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The inhibition of electron transfer steps in mitochondrial respiration have been studied. The effects of polyaromatic hydrocarbons such as benzene or acenaphthene, the local anesthetic dibucaine, and the cardiac beta-blocker propranolol on CO recombination to cytochrome oxidase following flash photolysis have been determined. The drugs and benzene/acenaphthene slow the rate of CO recombination. The energies of activation of CO recombination are altered. The most significant finding is the induction of bi-phasic kinetics by the drugs or solvents such as alcohol and the pH-dependence of those kinetics. A mechanism for the inhibition of CO recombination and the pH dependency of the kinetic parameters in both the control and drug-treated samples is discussed. The effects of local anesthetics dibucaine, tetracaine, and procaine and the beta blocker propranolol on ubiquinol-cytochrome c reductase activities has determined that dibucaine and propranolol inhibit electron transfer between cytochromes c_1 and c at all pH values; the drugs inhibit electron transfer between cytochrome b_{562} and cytochrome c_1 at pH values below 6.5. This study elaborates on the pH-dependency of the inhibition of electron transport reactions since proteins exposed on the cytoplasmic face are exposed to acidic conditions while those on the matrix face are exposed to alkaline conditions during oxidative phosphorylation and proton translocation. An understanding of the pH-effects of inhibitions by hydrophobic compounds is essential to understand their deleterious effects.

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EFFECT OF HYDROPHOBIC MOLECULES ON MITOCHONDRIA AND MITOCHONDRIAL PROTEINS

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Bradley K. Stringer, graduate student, August 1989-October 1990.

PRESENTATIONS AT SCIENTIFIC MEETINGS

1990 Effect of pH on CO Recombination in Cytochrome Oxidase. B.K. Stringer and H. James Harmon. Biophysical Society. Baltimore, MD Feb. 18-22, 1990.

1990 pH-Dependent CO Recombination in Cytochrome Oxidase. H. James Harmon and B.K. Stringer. American Society for Biochemistry and Molecular Biology (FASEB) New Orleans, LA June, 1990.

1991 pH-Dependent and Energy-Linked CO Binding to Cytochrome Oxidase in Intact Mitochondria. H. James Harmon and B.K. Stringer. Biophysical Society. San Francisco, CA, February 24-28, 1991.

PUBLISHED ARTICLES

1. "Inhibition of Cytochrome Oxidase by Dibucaine". Bradley K. Stringer and H. James Harmon. Biochemical Pharmacology, 40, 1077-1081 (1990) based in part on AFOSR 84-0264 findings.

2. "Effect of pH on CO Recombination to Cytochrome Oxidase in Intact Mitochondria". H. James Harmon and B.K. Stringer. FEBS Letters, 267, 167-170 (1990).

ARTICLES IN REVIEW

"Site of Inhibition and Additive Effects of Benzene, Naphthalene, Acenaphthene, and 1-Chloronaphthalene on Mitochondrial Respiration in Vitro" Andrew C. Beach and H. James Harmon. Chemical Research in Toxicology. In revision.

ANTICIPATED ARTICLES

1. "Effects of Benzene and Acenaphthene on CO Recombination to Cytochrome Oxidase"

2. "Effect of the Uncoupler CCCP on CO Recombination to Cytochrome Oxidase"

3. "Site of Inhibition of Dibucaine and Propranolol in Ubiquinol-cytochrome c Reductase (Complex III) in Mitochondria".

4. "Differences in CO Recombination to Cytochrome Oxidase in Intact Mitochondria and Submitochondrial Particles".

GOALS, AIMS, AND OBJECTIVES

The goals of this research were to identify the sites of action of hydrophobic molecules in the mitochondrial respiratory chain. The hydrophobic molecules include polyaromatic hydrocarbons such as naphthalene or benzene (which are present in the environment as pollutants but which serve an economically important role as a solvent and are found in fuels, fluids, etc.) and drugs such as local anesthetics or cardiac "beta blockers" which have an efficacious health usage but are also toxic to cells in high concentrations.

The goals were to determine the site of action as well as to determine possible mechanisms of inhibition and interactions with components of the mitochondrial respiratory chain.

STUDIES ON CYTOCHROME OXIDASE

CHARACTERISTICS OF CYTOCHROME 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 a_3 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 a_3 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 a_3 (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 a_3, 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 a_3 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 a_3 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).

Chance and co-workers defined functional oxygen intermediates in the mid 1970's (15-18); this applicant was fortunate to have been a post-doctoral researcher with Chance on this project. Oxygen intermediates have been recently reviewed (18,19) but will be quickly summarized below.

Following flash photolysis of carboxy-oxidase in the presence of oxygen, oxygen binds in place of CO. This intermediate with molecular oxygen bound but not reduced is termed Compound A and is analogous to the carboxy-oxidase. The spectrophotometric characteristics of Cmpd. A are similar to those of CO-oxidase with absorbances at 430 and 590 nm. The binding of O_2 is approximately 10-fold faster than binding of CO, however.

The transfer of electrons from the oxidase to O_2 results in the formation of "peroxy" compounds B; originally only one Cmpd B was noted, although multiple Compds. B exist corresponding to the number of electrons transferred to the oxygen (2). The rate of formation is measured by monitoring cyt a or the CuA signals at 604 nm, and 720 and 840 nm (cyt a and CuA, respectively).

Cytochrome oxidase is also the thermodynamic location of the third site of oxidative phosphorylation, the energy change (600 mV) associated with the oxidation of cytochrome c and the reduction of water being used for the ultimate phosphorylation of ADP. ADP is phosphorylated via the dissipation of the pH gradient and/or membrane potential generated by the respiratory reaction. Wikstrom (20,21) has deftly demonstrated that cytochrome oxidase is indeed a proton "pump," capable of translocating protons from the matrix to the cytoplasmic space, although the detailed mechanism of the translocation is as yet unknown. Two general models exist. The first is a "black box" pump that is energetically connected to the oxidase; the components of the pump are not necessarily components of the cytochrome oxidase redox system. The second model is where some pump components and respiratory

components are common; oxidation/reduction of a component involves the binding/release of protons on opposite membrane faces. The precedent of the latter translocating mechanism is the proton-motive Q-cycle in the cytochrome b-c_1 region of the "second site" of phosphorylation in which ubiquinone migrates from matrix fact to cytoplasmic in protonated form and migrates from cytoplasmic to matrix in non-protonated form, effectively translocating protons (22). Removal of subunit III of the oxidase reduces the proton-translocation of the oxidase by 50% (23), suggesting that part of the "pump" mechanism is intimately associated with, if not part of, the oxidase itself (yet the abolishment of only 50% suggests a "black box pump" may also be operative).

The involvement of a subunit of cytochrome oxidase suggests that proton translocation involves binding/release of protons from the oxidase subunits and that this may involve or result in kinetic altering conformational changes in the oxidase itself.

CYTOCHROME OXIDASE-OXYGEN INTERMEDIATES

The study of the formation of oxygen intermediates of the oxidase at low temperatures was begun by Chance and co-workers (11,16,17,24). (The applicant was a post-doctoral researcher with Chance in the mid-70's). To date, studies on the effects of pH on the formation of oxygen intermediates have not appeared in print although numerous articles on the spectrophotometric characteristics of the oxygen intermediates have been published. (see 19,25 for review).

Only a handful of workers are investigating the oxygen intermediates; nomenclatures of the oxygenate compounds vary as do the names of the different states of the enzyme prior to its use. Chance and co-workers favor the use of Compounds A, B, etc., while others favor use of compound 1, etc. We will adopt the Chance nomenclature since the applicant is familiar with that terminology. The original works (15-17) listed three compounds A-C. Compounds A are the oxygen-bound but not reduced intermediate, analogous to carboxy oxidase. Compound A_1 is formed from fully ferrous oxidase while Compound A is formed from the mixed valence form (ferricyanide oxidizes cyt a and CuA after CO is added). The spectrophotometric characteristics of Cmpds A are almost identical to those of the carboxy compound with an absorbance at 590 nm. Unlike carboxy oxidase, compound A is not readily photolyzable. In addition, the rate of formation of Cmpd A is faster than that of CO-oxidase although the energies of activation are similar (about 9.5 kcal/mol).

ENERGY-LINKED ASPECTS OF OXIDASE-OXYGEN INTERMEDIATES

Harmon and Sharrock (26) and Harmon and Wikstrom (27) published the effects of energization and uncoupling of respiration from oxidative phosphorylation on the rates of CO and oxygen binding in intact coupled pigeon heart mitochondria. The conclusions from that work are:

a. Energization of the membrane by ATP hydrolysis results in a doubling of the rate of CO or oxygen binding.

b. This rate increase is not due to a change in the energy of activation of the ligand binding, but an increase in the number of ligand molecules located immediately adjacent to the heme iron (the greater the concentration of ligand, the faster the rate of binding!)

c. The authors assumed that an energy-linked conformational change was responsible for the data.

These data have been repeated in this study in intact beef heart mitochondria; uncoupling changes the rate of binding by changing the regions near the heme. The 1977 studies, however, did not consider any effects due to the binding of protons to sites on the "proton pump" and/or any conformational proton- (and hence pH-) dependent changes.

In addition, Harmon and Wikstrom (27) demonstrated that membrane energization accelerated the rate of electron transfer from the oxidase (oxidation of oxidase centers) to the bound oxygen. Specifically, the rate of compound B formation involving the oxidation of cytochrome a and its associated visible copper center are accelerated as a result of ATP hydrolysis. Again, the role of protons are ATP hydrolysis-generated pH differences across the membrane or ATP hydrolysis generated proton binding/release was not considered.

The current level of understanding of the kinetic rates at low temperature is made possible by the use of computerized acquisition of spectrophotometric changes and the ability to fit the raw data to a mathematical function using computer programs. While we were able to obtain kinetic rates at 5-7 different temperatures in 1977, we routinely obtain computer-fitted data at approximately 30 different temperatures, allowing 1-2 degree temperature resolution (using the same sample). As a result, our current data allows interpretation of the binding kinetics at a level not possible 15 years ago; our experience with the low temperature kinetics (obtained while a post-doc with Dr. Chance), our experience in isolating highly active highly oriented membrane vesicles, and our computerized spectrophotometric abilities place this facility in a unique position to obtain these data on proton interactions with cytochrome oxidase.

PROCEDURES

MITOCHONDRIA AND PARTICLE ISOLATION

Mitochondria were isolated from heart tissue by the method of Harmon and Crane (8). Submitochondrial particles were isolated from beef heart mitochondria as described by Harmon (28); uncoupled totally inverted electron transport particles (ETP) will be isolated as before (8) and used to determine topographically-dependent pH-dependent kinetics without the contribution of and problems presented by a coupled respiratory membrane.

CO RECOMBINATION

CO binding to fully reduced oxidase was performed as described previously (26,29,30). CO recombination to reduced oxidase was measured at 446 nm with a Gilford 252 single beam spectrophotometer coupled to a 16-bit A/D converter in an IBM PC computer. A clear glass dewar with a 2 mm light path plexiglas sample holder as used previously (26,29,30) was fitted in place of the normal cuvette holder. The sample is suspended above liquid nitrogen in the dewar; the temperature is measured by a copper-constantan thermocouple in the sample and regulated by a small heater coil in the liquid nitrogen.

Low temperature kinetics are initiated by a single flash of 1500 BCPS xenon flash tubes. For measurement in the Soret (400-450 nm) region, the xenon tubes are fitted with Wratten #9 and #15 filters to pass light of 500 nm or longer light to the sample. A Corning 5113 filter is placed in front of the photomultiplier to block wavelengths above 500 nm (from the flash) but pass the 446 nm measuring beam light. This system has been used extensively and successfully in this laboratory (29,31,40,41).

