acta* 368, 318-338.

20. Wever, R., Muijsers, A.O., Van Gelder, B.F., Bakker, E.P., and Van Buuren, K.J.H. (1973) *Biochim. Biophys. Acta* 325, 1-7.
21. Wilson, D.F. and Chance, B. (1967) *Biochim. Biophys. Acta* 131, 421-430.
22. Wever, R., Muijsers, A.O., and Van Gelder, B.F. (1973) *Biochim. Biophys. Acta* 325, 8-15.
23. Nicholls, P. and Hildebrandt, V. (1978) *Biochem. J.* 173, 65-72.
24. Dutton, P.L. and Wilson, D.F. (1974) *Biochim. Biophys. Acta* 346, 165-212.
25. Schroedl, N.A. and Hartzell, C.R. (1977) *Biochemistry* 16, 4961-4965.
26. Hartzell, C.R. and Beinert, H. (1976) *Biochim. Biophys. Acta* 423, 323-338.
27. Wilson, M.T., Jensen, P., Aasa, R., Malmstrom, B.G. and Vanngard, T. (1982) *Biochem. J.* 203, 483-492.
28. Sekuzu, I., Taremore, S., Orii, Y., and Okunuki, K. (1959) *Biochim. Biophys. Acta* 37, 64-71.
29. Sekuzu, I., Takemori, S., Yonetani, T. and Okunuki, K. (1959) *J. Biochem. (Japan)* 46, 43-49.
30. Williams, R.J.P., Lemberg, R., and Cutler, M.E. (1968) *Can J. Biochem.* 46, 1371-1379.
31. Tiesjema, R., Muisjers, A.O. and Van Gelder, B.F. (1972) *Biochim. Biophys. Acta* 256, 32-42.
32. Muisjers, A.O., Tiesjema, R., and Van Gelder, B.F. (1971) *Biochim. Biophys. Acta* 234, 481-492.
33. Babcock, G.T., Vickery, L.E., and Plamer, G. (1976) *J. Biol. Chem.* 251, 7907-7919.
34. Lemberg, R. and Gilmour, M.V. (1967) *Biochim. Biophys. Acta* 143, 500-517.
35. Brittain, J. and Greenwood, C. (1976) *Biochem. J.* 155, 453-455.
36. Jensen, P., Wilson, M.T., Aasa, R. and Malmstrom, B.G. (1984) *Biochem. J.* 224, 829-837.
37. Antonini, E., Brunori, M., Colosimo, A., Greenwood, C. and Wilson, M.T. (1977) *Proc. Nat. Acad. Sci.* 74, 3128-3132.
38. Brunori, M., Colosimo, A., Rainoni, G., Wilson, M.T., and Antonini, E. (1979) *J. Biol. Chem.* 254, 10769-10775.
39. Shaw, R.W., Hansen, R.E., and Beinert, H. (1978) *J. Biol. Chem.* 253, 6637-6640.

40. Chance, B., Kumar, C., Powers, L., and Ching, Y.C. (1983) *Biophys. J.* 44, 353-363.
41. Wikstrom, M.K.F., Krab, K., and Saraste, M. (1981) in "Cytochrome Oxidase: A Synthesis" Academic Press, New York, pp 55-87.
42. Young, L.J. and Palmer, G. (1980) *J. Biol. Chem.* 261, 13031-13033.
43. Witt, S.N., Blair, D.F., and Chan, S.I. (1986) *J. Biol. Chem.* 261, 8104-8107.
44. Chance, B., Saronio, C., and Leigh, J.S., Jr. (1975) *Proc. Nat. Acad. Sci.* 72, 1635-1640.
45. Chance, B., Saronio, C., and Leigh, J.S., Jr. (1975) *J. Biol. Chem.* 250, 9226-9237.
46. Erecinska, M. and Chance, B. (1972) *Arch. Biochem. Biophys.* 151, 304-315.
47. Chance, B., Harmon, J., and Wikstrom, M.K.F. (1975) in "Electron Transfer Chains and Oxidative Phosphorylation" (Quagliariello, E., et al., eds.) North Holland, Amsterdam, pp 81-95.
48. Clore, G. M., Andreasson, L.-E., Karlsson, B., Aasa, R. and Malmstrom, B.G. (1980) *Biochem. J.* 185, 135-154.
49. Clore, G.M., Andreasson, L.-E., Karlsson, B., Aasa, R. and Malmstrom, B.G. (1980) *Biochem. J.* 185, 155-167.
50. Wever, R., Van Drooge, J.H., Van Ark, G., and Van Gelder, B.F. (1974) *Biochim. Biophys. Acta* 347, 215-223.
51. Boelens, R., Rademaker, H., Pel, R., and Wever, R. (1982) *Biochim. Biophys. Acta* 679, 84-94.
52. Denis, M. and Richaud, P. (1985) *Eur. J. Biochem.* 147, 533-539.
53. Brudvig, G.W., Stevens, T.H., Morse, R.H. and Chan, S.I. (1981) *Biochemistry* 20, 3912-3921.
54. Singer, M.A. (1980) *Biochem. Pharmacol.* 29, 2651-2655.
55. Casanovas, A.M., Nebot, M.F.M., Courriere, P., and Oustrin, J. (1983) *Biochem. Pharmacol.* 32, 2715-2719.
56. Singer, M.A. (1981) *Biochem. Pharmacol.* 31, 527-534.
57. Chazotte, B. and Vanderkooi, G. (1981) *Biochim. Biophys. Acta* 636, 153-161.
58. Jost, P., Griffith, O.H., Capaldi, R.A. and Vanderkooi, G. (1973) *Proc. Nat. Acad. Sci.* 70, 480-484.
59. Yu, C.A., Yu, L., and King, T.E. (1975) *J. Biol. Chem.* 250, 1383-1392.

60. Harmon, H.J. and Crane, F.L. (1974) *Biochem. Biophys. Res. Common.* 59, 326-333.
61. Kornblatt, J.A. and Hui Bon Hoa, G. (1982) *Biochemistry* 21, 5439-5444.
62. Karlsson, B., Lanne, B., and Malmstrom, B.G. (1977) *FEBS Lett.* 84, 291-295.
63. Beinert, H. and Shaw, R.W. (1977) *Biochim. Biophys. Acta* 462, 121-130.
64. Karlsson, B. and Andreasson, L.-E. (1981) *Biochim. Biophys. Acta.* 635, 73-80.
65. Sharrock, M. and Yonetani, T. (1975) *Biochim. Biophys. Acta* 434, 333-344.
66. Harmon, H.J. and Sharrock, M. (1978) *Biochim. Biophys. Acta* 503, 56-66.
67. Harmon, H.J. and Wikstrom, M.K.F. (1978) *Biochim. Biophys. Acta* 503, 67-77.
68. Yoshikawa, S. and Caughey, W.S. (1982) *J. Biol. Chem.* 257, 412-420.
69. Young, L.J. and Caughey, W.S. (1986) *Biochemistry* 25, 152-161.
70. Kumar, C., Naqui, A., and Chance, B. (1984) *J. Biol. Chem.* 259, 2073-2076.
71. Ansari, A., DiIorio, E.E., Dlott, D.D., Frauvenfelder, H., Iben, I.E.T., Langer, P., Roder, H., Sauke, T.B. and Shyamsunder, E. (1986) *Biochem.* 25, 3139-3146.
72. Rachmilewitz, E.A., Peisach, J., and Blumberg, W.E. (1971) *J. Biol. Chem.* 246, 3356-3366.
73. Peisach, J. and Gersonde, K. (1977) *Biochemistry* 16, 2539-2545.

Table 1. Effect of Hydrophobic Molecules on Cytochrome c Oxidase

<u>Assay Condition</u>	<u>K_m (μM cyt c)</u>	<u>V_{max} (nmole O₂/min/mg)</u>
ETHANOL BASED		
Control	13.1	383
15 ppm Acenaphthene	12.45	372
15 ppm Naphthalene	26.6	652
5 mM Lidocaine	27.6	620
15 ppm Benzene	6.5	225
WATER-BASED		
Control	21.1	574
1 mM Verapamil	37.8	894
5 mM Tetracaine HCl	74	482
5 mM Procainamide HCl	19.4	503
50 mM Procaine HCl	75	620

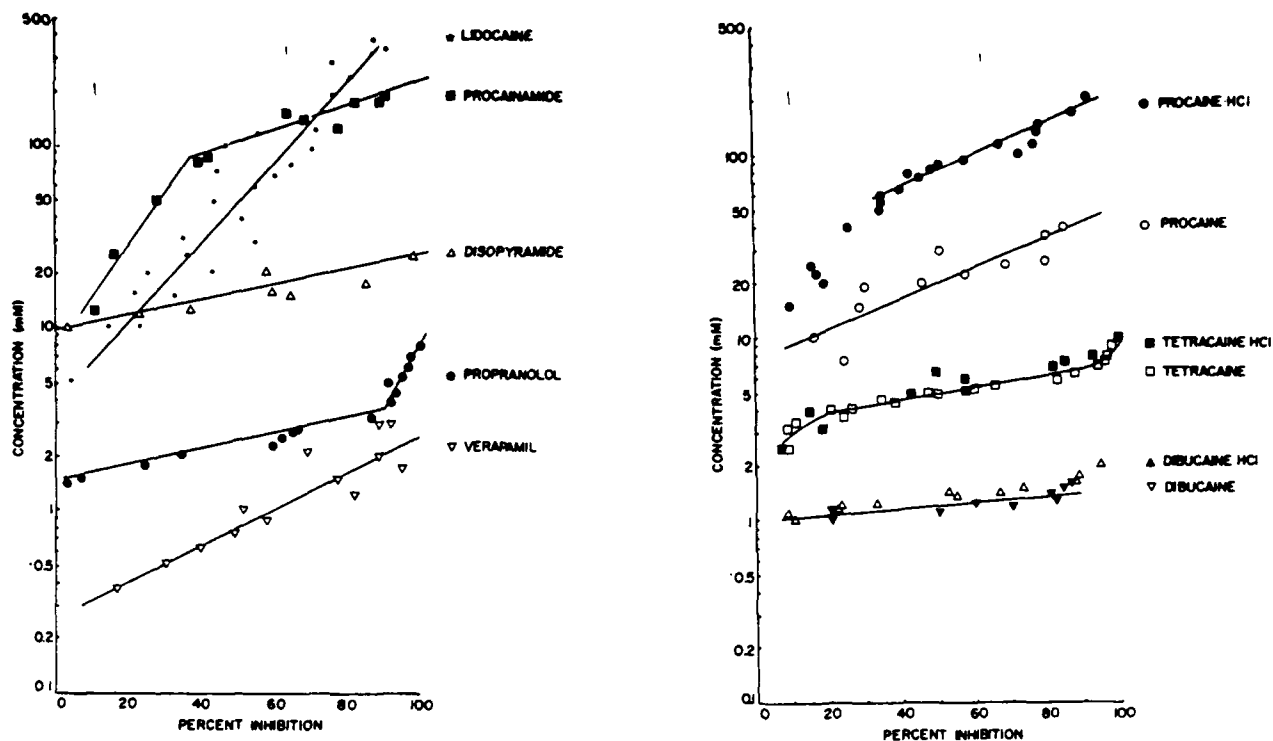


Figure 1. Concentration dependence of inhibition of mitochondrial succinate oxidase activity by local anesthetics and cardiac drugs.

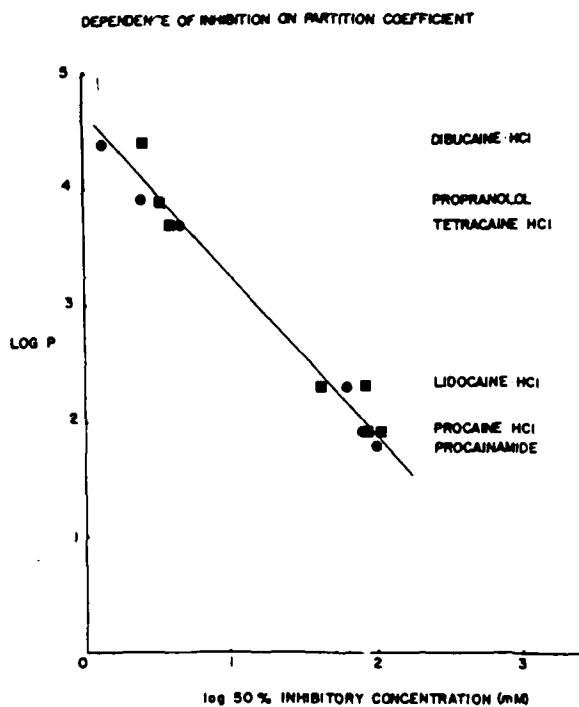


Figure 2. Dependence of the concentration of drug needed to achieve 50% inhibition of succinate inhibition on the partition coefficient of the drug.

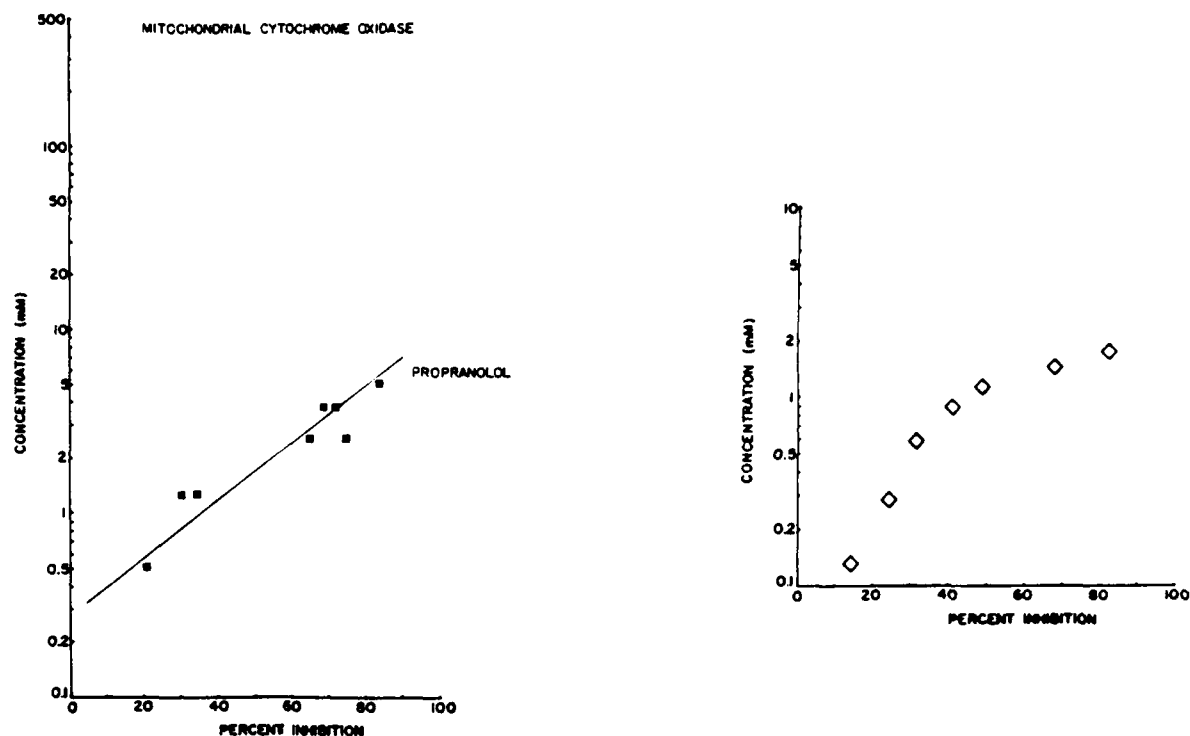


Figure 3. Effect of increasing concentrations of propranolol on inhibition of ascorbate + TMPD driven cytochrome oxidase activity in intact mitochondria.