Fully reduced mitochondria are formed by the addition of 5 µgm TMPD, 90 mM ascorbate, and 20 mM succinate to a 1% or 100% CO-flushed mixture of cytochrome c and mitochondria in 0.25 M sucrose-50 mM sodium phosphate buffer (pH 0.25 m). The mixture is bubbled with CO in the dark for 20 minutes and then loaded in to the sample holder in the dark prior to freezing in liquid nitrogen.

Following flash photolysis of the oxidase-CO complex at time zero, CO recombines to the oxidase, resulting in a decrease in 446 nm absorbance (ferrous cyt a_3 absorbs at 446 nm).

$$a^{2+}a_3^{2+}CO <-----> a^{2+}a_3^{2+} + CO$$

The computed least squares fit of the data indicates that the time-dependence of the curves is exponential and can frequently 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; k is proportional to the rate of recombination. The energy of activation will be determined by plotting log k vs. inverse temperature. The occupancy (number of CO molecules that can be held in an intermediate region near the heme) will be determined from the dependency of the rate constant on the CO concentration as described previously by measuring CO rebinding the presence of 1% and 100% CO (26).

Some kinetic measurements cannot be fitted adequately to a single exponential function (as determined by plotting the autocorrelation function of the residuals of the fit vs. time) and are fitted to a double (or if needed, triple) exponential function

$$f(t) = e^{-k1t} + e^{-k2t}$$

where k_1 and k_2 represent the rate constants of the two separate kinetic phases of the reaction. All fitting of raw data is performed using the KINFIT program of On-Line Instrument Systems, Inc. of Jefferson GA.

EXPERIMENTAL FINDINGS

The reduction of oxygen requires the close proximity of three substrates: electrons delivered to oxygen by the cytochrome a_3 /CuB binuclear center, oxygen which is bound to the iron of cytochrome a_3, and protons. Since the role of the active site is to spatially concentrate the substrates to enhance their collisional interaction, it is logical to expect that protons will interact at or very near the heme. This provides the basis for our initial studies on the effects of pH on the binding of CO, an oxygen analog, to cytochrome a_3. While a proton is not consumed as a substrate in the binding of CO/oxygen, we cannot rule out that protonation/deprotonation of a nearby residue will not alter the binding kinetics or, alternatively (and supported by preliminary data) that a proton binds to the oxidase in conjunction with, or possibly as a result of, ligand (CO/oxygen) binding.

EFFECT OF pH ON CO RECOMBINATION IN INTACT MITOCHONDRIA

CO recombination to oxidase has previously been thought to be independent of the pH of the suspending medium. We now know that this view is not correct [Harmon and Stringer, FEBS Letters 267, 167-170 (1990) enclosed as reprint]. Figures 1-3 show the

Arrhenius plots of CO recombination to reduced oxidase at pH 7.4, 5.5, and 9.0, respectively, in intact mitochondria. A pH 7.4 (Fig. 1), a single slope is observed in the plot of log rate constant k vs. 1/T in the presence of 1% CO and 100% CO. The two slopes convey different pieces of information concerning CO recombination. In the presence of 1% CO, only one CO is present per oxidase on average; after photolysis, recombination of the single (previously bound) CO with the heme iron requires crossing thermodynamic barriers separating intermediate CO-holding (not binding) regions. The rate of CO movement from one region to another is a function of the concentration of CO present as well as the height of the energy barriers encountered. Increasing the number of CO molecules in a region will increase the concentration of CO and the rate of CO migration from that region, assuming the barrier heights are not altered by the increased CO concentration. The concentration dependence of the value of k indicates the number of CO molecules present in each intermediate region (the occupancy). If a region can hold 2 CO, then the rate of CO migration from that region will be proportionally faster. The binding of CO to the oxidase is not truly second order, since a 100-fold increase in CO concentration results in only a two-fold increase in binding rate. Thus the Arrhenius plot in the presence of 1% CO indicates a minimum number of barriers and defines their heights while plots in the presence of 100% CO indicate the occupancy or number of CO molecules in the intermediate regions separated by the barriers. This interpretation of the data is consistent with previous interpretations by this and other investigators [28,30,31] with cytochrome oxidase.

We interpret the results by the reaction coordinate models (included as insets of Figures 1-3) as in previous reports with cytochrome oxidase [26,29,32] and myoglobin [33,34]. CO migration to the heme at pH 7.4 entails crossing one or more barriers of 10.5 kcal/mole from one or more intermediate regions that can hold 2 CO maximally. Since the kinetics we would observe with one barrier/region are indistinguishable from those with multiple identical barriers/regions, we will assume 3 barriers and regions (other than the heme and bulk solvent denoted "M", the barrier to which is temperature-dependent) to be consistent with the number of barriers observed at pH 9.

At pH 5.5, CO must cross at least three barriers. The innermost barrier (and the last to be frozen out at low temperatures) is 11.3 kcal in height and separates the Fe from the innermost region I; region I can contain 2 CO. Between 205K and 210K, CO molecules migrate from region I_2 which has an occupancy of 1 CO since the values of k are the same in 1% and 100% CO; additional identical regions may be present. An outermost region I_3, which because of its larger k-value in 100% CO has an occupancy of 2, is separated from I_2 by a 7.1 kcal barrier. Migration to the heme from I_3 via the other intermediate regions occurs above 215K.

At pH 9, the presence of up to three slopes with 1% CO indicates a minimum of three energy barriers. The values of k are greater with 100% CO only above 210K. Thus inner regions I, I_2, I_3 can each hold 1 CO and are separated by 17.5 (Fe-I) and 9.6 kcal/mole (I-I_2 or I-I_3) barriers. Regions I_2 and I_3 are distinguishable because of their difference in occupancy, not their difference in barrier heights.

The pH-dependent changes observed here are likely due to pH-dependent conformational changes and not due to translocated protons since proton translocation cannot occur without enzymic turn-over. Alternatively, the changes observed may be related to the binding of substrate protons at alterations in barrier size and region occupancy could be due to protonation/deprotonation of 1 or more amino acid residues in the pocket or channel to the protein exterior. Dost et al [34] observed pH-dependent CO binding to myoglobin and the beta-chain of hemoglobin, attributed to a single titratable residue with a pK of 5.7, likely the distal histidine. That the plots between pH 6 and 8.5 are not different

than those observed at pH 7.4 (in agreement with studies by Wohlrab and Ogunmola [35]) suggests that the change at pH 5.5 may be due to deprotonation of a histidine residue. Fabian and Malmstrom [36] and Papodopoulos et al [37] have suggested that changes in Soret absorbance maxima may be due to protons associating with the histidine of cytochrome a3 .

Oliveberg et al [38] reported that the effects of pH on intra-oxidase electron transfer in mixed valence isolated oxidase were not due to pH dependence on the formation of the "oxy" form, although the authors conceded a large experimental uncertainty in their measurement of rates from room temperature flow-flash experiments. The stimulation of both CO and oxygen binding to reduced pigeon heart mitochondrial oxidase following "energization" by ATP hydrolysis suggests that oxygen binding closely parallels CO binding [6,27,28]; we thus expect that the formation of the oxygenated ferrous intermediate compound A [15] would also show pH-dependent rates of formation at low temperatures; those experiments are being performed.

It is unlikely that the binding of CO involves a "substrate" proton, but it is possible that one or more protonatable groups are present in the CO-binding path in the oxidase; during oxygen reduction protonation/deprotonation may induce an actual physical barrier as seen by Frauenfelder and co-workers with myoglobin [34]. A second alternative is that a pH-dependent conformational change may be responsible for the kinetic differences.

EFFECT OF UNCOUPLER ON CO RECOMBINATION IN MITOCHONDRIA

The results obtained in intact mitochondria (BHM) at different pH values could represent energy-linked effects as well as non-coupled kinetics. To measure the energy-linked effects, uncoupled rates were measured after the addition of 0.84 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) in ethanol (with EtOH in "control"). The uncouplers will also abolish transmembrane pH differences. The matrix pH will correspond to that of the suspending medium: thus the effect of pH on both membrane surfaces (instead of just the exterior surface) can be determined. Differences in kinetic parameters between control and uncoupled states will represent energy-linked effects as well as conformational and substrate effects of protons on the now accessible M-face.

Data in Figures 4-11 indicate that the raw absorbance vs. time data can be fitted to either a single exponential (with non-linear residuals) or to 2 simultaneous exponential changes with respect to time. If we fit to 2 exponentials, we note a 5-fold (minimum) difference in rate constants between a slower and a faster phase. Less than 30% of the total absorbance change in BHM can be attributed to the fast phase. Hence, rate constants of the single exponential and the slow phase of the 2 exponential fits are similar. We shall examine the data first in a simplistic single phase/exponential manner and then as a two phase/exponential process.

RESULTS BASED ON SINGLE PHASE KINETICS

The value of the absolute rate constants at 200 K are plotted vs pH in Fig. 4. In general, the rate of CO recombination is faster at lower pH values in both the presence and absence of uncoupler CCCP, although the decrease is grater at pH 5.5. While the k-values are pH-dependent, the energy of activation (Ea) in the absence of CCCP is independent of pH. In the presence of 0.84 μM CCCP, an increase in Ea occurs only at pH 9.0 (cf. Fig. 5). Overall, the data indicate that the rate of CO recombination is higher at lower pH. In the presence of CCCP, CO recombination is disfavored at pH 9 by the combined effects of a lower rate constant and higher Ea than at lower pH values.

RESULTS BASED ON TWO PHASE EXPONENTIAL KINETICS

Slow Phase

Because at least 70% of the raw data is possibly due to the slower phase, the single phase and slow phase results are similar. In both the presence and absence of CCCP, the rate at 200K is pH-dependent with faster rates at lower pH (cf. Fig. 6); the rates in the presence of uncoupler are approximately 50% of the control (no CCCP) rates. While differences in k-values with 1 and 100% CO present are not observed, CO-concentration dependent differences in Ea are observed In control samples, the Ea at pH 9.0 in the presence of 1% CO is greater than at pH 5.5 or 7.4 (cf. Fig 7); in contrast, the Ea in 100% CO at pH 5.5 is markedly greater than in 1% CO or at pH 7.4. In the presence of CCCP, CO concentration dependent differences are not observed; Ea's at pH 5.5 and 7.4 are similar to each other while Ea at 9.0 is about 10% higher. These data are similar to the single phase kinetics in the presence of CCCP (Fig. 5).

Fast Phase

The k-values of the fast phase in mitochondria are 5-fold greater than k-values of the slow phase. In the absence of CCCP, the k-values are reasonably similar except for the marked decrease in k-values in 1% CO at pH 9.0, indicating the reaction is disfavored at pH 9 (cf. Fig. 8). The occupancy of the fast phase is at least 2 and increases to 4 at temperatures above 230 K at pH 9. The k-values are smaller in the presence of CCCP, show a slight increase with decreasing pH, and exhibit no CO concentration-dependent differences, the rates in 1% and 100% CO being the same (cf. Fig. 9).

The energies of activation in the EtOH control are not constant with pH; significant Ea increases are seen in 1% and 100% CO at pH 9, as seen in single phase kinetics (Fig. 10). In contrast, the marked increase in Ea at pH 9.0 is not observed in the presence of CCCP and is not affected by CO concentration (cf. Fig. 11).

The simplest interpretation of these findings will come from the single phase data and are supported by the two phase findings. In general, CO recombination is more rapid at low pH in the presence/absence of uncouplers. Ea is generally higher at pH 9; CCCP abolishes the Ea increase in the slow phase. The single and double phase data differ in the effect of CCCP at high pH, due either to uncoupling, the conformational effect of pH on the M-side of the oxidase when CCCP allows pH equilibration with the matrix, or both.

The increase in rate at low pH could be due to:

1. A pH-induced conformational change (independent of proton pumping or proton substrate)

2. Binding of a proton that is ultimately involved in ligand binding and oxygen reduction, or proton pumping.

The increase in Ea at pH 9 in the presence of CCCP could be due to the contribution of matrix-based pH effects now that matrix and medium (cytoplasmic) sides are at the same pH.