Figure 3B. Effect of increasing dibucaine-HCl concentration on inhibition on ascorbate +TMPD driven cytochrome oxidase activity in intact mitochondria.

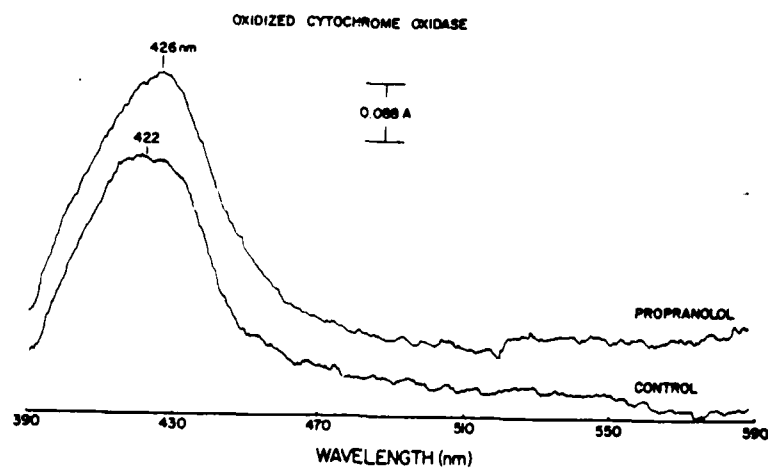


Figure 4. Effect of 25 mM propranolol on Soret absorbance of isolated ferric cytochrome oxidase.

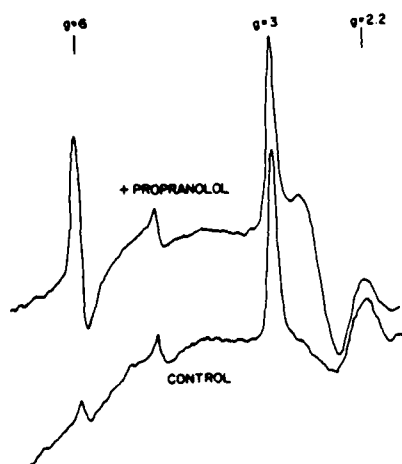


Figure 5. EPR spectrum at 9 K of formation of high spin cytochrome oxidase by addition of 25 mM propranolol to ferric isolated oxidase.

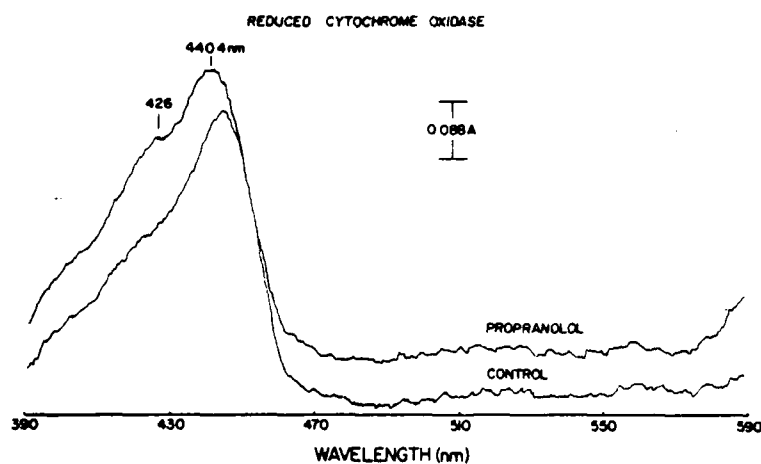


Figure 6. Effect of 25 mM propranolol on the Soret absorbance spectrum of ferrous (dithionite reduced) isolated oxidase.

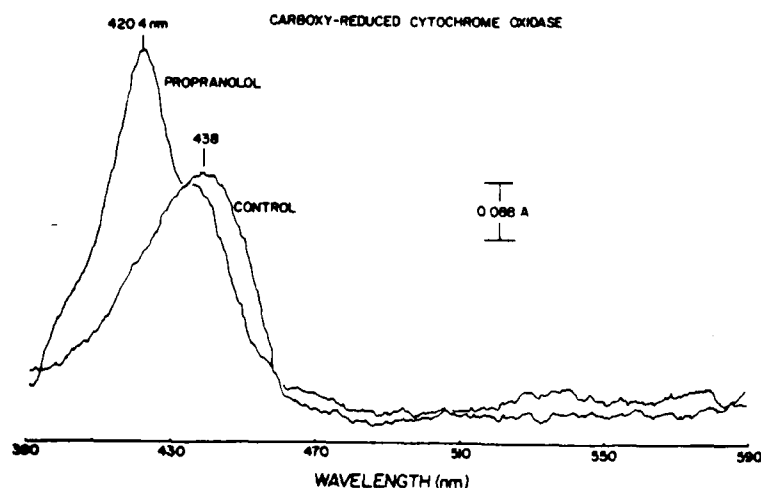


Figure 7. Soret spectrum of ferrous carboxy cytochrome oxidase. 100% CO was bubbled through the sample used for Figure 6. This sample contains a mixture of 427 nm normal carboxy oxidase and 418nm propranolol-treated high spin oxidase.

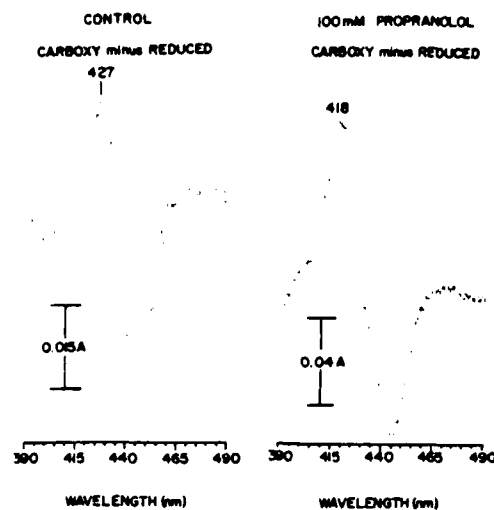


Figure 8. Ferrous carboxy MINUS ferrous cytochrome oxidase difference spectra of control and propranolol-treated oxidase to indicate that complete conversion to the 418 nm form occurs at high drug concentrations. Similar difference spectra are obtained with 25 mM tetracaine-HCl or 25 mM dibucaine-HCl.

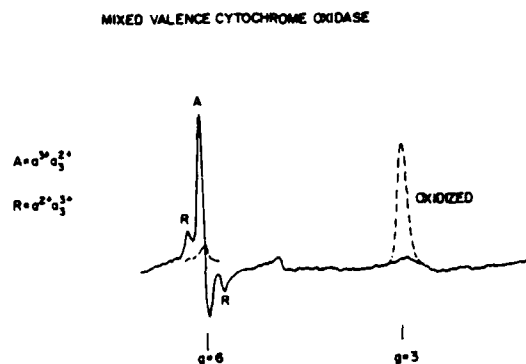


Figure 9. EPR spectrum at 9 K of ferric (oxidized) and mixed valence cytochrome oxidase. Mixed valence oxidase was made by oxidizing CO-bound reduced oxidase with ferricyanide to oxidize cytochrome a and then photolyzing the sample to yield the mixed valence unliganded oxidase. Both rhombic and axial $g=6$ signals are observed. These signals should be compared to the high spin signals observed in Figure 5, where only axial high spin is seen.

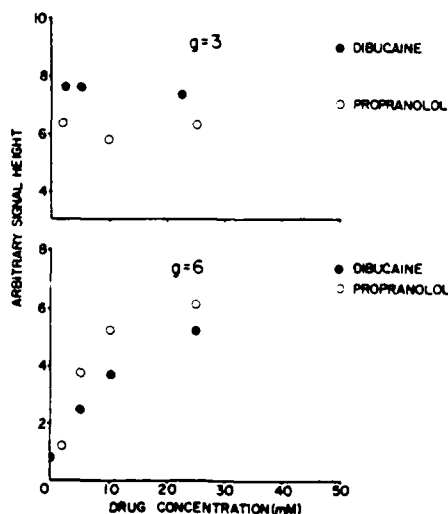


Figure 10. Effect of increasing dibucaine and propranolol concentrations on the intensity of the low spin $g=3$ and high spin $g=6$ signals. The $g=3$ intensity is unaffected while $g=6$ intensity increases with increasing drug concentrations. High spin is not formed at the expense of the $g=3$ signal.

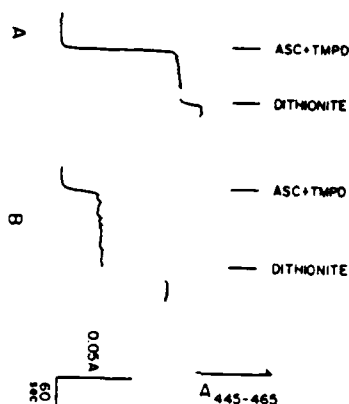


Figure 11. Effect of 1.5 mM dibucaine on the reduction of cytochrome oxidase in intact mitochondria measured at 445 vs. 465 nm using ascorbate+ TPMD+ cyt c as reductant. Cytochrome c-depleted intact mitochondria were suspended at 2 mg/ml in 0.25M sucrose-50 mM NaPi (pH 7.4) buffer. 5 mM ascorbate, 12.5 μ gm TMPD and 100 μ gm cyt c were used as reductant.

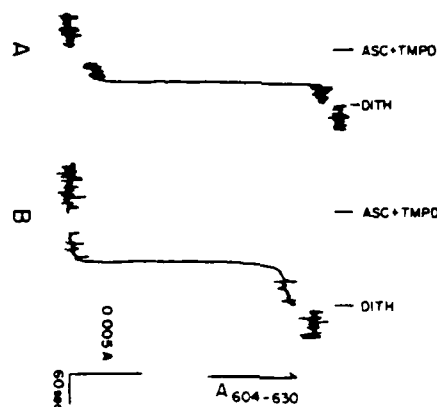


Figure 12. Effect of 1.5 dibucaine-HCl on reduction of cytochrome oxidase as measured at 604 minus 630 nm. Conditions as in Figure 11.

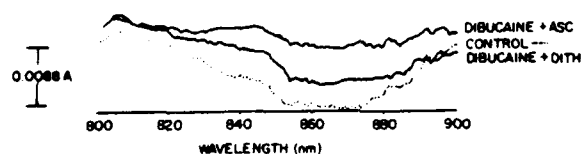


Figure 13. Reduced MINUS oxidized difference spectra of the near-IR region in intact mitochondria. Control mitochondria were reduced with ascorbate+TMPD + cyt c as described in Figure 11. Dibucaine-HCL- treated mitochondria were treated with both ascorbate+ TMPD and with dithionite as labelled. The 860 nm trough is not caused by presence of either Hb/Mb or adventitious copper. The mitochondria were washed twice with 0.15 M KVL and then with bathocuproine- and bathophenanthroline-sulfonate to remove adventitious metals. The 860 trough is also seen in isolated oxidase as well and represent the copper center. The copper center is not reduced by ascorbate + TMPD in dibucaine treated oxidase; it can be subsequently reduced by dithionite, however, indicating that it is not lost or destroyed by the drug.

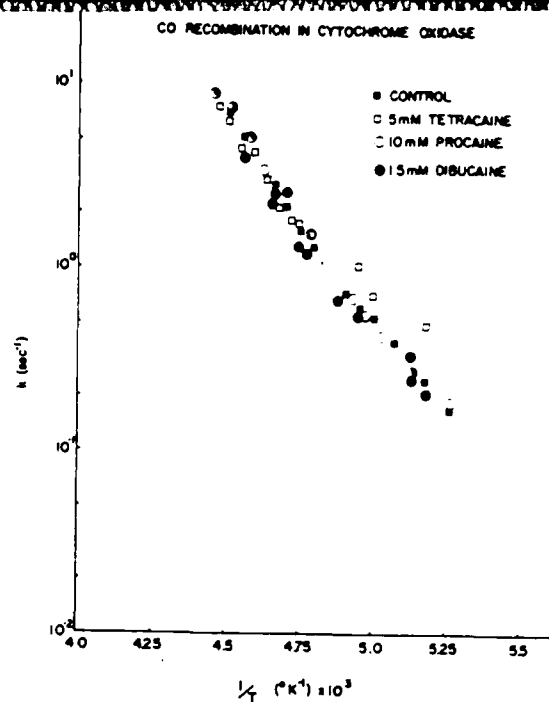
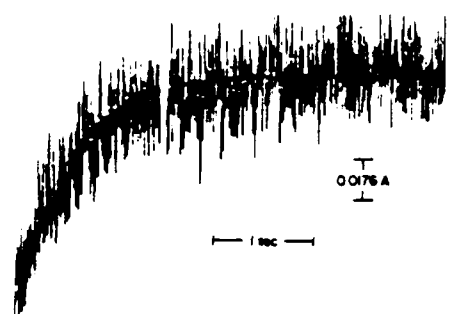


Figure 14. Plot of the change in 448 nm absorbance (increase in amount of cytochrome oxidase-CO complex in upward direction) vs. time. The plot represents raw data acquired by the computer. Following flash photolysis, CO rebinds to cytochrome oxidase. The computed least squares fit of the data indicates the time dependence of the curve is exponential and can be described by the equation $f(t) = e^{-kt}$ where $f(t)$ is the fraction unbound at time t after the flash.

Figure 15. Plot of the log k for CO recombination vs. inverse temperature in the presence of tetracaine, procaine, and dibucaine. The energy of activation is approximately 9.3 kcal/mole. The intensity of the absorbance change (Figure 11) decreases with increasing drug concentration. This plot measures only the fraction of oxidase not altered by the drugs.

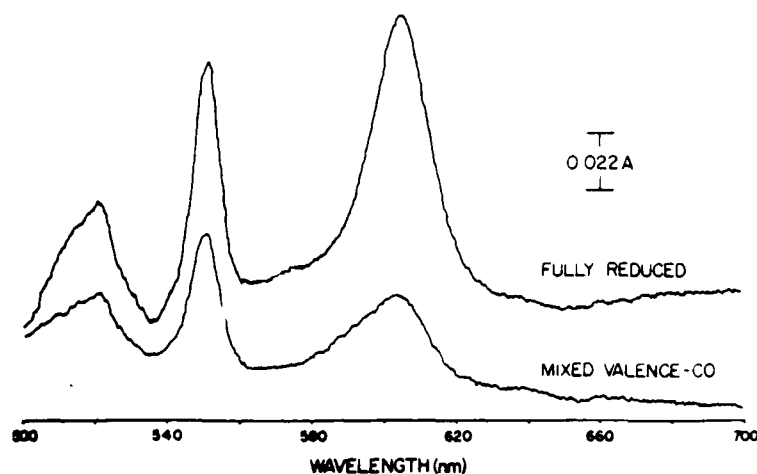


Figure 16. Absorbance difference spectra (reduced MINUS oxidized) of a 1:1 cytochrome c -purified cytochrome oxidase complex at room temperature. The mixed valence +CO spectrum was obtained by the addition of ascorbate +TMPD as reductant. The fully reduced spectrum was obtained by addition of dithionite to an identical sample in the absence of CO.