The possible contribution of pH effects on oxidase components exposed on the matrix face can be determined by examining the effects of altering pH on CO recombination in coupled and uncoupled submitochondrial particles (SMP), as follows.

pH-EFFECTS ON CO RECOMBINATION IN SUBMITOCHONDRIAL PARTICLES

Preliminary data on CO binding kinetics in SMP at pH 5.5, 7.4, and 9.0 in the presence/absence of uncoupler show a very different picture than in mitochondria. While addition of CCCP to BHM results in no change in Ea and a less than 10% decrease in rate constant at 200 K at pH 5.5 and 9, CCCP doubles the rate at pH 5.5 (but not at 9.0) in SMP. At pH 9 and 5.5, CCCP decreases the Ea approximately 15%, unlike the effect on BHM. The rate constants at pH 9 are approximately the same in SMP and BHM, but the control (absence of CCCP) rate at pH 5.5 is approximately 1/3 than in BHM.

Table 1
Effects of pH on CO Recombination in SMP

	pH 5.5		pH 7.4		pH 9.0	
	-CCCP	+CCCP	-CCCP	+CCCP	-CCCP	+CCCP
1% CO E_a (kcal/mol)	9.8	8.8	9.8	N.D.	10.6	8.5
k_{200k} (sec^{-1})	0.075	0.15	0.07		0.054	0.057
100% CO E_a (kcal/mol)	10	8.8	---	10.1	10.5	9.6
k_{200k}(sec^{-1})	0.09	0.14	---	0.07	0.07	0.065

These results reinforce the findings we obtained from BHM and indicate topographical differences in the kinetics, particularly at pH 5.5. In BHM, addition of CCCP slightly decreases the rate constants at 200 K. The rate in coupled SMP is approximately 35% that in BHM and the rate in the presence of CCCP approaches the rate in BHM with CCCP. When the matrix face of BHM is allowed to be pH 5.5 due to uncoupler, the rate decreases due to the contribution of the slower kinetics of the M-face (SMP). Addition of CCCP to SMP allows the faster C-side (BHM) kinetics to contribute to the overall kinetics, increasing the rate constant.

This data and the conclusions may explain the existence of the double phase kinetics; kinetics of one face is slower than the other. The data support our earlier conclusion that proton binding on the C-side results in faster CO binding kinetics; we expect to observe accelerated oxygen binding kinetics as well (based on earlier similarities between CO and oxygen binding kinetics; cf. refs 28,29) that could result in accelerated formation of compound B (assuming the electron transfer rates in the oxidase are pH-independent; this is unlikely).

Recently acquired data on the effect of pH on CO recombination in uncoupled non-energy-linked electron transport particles await detailed calculation and analysis.

EFFECT OF POLYAROMATIC HYDROCARBONS ON MITOCHONDRIAL RESPIRATION

This work was performed in the time period betwen the end of AFOSR 84-0264 and the initiation of the current AFOSR 89-0458 grant. Certain aspects of the data analysis were perfomed during the AFOSR 89-0458 time period and the manuscript (enclosed as a pre-print) is included in this report.

The research investigated the effects of benzene, acenaphthene, naphthalene, and 1-chloronaphthalene on mitochondrial respiration. The results can be summarized below (data in the enclosed pre-print):

1. All 4 compounds inhibit respiration in the ubiquinol-cytochrome c reductase (Complex III) portion of the mitochondrial respiratory chain.

2. The concentration needed for 50% inhibition is linearly dependent on the log of the partition coefficient.

3. Acenaphthene, naphthalene, benzene, and 1-chloronaphthalene inhibit respiration at coenzyme Q (ubiquinone).

4. Except for benzene, the alteration of the UV absorbance spectrum of ubiquinone is altered at the a ratio of perturbant to ubiquinone corresponding to the perturbant/ubiquinone ratio found in mitochondria at the 50% inhibitory concentration of the PAH.

5. We conclude that naphthalene, acenaphthene, and 1-chloronaphthalene inhibit by altering the electron distribution in ubiquinone, altering its ability to accept/donate electrons in the respiratory chain.

EFFECT OF BENZENE ON CO RECOMBINATION IN MITOCHONDRIA

In a continuation of studies begun in AFOSR 84-0264, the effects of 15 ppm benzene dissolved in ethanol were measured at pH 7.4 (cf. Table 2). The results are summarized below:

1. The occupancy of the oxidase is unity. The occupancy in the presence of ethanol is also one; there is an apparent solvent effect that changes the occupancy of the regions but not the energies of activation (compare no ethanol to ethanol-containing controls).

2. The energies of activation in 1% and 100% CO increased markedly to 11.3 kcal/mole (average).

3. The rate constants are 50% those in the presence of ethanol only; the rate of CO recombination is slower.

4. A fast recombination phase is present but represent only <10% of the total activity and is not considered significant.

5. From previous studies (AFOSR 84-0264) the K_m of the oxidase for cytochrome c as substrate decreases 50% (from 13.1 uM to 6.5 uM) while V_{max} decreases 40%.

TABLE 2. EFFECTS OF ACENAPHTHENE, BENZENE, AND BATHOCUPROINE ON CO RECOMBINATION TO CYTOCHROME OXIDASE IN MITOCHONDRIA

		pH 5.5		pH 7.4				pH 9.0
		EtOH	Bathocuproine	Bathocuproine	EtOH	Acenaphthene	Benzene	EtOH
Single Phase								
	E_a 1%	7.47			8.21	10.76	11.2	9.42
	k_5 1%	0.27			0.13	0.053	0.049	0.063
	occ cold	1			1	1	1	1
	occ warm	1			1	1	1	1
	k_5 100%	0.215	0.42	0.112	0.12	0.066	0.056	0.07
	E_a 100%	8.76	7.7	9.51/7.8	9.22	10.56	11.4	10.05
Double Phase			No			No		
1%	Slow %	~70%			~75%			>80%
	E_a slow	8.23			8.91			9.96
	E_a Fast	6.89			6.93			8.85
	k_5 Slow	0.165			0.075			0.043
	k_5 Fast	1.1			0.7			0.31
	Fast/Slow	6.7			9.3			7.2
100%	Slow %	~75	65		~80%			~85%
	E_a Slow	9.7	7.77	8.58	10.53			11.7
	E_a Fast	8.29	5.65	7.1	9.6			14.81
	k_5 Slow	0.15	0.28	0.074	0.066			0.058
	k_5 Fast	0.85	2.1	0.62	0.6			0.82
	Cold OCC	1			?			?
	Warm CCC	1						
	Fast/Slow	5.7			9			14

E_a 1% = energy of activation with 1% CO
E_a 100% = energy of activation with 100% CO
k_5 = rate constant at 200K; units are sec^{-1}

Thus the decrease in V_{max} of oxygen reduction is substantiated by the parallel decrease in CO binding rates. Since CO binding is analogous to oxygen binding and thus far the effects are the same with either ligand, the decrease in oxidase activity is due to a decreased rate of ligand binding to the oxidase.

EFFECT OF ACENAPHTHENE ON CO BINDING

The effects of 15 ppm acenaphthene dissolved in ethanol were also measured at pH 7.4; the results are summarized below (cf. Table 2).

1. No double exponential 2-phase kinetics were observed.

2. The energy of activation increased by 2.5 kcal/mole.

3. The occupancy of all intermediate region(s) is one (1).

4. The values of k are decreased to 40-50% that in ethanol; the rate of CO binding is decreased.

5. From previous studies (AFOSR 84-0264) we observed that the values of K_m and V_{max} are unchanged. This means that the rate of electron transfer within the oxidase must be stimulated by acenaphthene to overcome the 50% inhibition in activity caused by the inhibition of ligand binding rate.

The net effect of benzene and acenaphthene on cytochrome oxidase is to decrease the rate of CO (or oxygen) binding to cytochrome a_3. The decrease in rate of oxygen binding will necessarily result in a decrease in oxygen reduction and electron transfer in the mitochondrial respiratory chain; that will result in a decrease in proton translocation and a decrease in ATP production in exposed cells.

WHY STUDY THE INHIBITION AT DIFFERENT pH VALUES?

The mitochondrion is not static. During oxidative phosphorylation, protons are translocated (extruded) from the mitochondria, alkalinizing the matrix space and acidifying the intermembrane space between the inner and outer membranes. Thus, during normal operation of the mitochondria, those proteins/redox centers on the cytoplasmic side will experience a different set of conditions than proteins on the matrix face. Our first indication of pH-dependent kinetics of the CO recombination to oxidase and the different extents of inhibition at different pH's suggested that pH would affect the proteins as much as it would affect the drugs/compounds (by virtue of their pK's).

Several mitochondrial redox centers, such as cytochrome a and the Rieske Fe-S center of complex III, are known to have pH-dependent oxidation-reduction midpotentials. Since the oxidase uses electrons, oxygen, and protons as substrate in the reduction of oxygen to water, it is believed that the oxidase is pH-dependent in its kinetics and structure (although both are unknown at this time).

Polyaromatic compounds such as benzene are hydrophobic and non-protonatable. Local anesthetics and drugs such as propranolol do indeed have pH-dependent groups with pK values of the nitrogenous groups in the pH 8.5-9 range. The observance of pH-dependent inhibitions in ubiquinol-cytochrome c reductase prompted us to look at other possible pH-dependent effects, particularly in the oxidase (where, we were surprised to

find large and kinetically-relevant effects). Thus, from the studies it will be apparent that, particularly in bioenergetic systems, the effect of exposure to hydrophobic or amphipathic molecules (such as the local anesthetics) is probably more a function of the pK of the proteins and their processes (which occur in the more physiological pH 5.5-8.5 range) that on the pK of the compounds (pK's often outside the physiological range).

EFFECTS OF DIBUCAINE AND PROPRANOLOL ON CO RE-BINDING KINETICS TO CYTOCHROME OXIDASE AT DIFFERENT pH VALUES

Previous data, presented in final report of AFOSR-84-0264 and published in part in Biochemical Pharmacology 40, 1077-1081 (1990) indicated the site of interaction and inhibition of the drug dibucaine (and tetracaine and propranolol; cf; technical report AFOSR 84-0-264) at the detectable CuA center associated with cytochrome a of the oxidase; interaction of the drugs with the IR-undetectable CuB center associated with the oxygen (and CO) -reactive binuclear center of the CuB and cytochrome a_3 could not be directly measured but was suggested on the basis of the formation of a high-spin g=6 EPR signal due to the disruption of the antiferromagnetic coupling between CuB and cytochrome a_3 that is responsible for the lack of spectral signal of the CuB and the limited spectral characteristics of cytochrome a_3. Since binding of oxygen and its analog carbon monoxide involves the participation of the CuB center, it was of interest to investigate the effects of the drugs on the CO recombination kinetics to determine the effect of the drugs on the copper and possibly utilize the effects of the drugs to better understand the operation of the oxidase.

The experiments described earlier with the uncoupler CCCP dissolved in ethanol required the use of a control assay with only ethanol present. The correct control for the experiments just described is the assay without ethanol since the hydrophilic water soluble hydrochloride forms of the drugs were used. In the absence of ethanol or other hydrophobic molecules, the rebinding of CO after flash photolysis follows a single exponential time course. In the presence of drugs under certain conditions, the data are best described by a double exponential expression.

PROPRANOLOL SINGLE EXPONENTIAL KINETICS

Propranolol (2 mM; concentration at which 70% inhibition of cytochrome oxidase activity occurs) causes a slight (< 1 kcal/mole) decrease in the energy of activation (E_a) of the CO binding at pH 5.5 and 9.0 and slightly increases the E_a at pH 7.4 (cf. Table 3). The rate of CO binding increases at pH 5.5 by approximately 30% compared to the control with little change at higher pH in the presence of 1% CO.

In the presence of 100% CO, only slight decreases in E_a are observed; a 20-25% decrease in rate (as defined by the rate constant k at 200 K) is observed such that the rates determined with 100% CO are very similar to those with 1% CO.