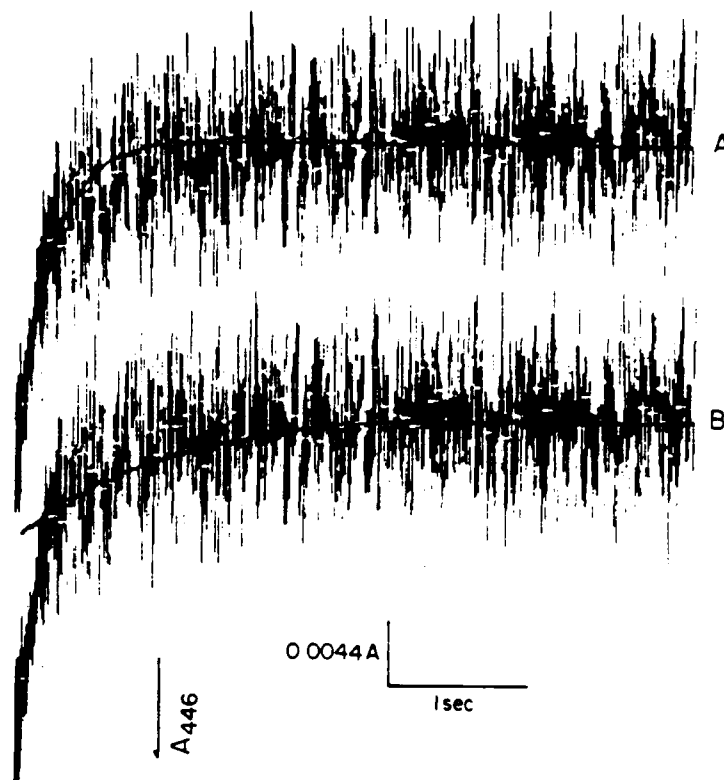


Figure 17. Recording of 446 nm absorbance changes vs. time following flash photolysis of mixed valence carboxy-cytochrome oxidase at -65°C . The computerized fit to a single exponential for the data in trace A in the first 1.5 seconds is shown and yields values of $k = 0.65/\text{sec}$ and a half-time of 1.07 seconds. For trace B (same raw data as trace A, the half-time is 3.9 seconds and the value of k is $0.178/\text{sec}$).

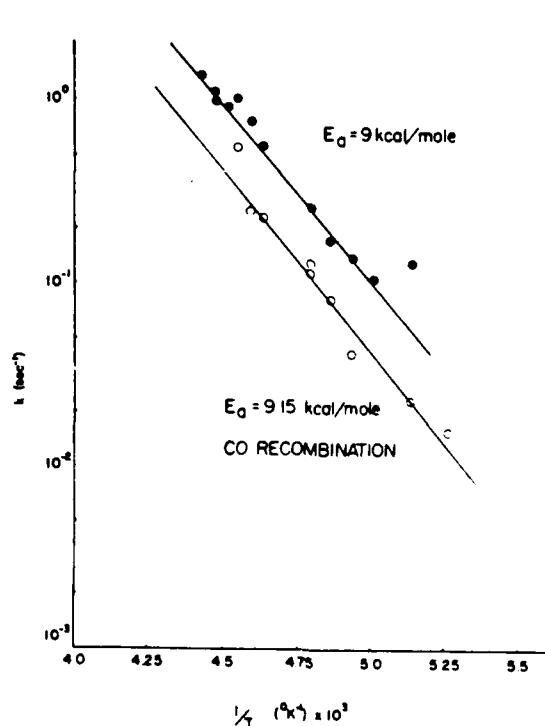


Figure 18. Plot of $\log k$ vs. inverse temperature for the fast (closed circle) and slow (open circle) phases of 446 nm absorbance changes following flash photolysis of mixed valence carboxy-cytochrome oxidase. The energy of activation of the fast phase is 9 kcal/mole while that of the slow phase is 9.15 kcal/mole. The slow phase data is very similar to that obtained from the monophasic 446 nm changes in fully reduced carboxy-oxidase.



Figure 19. Recording of 550 nm absorbance changes (increase upward) vs. time following flash photolysis of 1:1 cytochrome *c*-mixed valence carboxy-oxidase complex at -60°C . The reduction/oxidation cycle is indicated in the region of the open arrow.

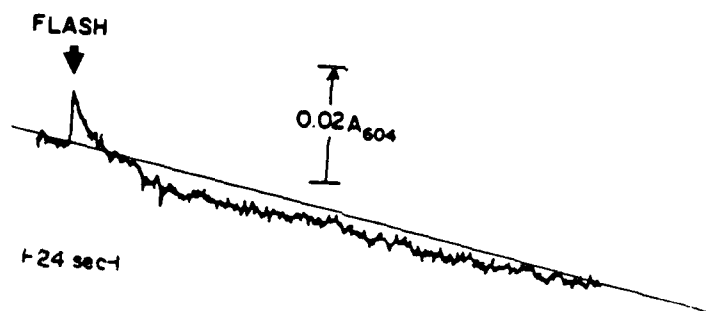


Figure 20. Plot of 604 nm absorbance vs. time following flash photolysis at -60°C in mixed valence carboxy-oxidase. The arrow denotes the direction of absorbance increase.

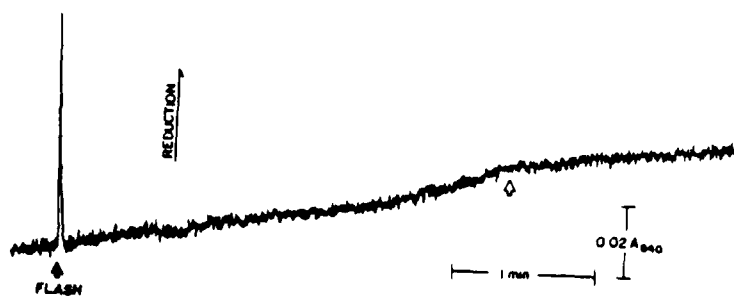


Figure 21. Recording of 840 nm absorbance decrease (upward) vs. time following flash photolysis of mixed valence carboxy-oxidase.

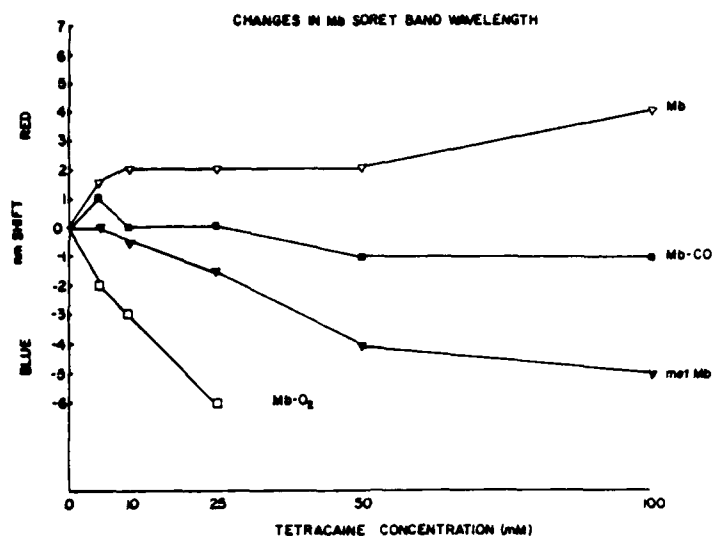


Figure 22. Effect of tetracaine on the Soret region wavelength maxima of different ligation states of myoglobin.