The overall effect is that the rates in the presence of 1% and 100% CO are similar. Data from the control samples indicates that the number of intermediate regions and the CO occupancy number of those regions is dependent on the pH of the suspending medium (See figures 1-4 and the enclosed FEBS Letters reprint). In the presence of propranolol, two intermediate regions other than the heme iron are hypothesized at all 3 pH values tested. The innermost region contains only 1 CO at all pH values, in contrast to the two CO held in this region at pH 5.5 and 7.4 in the untreated control samples; the occupancy of the innermost region at pH 9 remains at unity. In the presence of the drug, the other outermost

TABLE 3. EFFECTS OF DIBUCAINE AND PROPRANOLOL ON CO RECOMBINATION TO CYTOCHROME OXIDASE IN BEEF HEART MITOCHONDRIA

		pH 5.5			pH 7.4			pH 9.0		
		H_2O Control	2mM Propranolol	2mM Dibucaine	H_2O Control	2mM Propranolol	2mM Dibucaine	H_2O Control	2mM Propranolol	2mM Dibucaine
Single Phase										
	E_a 1%	10.5	9.45	9.74	9.15	9.43	9.67	10.14	9.85	9.32
	k_5 1%	0.055	0.07	0.064	0.066	0.064	0.062	0.06	0.05	0.069
	occ cold	3	1	1	2	1	1	1	1	1
	occ warm	2	1	1	2	2	1	2	2	2
	k_5 100%	0.096	0.08	0.076	0.09	0.077	0.084	0.084	0.066	0.091
	E_a 100%	11.1/7.6	10.3	10.1	10.65	10.26	9.87	10.94	10.59	10.03
Double Phase										
1%	Slow %	---	65		---	---	---	---	---	---
	E_a slow	---	9.72	9.74	---	---	---	---	---	---
	E_a Fast	---	7.15	5.57	---	---	---	---	---	---
	k_5 Slow	---	0.045	0.052	---	---	---	---	---	---
	k_5 Fast	---	0.26	0.34	---	---	---	---	---	---
	Fast/Slow	---	~5	~5	---	---	---	---	---	---
100%	Slow %	---	40%	65%	---	---	55%	---	---	80
	E_a Slow	---	10.5	10.3	---	---	11.1	---	---	10.79
	E_a Fast	---	8.1	9.36	---	---	9.05	---	---	7.13
	k_5 Slow	---	0.051	0.065	---	---	0.056	---	---	0.05
	k_5 Fast	---	0.265	0.305	---	---	0.225	---	---	0.404
	Cold occ	---	1	1	---	---		---	---	
	Warm occ	---	1	2?	---	---		---	---	
	Fast/Slow	---	~5	~5	---	---	~4	---	---	~8

E_a 1% = energy of activation with 1% CO
E_a 100% = energy of activation with 100% CO
k_5 = rate constant at 200K; units are sec^{-1}

region closest to the medium can contain 2 CO just as in the control, but only 1 CO is found in the outermost region at pH 5.5 (2 CO are held there in the control).

DIBUCAINE SINGLE EXPONENTIAL KINETICS

The size of the energy barriers (discerned form the E_a's in the presence of 1% CO) decreases < 1 kcal/mole at pH 5.5 and 9.0 and increase about 0.5 kcal/mole at pH 7.4. The rate of CO binding in 1% CO at pH 5.5 and 9.0 is stimulated about 15% compared to the control (cf. Table 3). In the presence of 100% CO, E_a's at pH 7.4 and 9 decrease < 1 kcal/mole while the value of k at 200 K decrease about 20% at pH 5.5, is relatively unchanged at pH 7.4, and increases only 10% at pH 9.0.

We interpret the results in terms of a 2 barrier/intermediate region model (barrier heights determined from the energy of activation with 1% CO and occupancy from the stimulation of rate constants in the presence of 100% CO). The lack of significant increase in rate of binding at pH 5.5 and 7.4 in the presence of 100% CO indicates that both the innermost and outermost non-Fe CO holding regions can hold only 1 CO (in contrast to the 2 CO in each in the control at pH 5.5 and 7.4). At pH 9.0, the inner region holds 1 CO and the outer region holds 2 CO, as in the control.

Thus, the main effect of these drugs is to alter the occupancy of the intermediate regions at pH 5.5 and 7.4 but not at pH 9.0; the effect is a pH-dependent alteration. The occupancy change at pH 5.5 and 7.4 could be due to either a conformational change in the heme pocket area that affects both inner and outer regions at pH 5.5 and 7.4 such that only sufficient volume is available for only 1 CO or that the drug binds near the heme and sterically occupies space that is normally available for the second CO in that area. In either case, an apparent pK of the effect seems to be in the pH 7.4-9 region, and may be due to the drugs binding at a tyrosine or cysteine residue. It is of interest that the copper of the oxidase is held via Cu-S ligands which would show a pK of approximately pH 8. It is likely that the drugs act at a deprotonated cysteine of the CuB of the binuclear center.

DOUBLE EXPONENTIAL KINETICS IN THE PRESENCE OF DRUGS

The most striking effect of treatment with the hydrophobic drugs is the inducement of 2-phase kinetics; 35-50% (depending on conditions) of the rebinding occurs at a rate about 5-fold faster than the slower more predominate phase. A typical single and double phase Arrhenius plot is shown in Fig 12; the data are presented in Table 3. Propranolol induces 2-phase kinetics at pH 5.5 only. Dibucaine induces 2-phase kinetics at pH 5.5 in 1% and 100% CO and at pH 7.4 and 9.0 only in the presence of 100% CO where the stoichiometry of CO/aa_3 is more than 1 (one) (in general, with 1% CO and the mitochondrial protein we use, 1 CO molecule is present per oxidase molecule; that one CO molecule is bound at the heme Fe).

A description of the effects of dibucaine and propranolol on inducing two-phase kinetics requires comparison to a control sample. Unfortunately, only single phase kinetics are seen with water as a control (the drugs are made in water). Thus we are forced to use an imperfect "control" in the form of the ethanol-containing control use with the assays involving uncouplers, benzene and acenaphthene. The results can be summarized below:

1. The fraction of activity due to the faster phase is greater in the presence of the drugs than with ethanol alone except at pH 9.0 where less than 20% of the activity is due to the fast phase, the remainder due to the slower phase (which resembles the single phase kinetics, as expected).

2. At pH 5.5, the values of k in 1% and 100% CO are about 30% those seen with ethanol; the drugs inhibit the rate of CO recombination. At pH 7.4 and 9.0, the value of k at 200K for the slow phase with 1% and 100% CO is similar to that seen with EtOH, but the k-value with 100% CO is 30-50% that seen in ethanol.

3. The energies of activation of the slow phase in 100% CO are similar in the presence of the drugs or EtOH at pH 5.5 and 7.4, but the energy of activation of the fast phase at pH 9.0 is twice as large as with ethanol or with dibucaine-HCl.

4. No double exponential fits are observed at pH 7.4 or 9 in the presence of 1% CO.

EFFECT OF BATHOCUPROINE ON CO BINDING KINETICS

Preliminary studies were begun (but have not yet been completed) on the effects of bathocuproine, a potent hydrophobic copper-specific chelator which has been shown previously by this investigator (Harmon and Crane, BBA 368, 125-129, 1974) to inhibit cytochrome oxidase at a concentration of about 14 μM by preventing the reduction of the detectable copper of the oxidase (and presumable by acting at the undetectable as well). In this manner it acts similarly to the dibucaine, tetracaine, and propranolol but at a much lower concentration (14 μM instead of 1-2 mM). It is of interest to see if the effects of the hydrophobic drugs is similar to the effects of bathocuproine. The effects are summarized below and in Table 2.

SINGLE EXPONENTIAL FITS

1. At pH 7.4, the values of k and the energy of activation are not altered (remember that this is in the presence of ethanol and the kinetics in the presence of 1% and 100% CO are the same (occupancy of 1).

2. At pH 5.6, the energy of activation is 1 kcal/mole less than in control, but the value of k at 200K has doubled; CO binding is faster!

DOUBLE EXPONENTIAL FITS (seen with 100% CO only)

1. At pH 7.4, energy of activation decreases about 2 kcal/mole for both the fast and slow phase but the k-values at 200 K are unaffected (but slower at colder temps due to decreased Ea).

2. At pH 5.6, energy of activation decreases 2-3 kcal/mole; k-values increase 2-2.5 fold.

The results with bathocuproine are clearly different on many aspects. We suspect that this metal chelator is acting directly at the Cu atoms while the drugs are acting at ligands to the coppers.

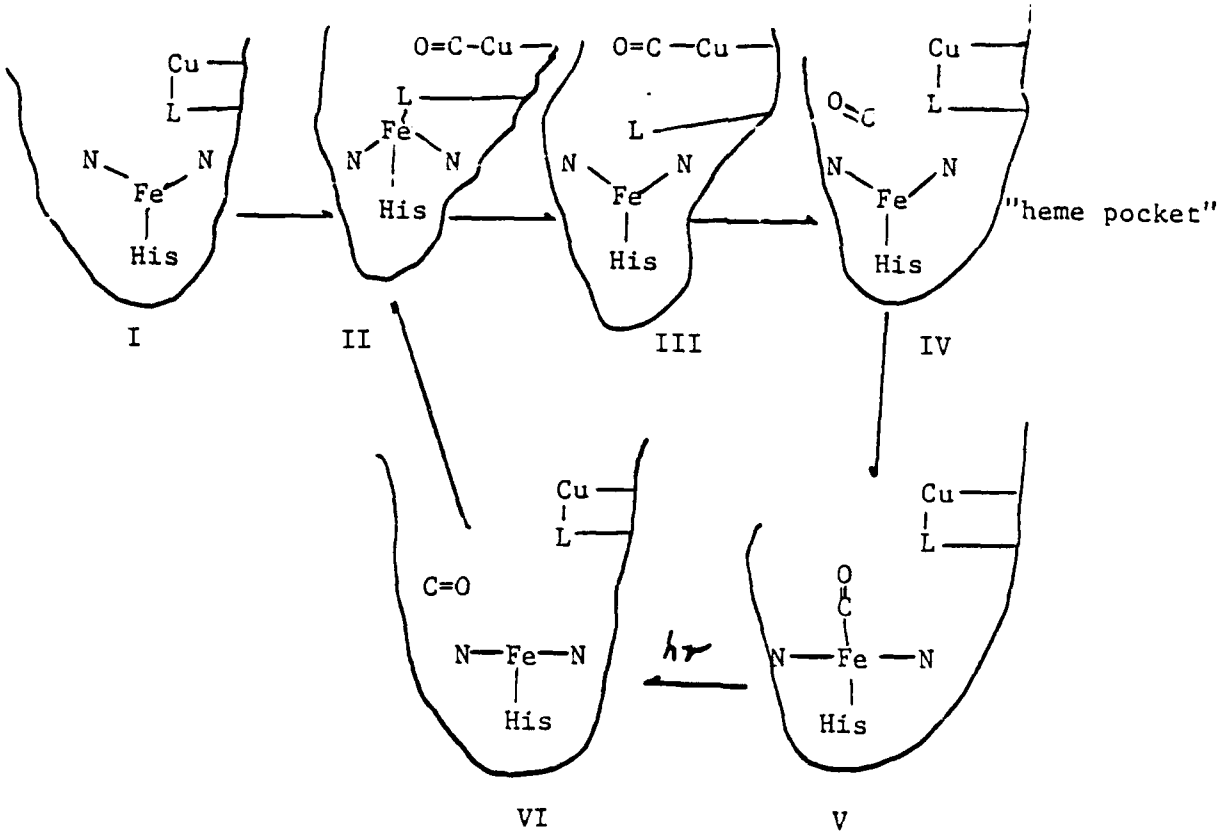
THE MODEL OF CYTOCHROME OXIDASE FUNCTION

Any attempt to understand the inhibition of the oxidase requires an understanding of the current models of cytochrome oxidase function. Since we are dealing here with effects

of hydrophobics on CO recombination, we need only worry about the mechanism of ligand binding at the CuB-a_3 binuclear center.

Rapid FTIR kinetics of the 1963 cm^{-1} infra-red CuB-CO stretching frequency indicate that following flash photolysis of the Fe-CO complex, a change in absorbance at 1963 cm^{-1} is observed, indicating that the photolyzed CO binds to the undetectable CuB center; at room temperature, the CO migrates from the CuB center to the iron of cytochrome a_3 within a picosecond (39,40,41). At temperatures below approximately 230 K, the photolyzed CO remains trapped within the heme pocket and does not communicate with the bulk suspending medium; this has been a fundamental aspect of the CO kinetic studies since 1974 and is verified from studies of the kinetics of CO binding by measuring changes of CuB (39-42)or the heme iron (26,32). Thus we concentrate on the environment of the heme pocket region and how changes in the pocket due to drugs and/or protons can alter CO binding kinetics and pathways.

A recent model has been proposed by Woodruff and co-workers (40,41) and is based on the rapid FTIR kinetics of CuB redox center following flash photolysis. It is shown below in an abbreviated form.



SCHEME OF WOODRUFF AND CO-WORKERS

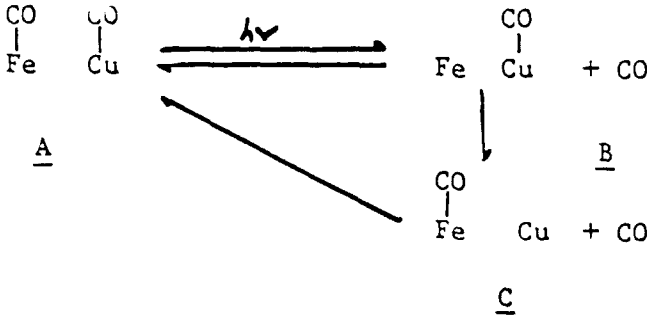
In this model, CO approaches form I and binds to CuB; CuB must break its bond to an unknown ligand L to bind the CO. The free ligand L (possibly a cysteine) binds to the heme iron, pulling it out of the heme plane as seen in form II. The Fe-L bond is broken in form III, allowing the Fe to settle back across the heme plane. CO dissociates from CuB (which reforms the Cu-L bridge) in IV and migrates to the Fe to yield structure V, the iron

carbonyl species. Upon flash illumination, CO is released from Fe (which moves into the heme plane) as in structure VI. CO rebinds to CuB to form structure II. Basically, the model states that CO binds to CuB first and is then transferred to the heme iron.

This model, however, is based upon the assumption that the heme pocket can accommodate one and only one CO, an assumption we now know to not be correct under all conditions. The basis for the incorrect assumption may lie in the fact that the FTIR studies and most other CO rebinding studies have been performed using isolated purified cytochrome oxidase while this investigator uses intact mitochondria. Studies in this laboratory (30) indicate that at pH 7.4, the maximal occupancy of the heme pocket of **isolated** oxidase is 1 (one) CO in agreement with work by Sharrock and Yonetani (32). Data presented here and published by this investigator indicate that 2 CO can occupy the heme pocket in intact oxidase.

That 2 CO can occupy the heme region alters the possibilities of CO binding, allowing (under certain conditions) for the binding of CO to the heme to occur by two different paths, one via the CuB site and another possibly to the heme directly.

In the presence of only 1 CO/heme a_3, the CO could follow the path proposed in the previous model. However, if as second CO is present, the CuB after photolysis may be liganded to one (the second) CO already (if two CO are in the pocket after photolysis, one rebinds to the Fe; the other could bind to CuB). The photolyzing flash used is in the visible light range and would photolyze the 594 nm Fe-CO bond, not the 1963 cm^{-1} Cu-CO bond. Rebinding of the photolyzed (now) unbound CO to the heme iron could occur either by CO recombining to the heme directly (CO binds to structure B below) or a CO transfer to the heme may occur via form C; the photolyzed CO would then rebind to the vacant CuB. That only one apparent exponential phase is observed may indicate that only one pathway is preferred. Alternatively, it will be difficult to separate two competing simultaneous reactions unless their rate constants are more than 5-fold different (if more similar, they blend as an "apparent" single exponential). The B-C-A pathway here may correspond to the path III- IV -V in the Woodruff model; the freed CuB binds the photolyzed CO (the CO would "swap" positions on Fe and CuB). Recombination via III-IV-V would be faster than the sequence II-III-IV-V and may account for the fast phase of recombination. Alternatively, the reaction could truly be second order with respect to CO; that is, 2 CO compete for the same CuB site, accelerating the rates, although only a 2-fold difference between fast and slow rates would be expected, nor would this explain fast and slow phase kinetics. (This assumes that either CuB or Fe, but not both, can bind CO).



The alternative double CO binding model assumes that under certain conditions, Fe and CuB can both bind CO. That biphasic kinetics at pH 7.4 and 9.0 in the presence of

dibucaine/propranolol are observed only in the presence of 100% CO when more than 1 CO is present per CuB/Fe suggests that a second binding site may be available.

Biphasic kinetics are observed in the presence of alcohol at all pH values. The alteration of Cu-CO spectra in the presence of alcohol has been reported (42) two IR Cu-CO bands in the 1960 cm^{-1} region have been reported (39,42) two Cu-CO bands are observed, suggesting that CuB resides in two environments, one more mobile and more perturbable that the other. It is possible that drugs like propranolol and dibucaine, which we have shown to act at the level of the copper in the oxidase, may bind to CuB or the ligand L and result in two CuB species, the normal CuB and the drug altered CuB*. The binding of CO to Fe from CuB would occur fairly normally and account for the strong similarity in parameters of the slow phase binding and the "apparent" single phase kinetics. If the lifetime of the CuB-CO were shortened due to interaction with the drugs or alcohol, then the fast phase would be observed in the presence of 1% CO; in 1%CO, some of the CO would rebind to a modified form II (II*) with faster kinetics. This model, while plausible, does not explain the occupancy or the basis of the observance of double phase kinetics only in 100% CO (but would explain 2 kinetic phases in 1% CO!).

In either single or double exponential kinetics, the rates of recombination are faster and the energies of activation are generally lower at lower pH values. This suggests that a proton is involved with either CO binding to Fe or with dissociation of CuB-CO (the step which limits the rate of CO binding to Fe). Woodruff and co-workers have suggested that the ligand L and the imidazole of the distal histidine on the heme can be protonated. The pK of the protonation of the imidazole group will be around 8.5. Any facilitation of CO binding to Fe due to protonation of the imidazole would be expected at pH 9.0 or 7.4, not at pH 5.5 as observed in our data. The presence of L, the protonated (cysteine) ligand may alter the rate of dissociation of LH from CuB to allow CO binding to CuB in species I, may increase the rate of dissociation of L(H) from Fe-L in species II (or may not allow the Fe-L ligation to occur, allowing the naked Fe to react with CO more easily and rapidly, or may be a steric block to the transfer of CO from metal to metal. The latter possibility is unlikely since rates are faster and the E_a lower in the protonated form.

If the histidine is protonated or perturbed to push the Fe into the heme plane and is manifested as a blue-shift in the heme Soret band as suggested by Woodruff and co-workers, then it is possible that the effect of propranolol/dibucaine may also be on the heme as well as on Cu or L. The addition of hydrophobic drugs to the oxidase was shown (AFOSR 84-0264) to induce blue-shifts in the Soret spectrum of the oxidase as well as break the antiferromagnetic spin coupling between Fe and CuB. Thus the slower kinetics in the presence of the drugs may be due to the alteration of the position of Fe in the heme plane or a steric hindrance at a metal site. Further experimentation is needed to determine the cause of the faster kinetics.

The fast phase kinetics show a pH-dependence and CO concentration as well as a temperature dependence. First, the extent of fast phase contribution to the overall activity is about 50% at pH 5.5 and less than 10% at pH 9.0, suggesting that a protonated species in responsible for the fast phase recombination (this would rule out the protonation of the distal histidine as the causative factor). Second, in the presence of the drugs, the contribution of the fast phase decreases with decreasing temperature. The energy of activation of the fast phase is generally less than the slow phase, but the fast phase kinetics "disappear" at cold temperatures. If, for example, 30% of the rate at 240K is due to fast phase, 0% of the overall reaction is fast at 190K; additionally, the total extent of the absorbance change observed on photolysis and rebinding is 30% less at 190K than at 240K. The fast phase is "frozen out" at cold temperatures while the slow phase is not.

This suggests that the fast phase is due to a far more mobile environment that can be "frozen out" at a warmer temperature than the slow phase and that the fast phase represents a process at a site located further from the heme than the slow phase (such as CuB).

Factors that would increase the extent and rate of dissociation of L from CuB either by protonation (to form LH), presence of propranolol or dibucaine (to break the Fe-Cu interaction and possibly prevent L-metal ligation, or bathocuproine (to bind Cu and prevent Cu-L ligation) would be expected to facilitate biphasic kinetics and the existence of an alternate CO binding sequence.

EXAFS (Extended X-ray Absorbance Fine Structure) STUDIES

In February, 1990, this investigator travelled to the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory to collaborate with investigators from the University of Pennsylvania and Utah State University at Logan UT to perform Cu- and Fe- edge EXAFS to determine ligand bond lengths, angles, and orientations within 5 A of the Fe (Fe- EXAFS) or Cu (Cu-EXAFS). The effects of dibucaine/propranolol on the oxidase and myoglobin were performed. The data obtained will require extensive calculation and manipulation to determine the three-dimensional ligation state around the copper of the oxidase and the iron of Mb and the oxidase. The programs and VAX/mainframe capabilities to handle the data are available at Utah State. Dr. Harmon is attempting to visit Utah State for several weeks to perform the necessary calculations and comparison of the EXAFS x-ray fluorescence pattern to molecules of known structure or model compounds. It is hoped that these experiments may shed some light onto the alterations of structure of Mb and the oxidase in response to changing pH and treatment with hydrophobic molecules.

STUDIES ON UBIQUINOL-CYTOCHROME C REDUCTASE

SITE OF INHIBITION OF HYDROPHOBIC DRUGS IN COMPLEX III (UBIQUINOL-CYTOCHROME c REDUCTASE) PORTION OF THE RESPIRATORY CHAIN.

Previous research identified a site of inhibition of tetracaine, dibucaine, and propranolol in cytochrome oxidase (data provided in the enclosed Biochemical Pharmacology reprint based on data obtained under AFOSR 84-0264 indicates that dibucaine inhibits cytochrome oxidase at the level of the coppers; tetracaine and propranolol have similar effects. The hydrophilic drugs such as procaine or procainamide were without effect.

Local anesthetics and propranolol inhibit mitochondrial succinate----> O_2 activity; the I_{50} or concentration to achieve 50% inhibition of activity was linearly dependent on the log of the partition coefficient (ie, hydrophobic drugs inhibit at a lower concentration).

To test the possibility that inhibitory sites other than cyrochrome oxidase may be present in the respiratory chain, we have investigated the presence of inhibitory sites in the ubiquinol-cytochrome c reductase (complex III) region of the respiratory chain. This complex houses the second site of oxidative phosphorylation and consists of a ubiquinone binding protein(s), a single polypeptide that contains two hemes [these are referred to as cytochrome b_{562} and b_{566} on the basis of their wavelength maxima; b_{562} is reducible by succinate (midpotential Em =30 mV) while b_{566} (Em=-30 mV) is reducible by NADH or strong reductants], an Fe-S protein described and isolated by John Rieske (43), and cytochrome c_1. Ubiquinol is the reductant; cytochrome c is the oxidant of the complex, accepting electrons from cytochrome c_1. Thus the reduction of four centers can be

determined spectrophotometrically: cytochrome b_{562} (562 nm), cytochrome b_{566} (566 nm), cytochrome c_1 (554 nm), and cytochrome c (550 nm). Measurements of the reduction level of centers whose wavelength maxima are separated by only 4 nm requires a dual wavelength spectrophotometer with narrow bandwidth (slits) and very high resolution. Subtraction of spectra by computer assists in determination of the wavelength maxima and the centers reduced.