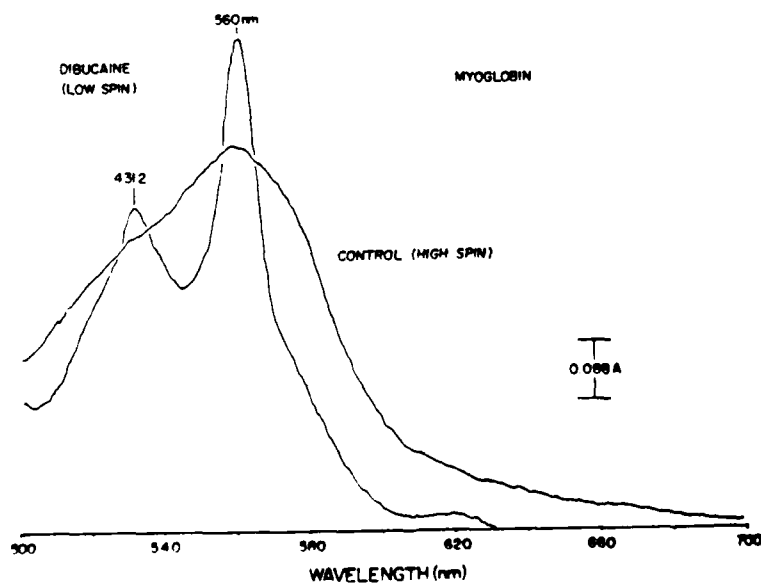


Figure 23. Alteration of the alpha band absorbance of myoglobin in the presence of local anesthetic (optical spectrum recorded at -162°C).



Figure 24. EPR spectrum at 9K of untreated myoglobin with characteristic high spin $g=6$ signal.

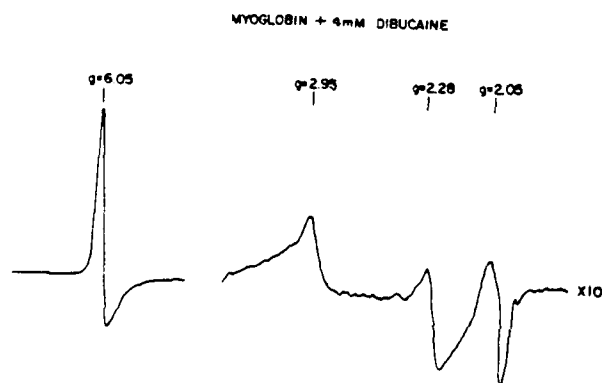


Figure 25. Effect of dibucaine on 9K EPR spectrum of myoglobin. Note the decrease in intensity at $g=6$ and the appearance of low spin hemichrome signals at $g=2.95$ and 2.28 .

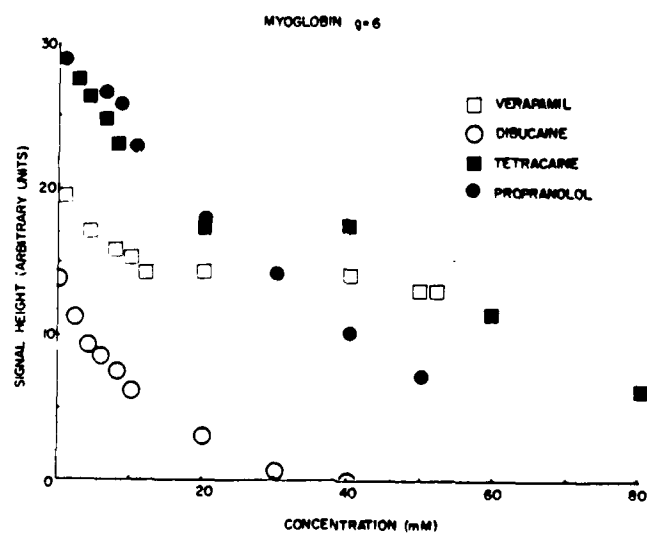


Figure 26. Effect of increasing dry concentrations on the intensity of $g=6$ signal and, thus, the amount of (normal) high spin myoglobin in the sample.

Effect of Naphthalene on Cytochrome Oxidase Activity

H. James Harmon

Department of Zoology, Oklahoma State University, Stillwater, Oklahoma 74078

Previous reports have demonstrated that naphthalene inhibits oxygen consumption in *Daphnia magna* (Crider et al. 1982), tissue culture cells (Harmon and Sanborn 1982), and intact mitochondria and submitochondrial particles (Harmon and Sanborn 1982). Struble and Harmon (1985) extended the studies to algal mitochondrial respiration as well as photosynthetic activity. We were able to demonstrate the specific site of apparent respiratory inhibition to be coenzyme Q (ubiquinone, UQ) (Harmon and Sanborn 1982) and later to demonstrate the molecular basis of this inhibition at ubiquinone (Struble and Harmon 1983).

Harmon and Sanborn (1982) could not demonstrate an effect of naphthalene on cytochrome oxidase activity. The observation by Struble and Harmon (1985) that naphthalene can stimulate respiration in algae prompted the reinvestigation of the effect of naphthalene on the kinetics of cytochrome oxidase. Cytochrome oxidase is a multi-subunit membranous protein responsible for the oxidation of cytochrome c and the reduction of molecular oxygen to water. Because of the complicated nature and mechanism of this enzyme, the potential exists for multiple and possibly opposite effects of naphthalene on its function.

MATERIALS AND METHODS

Beef heart mitochondria were isolated by the procedure of Crane et al (1956). Lipid-depleted cytochrome oxidase was purified from beef heart mitochondria by the procedure of Yu, Yu, and King (1975) except that 20% neutralized cholic acid (Aldrich) was added at the levels of 3.75 ml/100 ml and 5.8 ml/100 ml protein suspension in the first and second detergent fractionations, respectively.

Cytochrome oxidase activity was measured at 25 C in a glass water-jacketed chamber fitted with a Clark oxygen electrode as described previously (Harmon And Crane 1976; Harmon and Sanborn 1982).

CO recombination kinetics were measured at 448 nm with a Gilford Model 252 spectrophotometer. A clear glass dewar with a 2 mm light

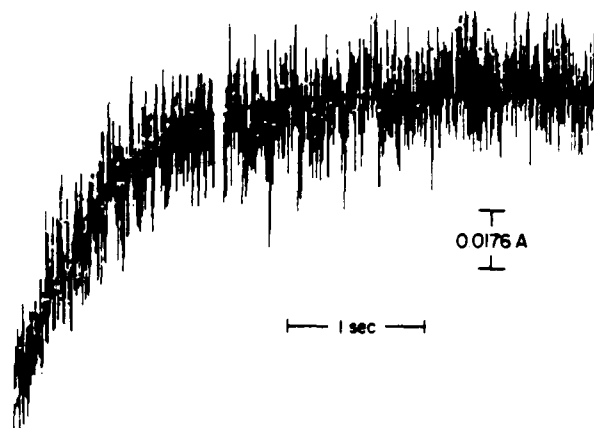


Figure 2. Plot of the change in 448 nm absorbance (increase in amount of cytochrome oxidase-CO complex in upward direction) vs. time. This plot represents typical raw data acquired by the computer. Following flash photolysis of the carboxy-oxidase at time=0, CO rebinds to cytochrome oxidase. The computed least squares fit of the data indicates the time dependence of the curves is exponential and can be described by the equation $f(t) = e^{-kt}$ where $f(t)$ is the fraction of unbound oxidase not recombined with CO at time t after the flash. k is proportional to the rate of recombination of cytochrome oxidase with CO after the dissociating flash.

sodium phosphate buffer (pH 7.4). This mixture was bubbled with 100% CO for 20 minutes in the dark and then loaded into the sample holder in the dark prior to freezing in liquid nitrogen.

RESULTS AND DISCUSSION

In the presence of 15 ppm (117 μ M) naphthalene, the values of K_m and V_{max} as derived from Lineweaver-Burk plots increase almost two-fold relative to the values in control samples (shown in Fig. 1). For the data shown in Fig. 1, the value of K_m increases from 13.1 to 26.6 M and V_{max} increases from 766 to 1304 ng-atom O/min/mg protein. From all experiments, the K_m values for control and naphthalene-treated oxidase were 17.1 ± 4.8 and 30.8 ± 7.8 (S.D.) M, respectively.