The general experimental protocol is as follows:

1. oxidized cytochrome c-depleted mitochondria suspended in buffer at desired pH in presence of drug or ethanol is placed in spectrophotometer cuvette with stirrer bar to keep the mitochondria in suspension. The spectrum of the oxidized spectrum is recorded.

2. Add succinate to final concentration of 2.5-5 mM to serve as substrate. Record subsequent spectra using the oxidized spectrum as reference to follow the reduction of the cytochromes. If no site of inhibition is present, absorbance increases at 562 and 554 nm will be recorded in the absence of cyt c (succinate reduces all centers); if exogenous cytochrome c is added, the absorbance increases at 562 and 552 (combination of 554 and 550 nm when 1:1 stoichiometric amount of cyt c added).

In the absence of cytochrome c, if a site of inhibition exists before (on the reducing side) of cyt b, no 562 or 554 nm absorbance will be seen. If the site of inhibition is between cytochromes b and c_1, 562 but not 554 nm absorbance increase will be seen.

In the presence of cytochrome c, the possibility exists of an inhibition site between cytochromes c_1 and c and can be determined as follows:

1. By observing the wavelength maximum of the cyt c's; if cyt c_1 only is reduced, the absorbance is at 554 nm; if both c's are reduced, the absorbance peak is shifted to 552 nm.

2. when the absorbance increase has reached its maximum value with time. dithionite is added to the solution to reduce all cytochromes completely This spectrum is recorded as has been the spectrum obtained with succinate alone as substrate. We can subtract the spectrum with succinate from the dithionite-reduced spectrum (to yield the dithionite MINUS succinate difference spectrum.

    a. If no 550 or 554 nm peak in the difference spectrum, then both c-type cytochromes were reduced by succinate.

    b. If 550 nm seen in difference spectrum, cyt c_1 but not cyt c was reduced by succinate.

    c. If a 552 nm peak is observed, neither c-type cytochrome was reduced by succinate.

The varying amounts of cyt c and c_1 reduced can be determined by de-convoluting the 550-554 nm region into two absorbance bands using the de-convolution programs of a program such as SpectraCalc (Galactic Industries).

From studies such as these we can summarize the findings.

1. Propranolol, dibucaine, and procaine do not prevent the reduction of cytochrome b_{562}. This indicates that the succinate dehydrogenase (Complex II) and ubiquinol---> cyt b pathways do not contain inhibitory sites.

2. At 2 mM levels, propranolol and dibucaine prevent the reduction of cyt c_1 (and hence cyt c) only when the pH of the mitochondrial suspension is below pH 6.5 (we cannot measure effects below pH 5.5 since the mitochondria congeal into a gelatin at lower pH). At pH 6.4- 6.46, 50% inhibition of cyt c_1 is observed with 2 mM concentrations (higher extents at higher concentrations). Tetracaine prevents cyt c_1 reduction at pH 7.4! This indicates a site of inhibition prior to cyt c_1 but after cyt b.

3. Cytochrome c is not reduced in the presence of dibucaine/propranolol at any pH even if cytochrome c_1 is fully reduced (as it is at pH 7 or above). This indicates that an additional site of inhibition exists between cytochrome c_1 and cyt c.

The transfer of electrons from succinate dehydrogenase to cyt c is not a linear sequence of SDH->Q->b->Fe-S ->c_1 -> c. Instead, the reduction/oxidation of cytochromes b occurs via the protonmotive or proton-translocating chemiosmotic "Q-cycle" that is responsible for the energy-linked translocation of 2 protons from the matrix to the cytoplasmic side of the membrane for every electron; this cycle is shown in Figure 13. Coenzyme Q is present on the matrix side as a semiquinone QH• that accepts an electron from succinate dehydrogenase and a proton from the medium. The reduced quinol form migrates to the C-side (cytoplasmic) where it is oxidized by the Rieske Fe-S center (which passes the electron to cyt c_1 and one proton is lost to the medium. The QH_c semiquinone is deprotonated and oxidized further by cyt b_{566} which transfers the electron to cyt b_{562} on the matrix face. The oxidized quinol migrates to the matrix face (the charged semiquinone is impermeant) where it is reduced at b_{562} and is protonated to yield the semiquinone and start the cycle anew. The net result is the transfer of one electron from the dehydrogenase (succinate or NADH) to the cyt c region and the translocation of 2 protons across the membrane.

An important aspect of this Q-cycle is that by virtue of its Em, cyt b_{566} (-30 mV) is not reducible by succinate (0 mV) unless an inhibitor such as antimycin A is present to block reduction of the matrix face semiquinone by cyt b_{562}. If the Fe-S and cyt c chain allows a path of oxidation for the quinol (such as to ferricyanide or oxygen) the quinol is oxidized to the semiquinone that has a low enough potential to reduce the -30 mV cyt b_{566}. If the cytochrome chain beyond Fe-S is inhibited or blocked (no oxidation pathway for the quinone), the oxidant-induced reduction of cyt b_{566} is not observed.

Measurements of the oxidant-induced reduction of cyt b_{566} indicate:

1. the drugs alone do not induce the oxidant-induced reduction. indicating that they do not act at a site between cyt b_{562} and the dehydrogenase as does antimycin A.

2. The drugs that prevent the reduction of cytochrome c_1 prevent oxidant-induced cyt b_{566} reduction when antimycin is present and an oxidation path for the quinol is available via the cytochrome oxidase.

This substantiates the presence of a site of inhibition on the oxidation side of the Q-cycle.

The pH-dependency of the inhibition of cytochrome c_1 reduction is curious. The Rieske Fe-S center may be the site of action since it is the site of inhibition by other hydrophobic compounds such as hydroxy-undecyl naphthoquinones (44) and undecyl hydroxy dioxobenzothiazol derivatives (the parent compound is UHDBT; ref 45). Both the naphthoquinones and UHDBT also prevent cytochrome c_1 reduction and block the oxidant-induced reduction of cyt b_{566}. As with the naphthoquinones (44) the inhibition occurs at a lower concentration of inhibitor at low pH values since the pK of the Rieske center is about pH 6.8- 7.0 and is exposed on the cytoplasmic side of mitochondria (is exposed to the medium). We suggest that the Rieske Fe-S center is a site of inhibition by the drugs.

If reduced cytochrome c is added to cyt c-depleted mitochondria, electron equilibration between the 270 mV cyt c and the 245 mV cyt c_1 center occurs (about 30% reduction of cyt c_1). In the presence of dibucaine and propranolol, this "reverse" electron transfer/equilibration (c-->c_1) is not observed, further indicating a site of inhibition between the 2 c-type cytochromes. The positive charge of the drugs may contribute to the inhibition of electron transfer between the c's since the drug will act as a competitive inhibitor for cyt c. This has been reported for the reaction with cytochrome oxidase (46,47). Cytochromes a and c_1 have negatively-charged domains to allow the binding of cytochrome c via its 13 exposed lysine residues; thus any positively charged molecule can compete for the binding site. While some of the effect may be due to competition for the cyt c binding site, we note that cytochrome c can still reduce cytochrome a either in these experiments or those published in Biochemical Pharmacology (48). Thus we find it unlikely that the inhibition of electron transfer between the cytochromes c is due to competitive binding. In addition, the high pK of the nitrogenous centers of the drugs would suggest that the charge and hence the inhibition would be less prevalent at high pH, which is opposite to what is observed. Thus we suggest that the cyt c--> cyt c_1 *inhibition is not based electrostatically.*

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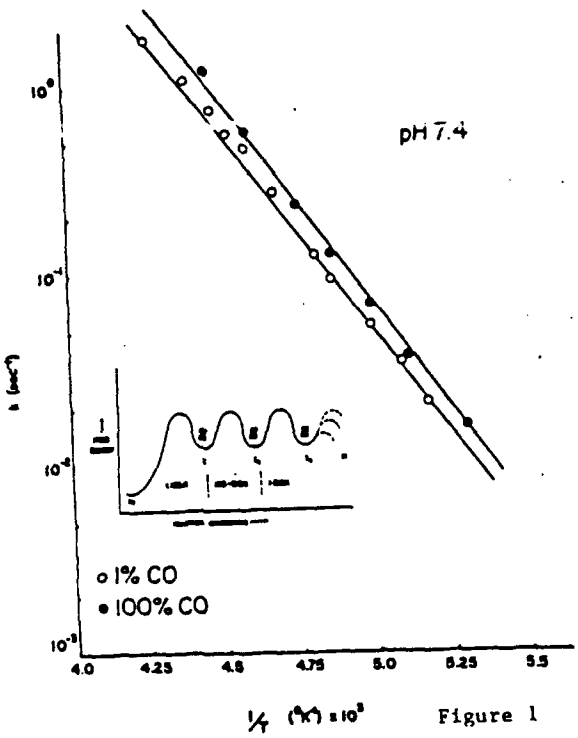


Figure 1

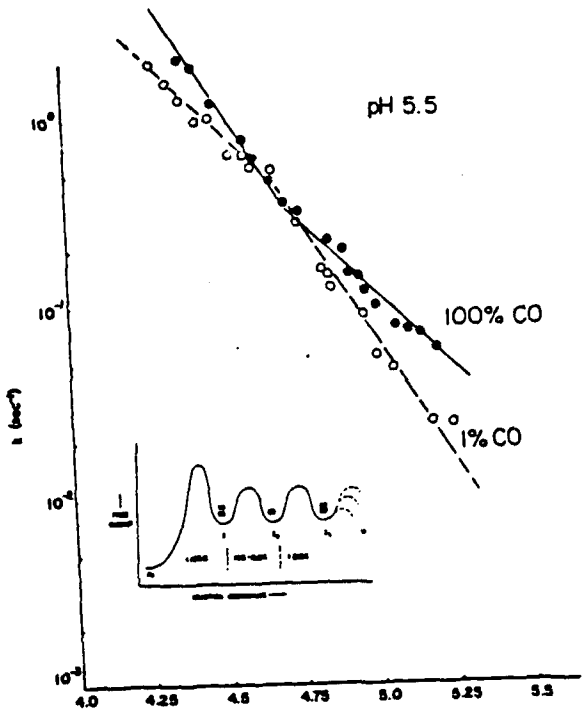


Figure 2

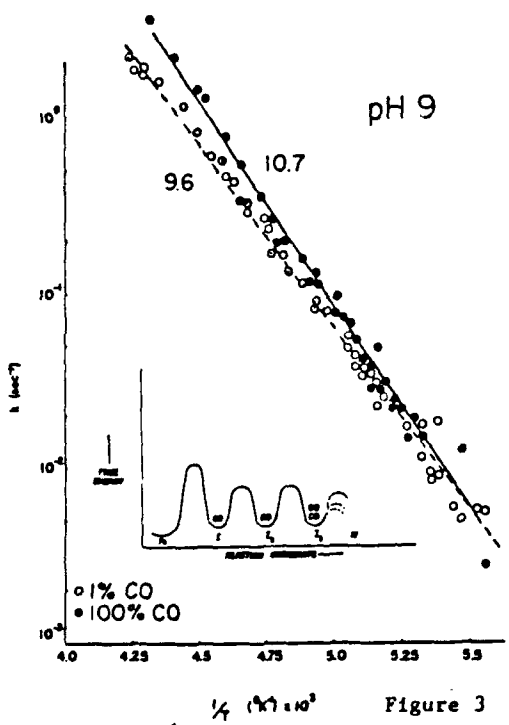


Figure 3

FIGURE LEGENDS

Figure 1. Plot of log k of CO recombination vs.inverse temperature at pH 7.4 in intact beef heart mitochondria. The calculated energies of activation are shown adjacent to the slopes. The fitted lines (least square fit) indicate energies of activation of 10.5 kcal/mole (coefficients of correlation for both lines are greater than 0.994). The standard error of measurements of k, unless shown as an error bar, lie within the data point dot shown on this and succeeding graphs.