Since $v/V_{max} = [S] / K_m + [S]$, at a given substrate concentration the v/V_{max} ratio will be smaller in the presence of naphthalene. Since the value of V_{max} is increased by naphthalene, the overall measured velocity of the reaction is essentially unchanged. Thus our previous report of overall apparent lack of effect on cyto-

APRIL 1985 FASEB ABSTRACT FORM

Mail to your society of membership (APS, ASBC, ASPET, AAP, AIN or AAI.) ASCB members send to ASPET; BMES, SEBM & SMB members send to APS.
9650 Rockville Pike
Bethesda, Maryland 20814

MAILING ADDRESS OF FIRST AUTHOR
(Please Print or Type. Provide full name rather than initials.)

H. James Harmon
Dept. of Zoology
Room 430 LSW
Stillwater, OK 74078

Office 405-624-5555

Phone: Home/Holiday 405-348-9985

PRESENTATION PREFERENCE (Check one)

- ☐ Poster ☐ Slide ☐ Minisymposium
☒ Indifferent

Final decision regarding presentation format is at the discretion of the programing society.

SELECT CATEGORY NUMBERS & TITLES (See Topic Category Lists)

10-MIN. ORAL OR POSTER PREFERENCE

1. 1129-2 HEME PROTEINS & PORPHYRINS
2. 1132-2 BIOGENESIS
3. _____

No. Title

MINISYMPOSIUM PREFERENCE

1. _____
No. Title

If AAI Topic List is Used. Choose one block only and list minisymposia choices and poster choices all from within same block: Block # _____

PUBLIC INFORMATION VALUE

- ☐ If requested, I will prepare a lay language summary to be used as the basis of a press release.

INTERACTION OF LOCAL ANESTHETICS WITH MITOCHONDRIAL CYTOCHROMES AND MYOGLOBIN H. James Harmon. Oklahoma State University, Stillwater, OK 74078

Inhibition of mitochondrial succinoxidase activity by the local anesthetics (LA) procaine, dibucaine, tetracaine, and lidocaine and the cardiac antiarrhythmic drugs procainamide, verapamil, and propranolol is proportional to the log of their partition coefficient. Alterations in the reduction of cyt cc_1 by dibucaine and cyt b by tetracaine and changes in peak wavelengths of mitochondrial cytochromes are observed. Lidocaine, but not other LA, decreases the midpotential of cyt c ; changes in EPR peaks, Soret extinction coefficients, and peak wavelengths are also observed. ^1H -NMR resonances of hydrophobic residues only in the heme pocket (Phe 48, Phe 36, Met 65 and Trp 59) are altered by lidocaine; procaine alters β -meso-CH of heme only. This data indicates that LA can interact with the hydrophobic protein interior as well as membrane lipid but that the interaction is mediated by factors other than partition coefficient. Further, effects on soluble proteins occur at concentrations below those needed to alter respiration but comparable to prescribed serum levels. The Soret bands of metmyoglobin and MbO₂ are blue-shifted by LA. Data suggests that O₂, but not CO binding to Mb is altered by tetracaine.

This research was supported by a Grant-in-Aid from the American Heart Association, Oklahoma Affiliate, and the Air Force Office of Scientific Research, Air Force Systems Command, USAF under Grant Number AFOSR 84-0264.

Blue lines are printer's cut lines; do not type on or outside of these lines.

All compounds that are designated by code or initial letters must be identified adequately in the abstract, e.g., MJ-1999; 4-(2-isopropylamino-1-hydroxyethyl) methanesulfonanilide hydrochloride.

IMPORTANT

- Prepare abstract carefully, on this form only.
- Also fill out:
 - Topic category selection
 - Public information value
 - Mailing address of first author
 - Signature block for member's signature
- Return to your society of membership by Dec. 3, 1984:
 - The original typed abstract form
 - 1 photocopy
 - Abstract handling fee
- See over for complete instructions.

All investigators must adhere to the guidelines as listed on the reverse of the abstract form.

APS, BMES, SEBM, SMB, ASPET & AIN members only:

Signing member, are you willing to chair a session? () yes, category # _____

APS, AAP & AIN members only: ☐ Submitted for special Society student or training award.

MEMBER'S AFFILIATION (Check one only):

☐ APS ☒ ASBC ☐ ASPET ☐ AAP ☐ AIN ☐ AAI ☐ ASCB ☐ BMES ☐ SEBM ☐ SMB

Submission of signed form indicates acceptance of rules including final withdrawal date of December 19, 1984.

H. James Harmon

Member's Name (Print or type)

Member's Signature

Member's Phone

REMITTANCE (NONREFUNDABLE)

ABSTRACT HANDLING FEE \$20

Payable to FASEB

(405) 624-5555

1986 ASBC/DBC,ACS Abstract Form

Mail to: ASBC/DBC,ACS Meeting Office
Beaumont House
9650 Rockville Pike
Bethesda, MD 20814

MAILING ADDRESS OF FIRST AUTHOR
(Please Print or Type. Provide full name rather than initials.)

H. JAMES HARMON
DEPT. OF ZOOLOGY
OKLAHOMA STATE UNIVERSITY
STILLWATER, OK 74078
Office 405/624-5555
Phone: Home/Holiday 405/348-9985

Effect of Cardiac Antiarrhythmics and Anesthetics on Cytochrome Oxidase Activity. H. James Harmon and J. Swartz. Oklahoma State Univ., Stillwater, OK 74078.

The effects of the cardiac antiarrhythmics and local anesthetics procaine, procainamide, dibucaine, tetracaine, lidocaine, verapamil, and propranolol on mitochondrial-bound cytochrome oxidase activity were measured. The concentration dependence of these compounds on succinate oxidase activity is linearly related to the log of their partition coefficients. At a fixed cyt c concentration, all compounds except verapamil and lidocaine are inhibitory to cyt oxidase to the expected extent based on their effect on succinoxidase. Lidocaine doubles the Vmax of the oxidase and the Km for cyt c; verapamil causes a 1.8-fold increase in Km and a 1.5-fold increase in Vmax, thus explaining their apparent lack of inhibition at fixed c concentrations. Procainamide does not alter Km or Vmax while procaine and tetracaine triple Km but do not alter Vmax. Procainamide is not a competitive inhibitor; procaine and tetracaine act as competitive inhibitors. Previous data (Harmon Fed. Proc. 44, 1779 abs.) indicates that procaine and tetracaine do not alter the characteristics of cytochrome c; thus these compounds affect cyt oxidase alone. The data suggest specific interaction of the drugs with proteins rather than non-specific detergent effects.

This research was supported by the Air Force Office of Scientific Research, Air Force Systems Command, USAF under Grant Number AFOSR 84-0264.

SELECT CATEGORY NUMBER & TITLES
(See Topic Category List)

Afternoon Symposia Preference

1. _____
No. Title

Poster/Poster-Discussion Preferences

1. _____
2. _____
3. _____
No. Title

Blue lines are printer's cut lines; do not type on or outside of these lines.

IMPORTANT

1. Prepare abstract carefully, on this form only.
2. Also fill out:
 - a. Topic category selection
 - b. Mailing address of first author
 - c. Signature block for member's signature
3. Return by January 14, 1986
 - a. The original typed abstract form
 - b. 1 photocopy
 - c. Abstract handling fee
4. See over for complete instructions.

All compounds that are designated by code or initial letters must be identified adequately in abstract, e.g., MJ-1999: 4-(2-isopropylamino-1-hydroxyethyl) methanesulfonanilide hydrochloride

Each abstract form submitted must be signed by a FASEB or ACS member. Signature provides assurance of adherence to the rules and the scientific validity of the presentation.

MEMBER'S AFFILIATION

☐ APS ☒ ASBC ☐ ASPET ☐ AAP ☐ AIN ☐ AAI ☐ ASCB ☐

H. James Harmon

Member's Name (Print or type)

Member's Signature

405/624-5555

Member's Phone No.