Figure 2. Plot of log k vs. inverse temperature at pH 5.5 The calculated energies of activation are shown adjacent to the slopes. The coefficients of correlation of fit of the drawnlines with 1% CO above and below 215K are both 0.99; the coefficients with 100% CO above and below 215K are 0.998 and 0.985, respectively.

Figure 3. Plot of log k vs. inverse temperature at pH 9.0. The energies of activation are shown. The coefficients of correlation of the fitted lines with 1% CO are and 100% CO 0.994 and 0.994, respectively.

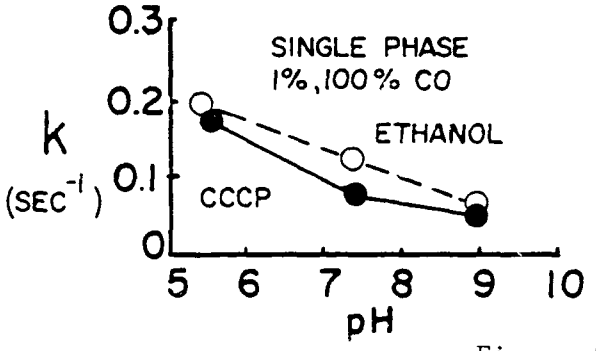


Figure 4

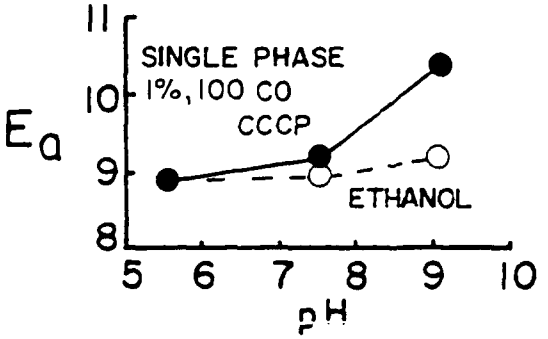


Figure 5

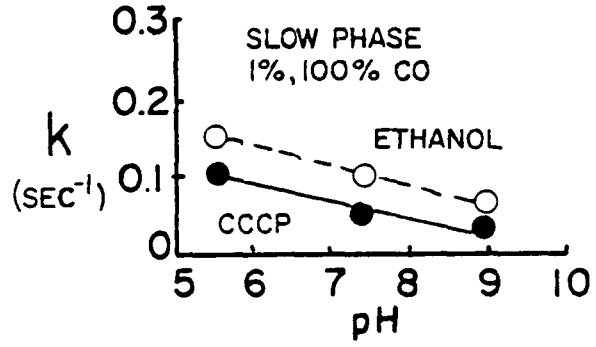
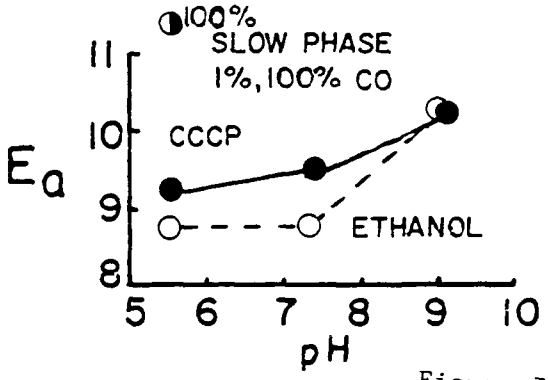


Figure 6



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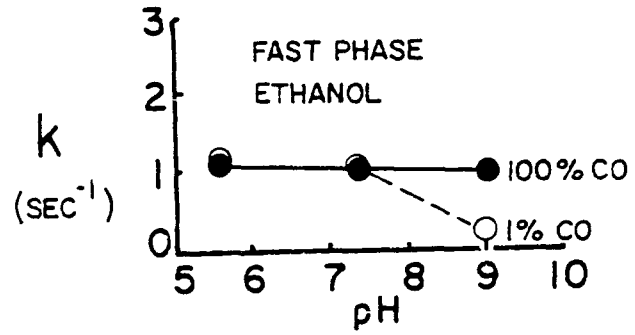

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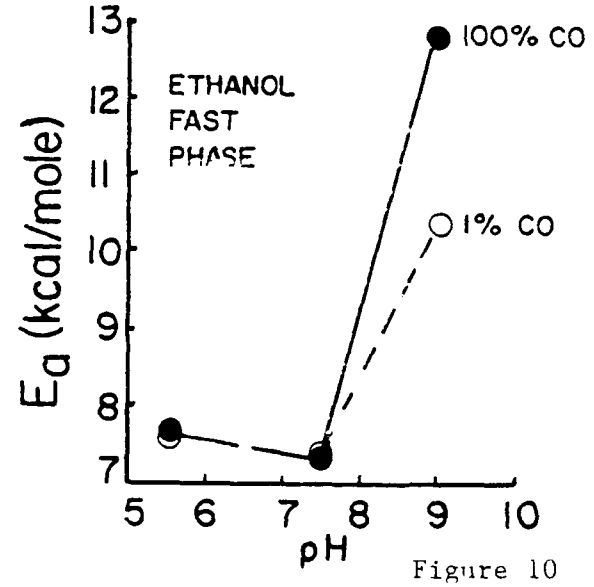

Figure 10

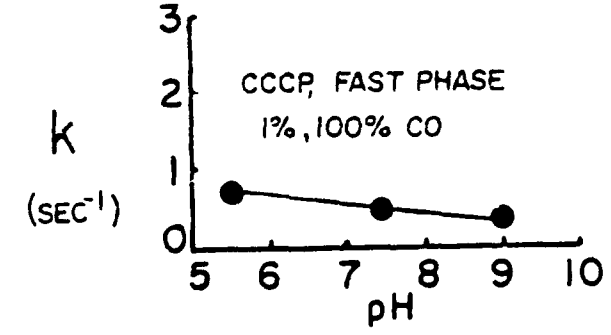

Figure 9

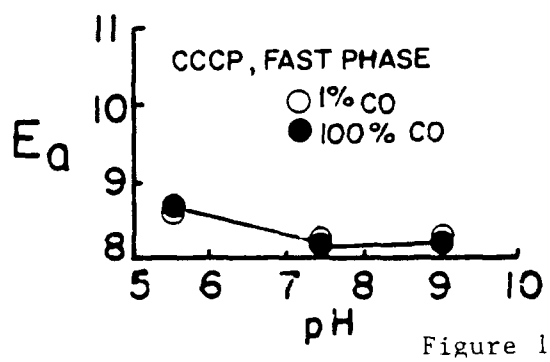

Figure 11

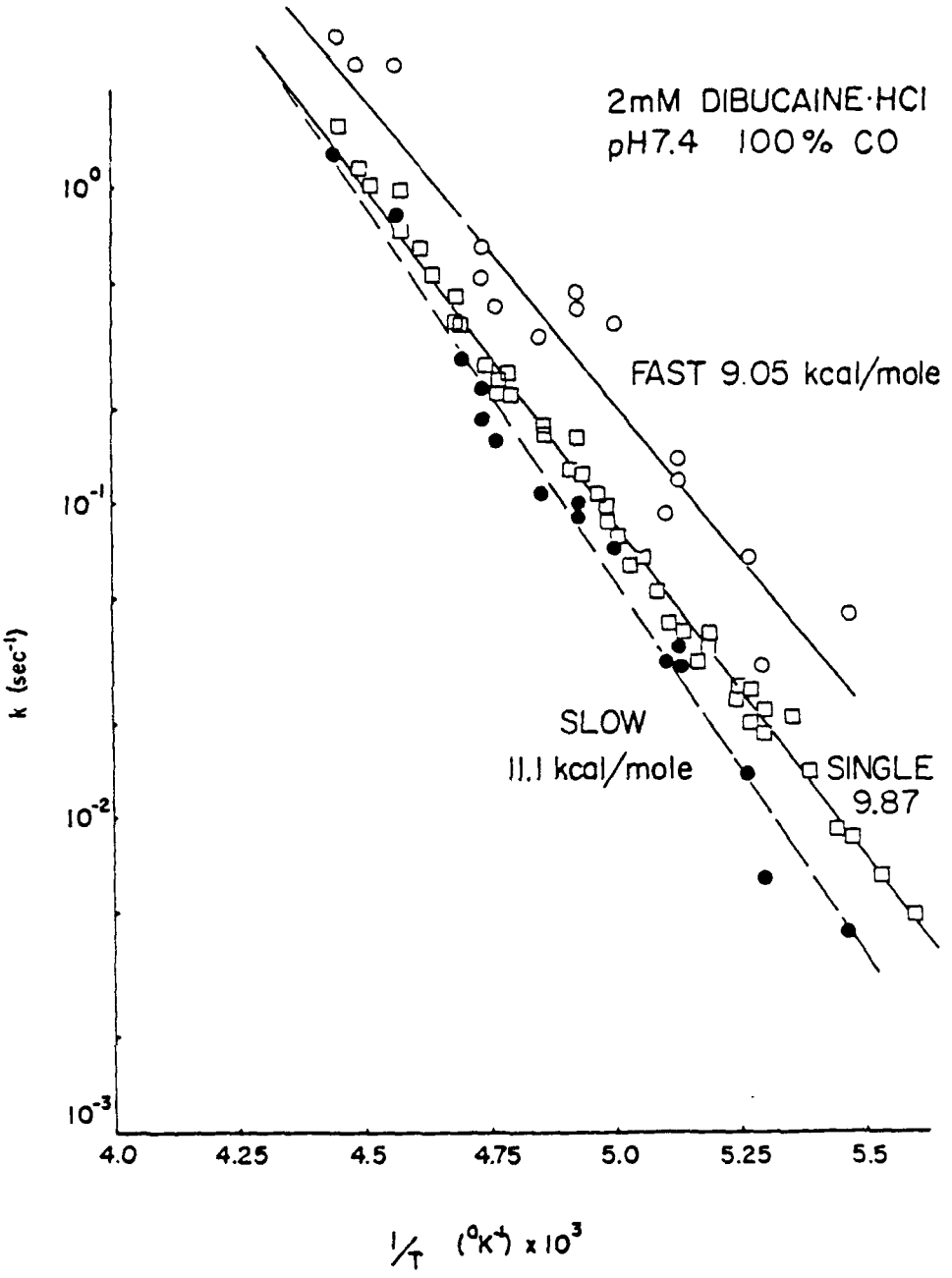

Figure 12. Plot of rate constant $\underline{k}$ vs. inverse temperature at pH 7.4 in the presence of 2mM dibucaine and 100 % CO. Raw data fit to a single exponential is shown by open boxes (Single). Raw data fit to double exponentials are denoted by open circles (fast phase) and closed circles (slow phase). Numbers next to the least-squares calculated slopes are the energies of activation. At 210 K, the fast phase accounted for 50% of the total activity under these conditions.