REMITTANCE

ABSTRACT HANDLING FEE \$25

Payable to ASBC

TUESDAY, JANUARY 13, 1987

1987 ASBC Abstract Form

Mail to: ASBC Meeting Office
Beaumont House
9650 Rockville Pike
Bethesda, MD 20814

MAILING ADDRESS OF FIRST AUTHOR
(Please Print or Type. Provide full name rather than initials.)

Dr. H. James Harmon
Dept. of Zoology
Oklahoma State University
Stillwater, OK 74078
Office (405) 624-5555
Phone: Home/Holiday (405) 348-9985

SELECT CATEGORY NUMBER & TITLES
(See Topic Category List)

Afternoon Symposium Preference

1. No. Title

Poster Preferences

1. 130 Bioenergetics
2. 29 Heme proteins and porphyrins
3. No. Title

ELECTRON REDISTRIBUTION IN CYTOCHROME OXIDASE AT LOW TEMPERATURES. H. James Harmon, Oklahoma State University, Stillwater, OK 74078.

CO recombination to either mixed valency or fully reduced mitochondrially-bound cyt oxidase follow monophasic kinetics when measured at 594 nm. Recombination of CO to mixed valence purified oxidase follows biphasic kinetics when measured at 448 nm. The oxidase was poised at +240 mV as determined by the extent of cyt c reduction with ascorbate+TMPD as reductant. A slow decrease in 448 nm absorbance with a $t_{1/2}$ of 3 seconds at -60 C and an E_{act} of approx. 9.5 kcal/mole corresponds to the binding of CO the cyt a_3^{2+} and is identical to CO recombination in fully reduced oxidase. A faster phase of 448 nm decrease with an E_{act} of approx. 12.5 kcal/mole is also observed. The biphasic 448 nm decrease could be due to two kinetically different CO binding reactions or due to oxidation of cyt a_3 (and reduction of other centers). After flash photolysis of partially reduced oxidase at approx. -60C, cyt a, cyt c, and the detectable copper undergo reduction as electrons are redistributed to these equipotential centers. These centers are then re-oxidized as electrons transfer to cyt a_3 to yield cyt a_3^{2+} .

This research was supported by the Air Force Office of Scientific Research, Air Force Systems Command, USAF under Grant Number AFOSR 84-0264.

IMPORTANT

1. Prepare abstract carefully, on this form only.
2. Also fill out:
 - a. Topic category selection
 - b. Mailing address of first author
 - c. Signature block for member's signature
3. Return by January 13, 1987:
 - a. The original typed abstract form
 - b. 1 photocopy
 - c. Abstract handling fee
 - d. Program Confirmation Card
4. See over for complete instructions.

Blue lines are printer's cut lines; do not type on or outside of these lines.

All compounds that are designated by code or initial letters must be identified adequately in the abstract, e.g., MJ-1999: 4-(2-isopropylamino-1-hydroxyethyl) methanesulfonamide hydrochloride.

Each abstract form submitted must be signed by a FASEB member. Signature provides assurance of adherence to the rules and the scientific validity of the presentation.

MEMBER'S AFFILIATION

☐ APS ☒ ASBC ☐ ASPET ☐ AAP ☐ AIN ☐ AAI ☐ ASCB

H. James Harmon
Member's Name (Print or type)

[Signature]
Member's Signature

(405) 624-5555
Member's Phone No.

REMITTANCE (NONREFUNDABLE)
ABSTRACT HANDLING FEE \$25
Payable to ASBC

No exceptions will be made.

Submission of signed form indicates acceptance of rules including final withdrawal date of February 12, 1987.

1988 FASEB ABSTRACT FORM

DO NOT FOLD THIS FORM

Mail to your society of membership
(APS, ASBMB, ASPET, AAP, AIN, AAI)
ASCB members send to ASBMB
BMES, SMB and SEBM members send to APS
9650 Rockville Pike
Bethesda, MD 20814

MAILING ADDRESS OF FIRST AUTHOR
(Please Print in black ink or Type. Provide full
name rather than initials.)

H. James Harmon
Zoology Department
430 LSW
Oklahoma State University
Stillwater, OK 74078

Office (405) 624-5555
Phone: Home/Holiday (405) 348-9985

PRESENTATION PREFERENCE (Check one)

☐ Oral ☐ Poster ☒ Indifferent

Final decision regarding presentation format is at
the discretion of the programming society.

SELECT CATEGORY NUMBERS & TITLES
(See Minisymposium, ASBMB Afternoon Symposia,
and Topic Category Lists)

1. 1130-2 BIOENERGETICS
 2. 1125-2 HEME PROTEINS & PEROXYGEN
 3. _____
 4. _____
- No. Title

If AAI Topic List is Used. Choose one block only
and list minisymposia choices and poster choices all
from within same block. Block # _____

Is first author graduate student? ☐ Yes ☒ No

IMPORTANT

1. Prepare abstract carefully, on this form only.
2. Also fill out:
 - a. Topic category selection
 - b. Mailing address of first author
 - c. Signature block for member's signature
3. Return to your society of membership by
December 8, 1987
 - a. The original typed abstract form
 - b. Two photocopies
 - c. Abstract handling fee
 - d. Program confirmation card
4. See over for complete instructions.

EFFECT OF PROPRANOLOL AND LOCAL ANESTHETICS ON MYOGLOBIN AND
CYTOCHROME OXIDASE. H. James Harmon and B. Lukas,
Oklahoma State University, Stillwater, OK 74078

Addition of the cardiac antiarrhythmic propranolol to
myoglobin causes a change in visible light absorbance,
intensifying the absorbance at 536 nm. Similar absorbance
changes are observed in the presence of the local anesthetics
tetracaine and dibucaine but not the more water-soluble lido-
caine or procaine. Addition of dibucaine, tetracaine, or
propranolol (DTP) changes the spin state of Mb heme iron from
high spin (g=6) to low spin (g=2.9). This spin state change
is likely caused by the binding of the E7 histidine residue at
the sixth position of the heme iron as a result of DTP-induced
conformational changes. Addition of DTP to heart mitochondria
cytochrome oxidase causes a low spin to high spin state change
in the heme of cytochrome a and the appearance of a new
absorbance band at 420 nm. These changes appear with alter-
ations in the K_m for cytochrome c and indicate that DTP acts
at or near the heme of cytochrome a. CO recombination in the
oxidase following flash photolysis is not affected by these
drugs suggesting they do not affect cytochrome a₃. These
conformational and spin-state changes will alter the binding
of oxygen to myoglobin and will alter the reduction of oxygen
by cytochrome oxidase. These effects may be related to the
adverse effects of these drugs.

This research was supported by the Air Force Office of
Scientific Research, Air Force Systems Command, USAF under
grant number AFOSR 84-0264.

Blue lines are printer's cut lines, do not type on or outside of these lines

All compounds that are designated by code or initial letters in the title must be identified
adequately in the abstract, e.g., MJ-1999; 4-(2-isopropylamino-1-hydroxyethyl) methanesul-
fonanilide hydrochloride.

All investigators must adhere to the guidelines as listed on the reverse of the abstract form.

APS, BMES, SEBM, SMB, ASPET & AIN members only:

Signing member, are you willing to chair a session? ☐ Yes, category # _____

APS, AAP & AIN members only:

☐ Submitted for special Society student or training award. Type of award _____

MEMBER'S AFFILIATION (Check one only):

☐ APS ☒ ASBMB ☐ ASPET ☐ AAP ☐ AIN ☐ AAI ☐ ASCB ☐ BMES ☐ SEBM ☐ SMB

Submission of signed form indicates acceptance of rules including final withdrawal date of December 28, 1987.
No exceptions will be made.

H. JAMES HARMON
Member's Name (Print or type)

[Signature]
Member's Signature
(405) 624-5555
Member's Phone

H. James Harmon

REMITTANCE (NONREFUNDABLE)

ABSTRACT HANDLING FEE \$25

Payable to FASEB