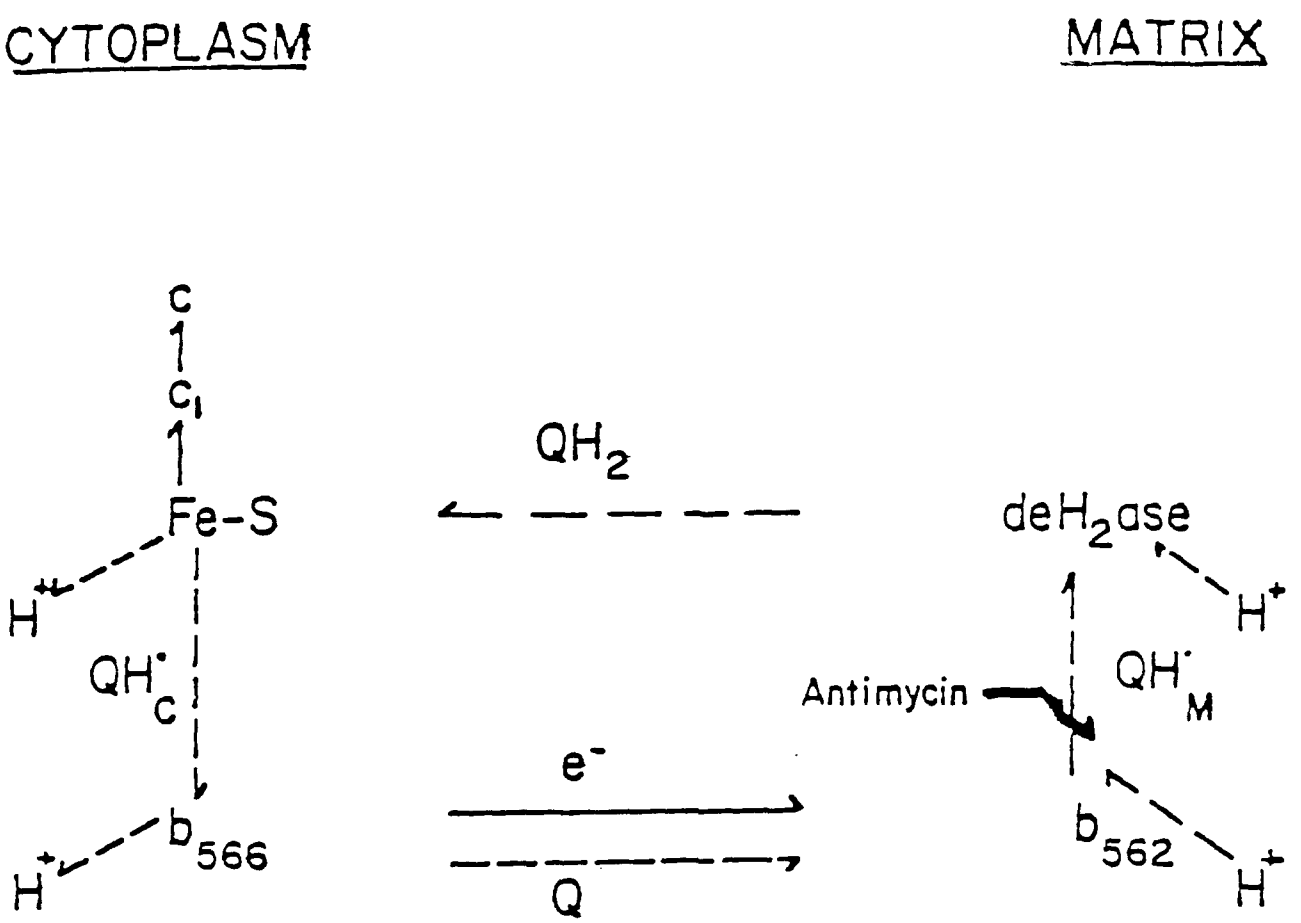

Figure 13. Diagram of proton-translocating "Q-cycle". $QH^{\cdot}_M$ and $QH^{\cdot}_C$ denote the semiquinone form on the matrix and cytoplasmic faces, respectively and are capable of lateral diffusion in the membrane only. QH_2 and Q can diffuse across the membrane. Solid arrows denote direct electron transfer.

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Effect of pH on CO recombination to cytochrome oxidase in intact mitochondria

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The rate of recombination of CO with fully reduced cytochrome oxidase in intact beef heart mitochondria was measured after flash photolysis at temperatures between 180 and 230K. At pH 7.4 a single Arrhenius slope corresponds to an apparent energy of activation (E_a) of 10.5 kcal/mol; the rate constants in 100% CO are twice those in the presence of 1% CO. At pH 5.5 with 100% CO, E_a's of 11.3 and 7.1 kcal/mol are observed above and below 210K, respectively, while E_a's of 7.4 and 11.1 kcal/mol are observed with 1% CO above and below 210K. At pH 9.0 E_a's of 9.2 (above 200K), 12.5 (190–200K), and 2.3 (below 190K) kcal/mol are observed with 1% CO; E_a's of 9.4, 13.4, and 2.4 kcal/mol are observed in the same temperature ranges with 100% CO present. The findings support a model with up to 4 energy barriers separating the heme region from the bulk medium with intermediate regions that can hold 1 or 2 CO, depending on pH.

Cytochrome oxidase; Copper; CO recombination; pH; proton

1. INTRODUCTION

Cytochrome oxidase (E.C. 1.9.3.1), a multi-subunit enzyme containing two hemes and two copper atoms, is responsible for the reduction of molecular oxygen to water in the mitochondrial respiratory chain. The reduction of oxygen requires three substrates to be present at cytochrome a_3: molecular oxygen, electrons donated from the respiratory chain via cytochrome *c*, and protons. Protons play three distinct roles in the operation of the oxidase: (i) substrate protons required in the reduction of oxygen; (ii) protons chemiosmotically translocated across the membrane in energy coupling; and (iii) those protons involved in or that determine the three dimensional conformation of the enzyme. Clearly, changes in proton conformation with varying pH may affect the utilization of protons in respiration and chemiosmosis.

The recent report by Fabian and Malmstrom [1] of the pH-induced spectral changes in oxidized oxidase lead those investigators to suggest that proton binds at or near cytochrome a_3. Proton binding near cytochrome a_3 may alter the kinetics of ligand binding as well. Data contained in the report of Brzezinski and Malmstrom [2] and earlier work from this group [3] suggested that CO recombination was not affected by changes in pH although a dedicated in-depth study was not performed. In this communication we report the pH-dependence of the energy of activation and number of CO molecules involved in CO recombination in cytochrome oxidase in intact beef heart mitochondria.

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2. MATERIALS AND METHODS

Intact beef heart mitochondria prepared by the procedure of Harmon and Crane [4] were suspended at 5 mg protein/ml in 0.25 M sucrose–50 mM sodium phosphate buffer at the desired pH (5.5, 7.4, or 9.0). The mitochondrial suspension was reduced by the addition of 28 μM tetramethyl-*p*-phenylenediamine dihydrochloride, 97 μM phenazine methosulfate, 50 mM succinate, 100 mM ascorbate (final concentrations), and 200 μg cytochrome *c* (Sigma type VI) while bubbling the solution with either 1% (99% N_2) or 100% CO. The suspension was bubbled with CO for 20 min in the dark and transferred to the sample holder; the loaded sample was allowed to sit in the dark for an additional 15 min and then frozen in liquid nitrogen.

CO recombination following flash photolysis was measured at 446 nm as described previously [5–7]. The resulting time-dependent absorbance changes were fitted to a single exponential curve using the Kinetics module of the Labcalc (TM) program (Galactic Industries) and the program KINFIT (On-Line Instrument Systems, Inc., Jefferson, GA). That lower standard deviations and higher Durbin–Watson values are obtained from single exponential than multiple exponential or power-law fits indicates that the re-binding can be described by a single exponential as before [5–7].

That the oxidase was totally reduced and ligated to CO was determined from reduced minus oxidized difference spectra measured with a Johnson Research Foundation (University of Pennsylvania) model DBS-3 scanning dual wavelength spectrophotometer.

3. RESULTS

Plots of the log of the rate constant *k* vs inverse temperature at pH 7.4, 5.5, and 9.0 are shown in Figs.

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The data points for the very low temperatures in Figure 3 could be interpreted to be not signifiantly different enough to warrant a 2.3 kcal barrier. All but the two lowest temperature points could lie on the major energy of activation slopes. Similary, the data at pH 5.5 could be interpreted to lie on a single slope line; the difference in slopes between 1% CO and 100% CO still indicates multiple occupancy in the outer region(s), however. My point is that while the individual data points are sound, the line(s) between them are open to some flexible interpretation.

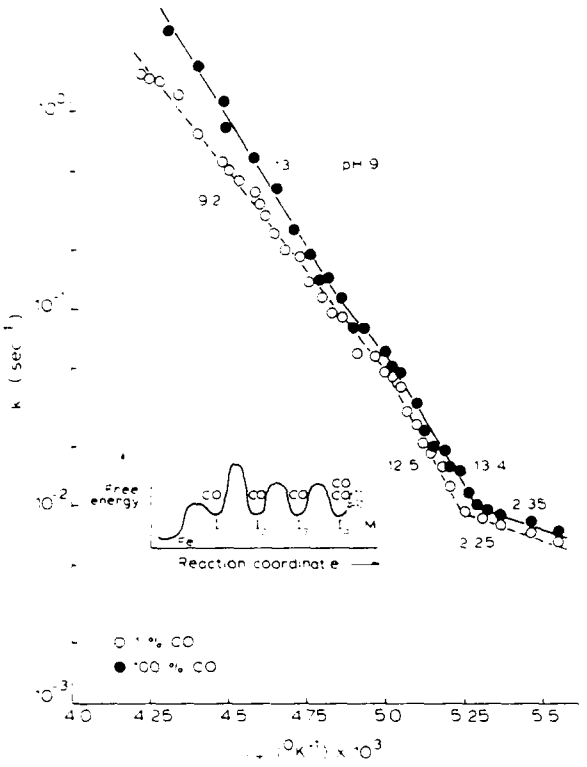

Fig. 3. Plot of log k vs inverse temperature at pH 9.0. The energies of activation are shown. The coefficients of correlation of the fitted lines with 1% CO are, from warmer to colder temperatures, 0.998, 0.982, and 0.995. The coefficients with 100% CO from warmer to colder temperatures are 0.992, 0.991, 0.966, and 0.986, respectively.

1% CO indicates a minimum number of barriers and defines their heights while plots in the presence of 100% CO indicate the occupancy or number of CO molecules in the intermediate regions separated by the barriers. This interpretation of the data is consistent with previous interpretations by this and other investigators [7,8] with cytochrome oxidase.

We interpret the results by the reaction coordinate models (included as insets of Figs. 1-3) as in previous reports with cytochrome oxidase [7,8] and myoglobin [9,10]. CO migration to the heme at pH 7.4 entails crossing one or more barriers of 10.5 kcal/mol from one or more intermediate regions that can hold 2 CO maximally. Since the kinetics we would observe with one barrier/region are indistinguishable from those with multiple identical barriers/regions, we will assume 4 barriers and regions (other than the heme and bulk solvent denoted 'M', the barrier to which is temperature-dependent) to be consistent with the number of barriers observed at pH 9.

At pH 5.5, CO must cross at least 3 barriers. The innermost barrier (and the last to be frozen at low temperatures) is 11.3 kcal in height and separates the Fe from the innermost region I; region I can contain 2 CO. Between 205K and 210K, CO molecules migrate from region I_2 which has an occupancy of 1 CO since the values of k are the same in 1% and 100% CO; additional identical regions may be present. An outermost region I_3, which because of its larger k-value in 100% CO has an occupancy of 2, is separated from I_2 by a 7.1 kcal barrier. Migration to the heme from I_3 via the other intermediate regions occurs above 215K.

At pH 9, the presence of three slopes with 1% CO indicates a minimum of three energy barriers. The values of k are greater with 100% CO only above 210K. Thus three inner regions I, I_2, I_3 can each hold 1 CO and are separated by 2.3 (Fe-I), 12.5 (I-I_2), and 9.2 kcal/mol (I_2-I_3) barriers. Region I_3 is separated from the outermost region I_4 (occupancy = 2) by a 9.2 kcal/mol barrier. Regions I_3 and I_4 are distinguishable because of their difference in occupancy, not their difference in barrier heights.

The pH-dependent changes observed here are likely due to pH-dependent conformational changes and not due to translocated protons since proton translocation cannot occur without enzymic turn-over. Alternatively, the changes observed may be related to the binding of substrate protons at the cytochrome a_3-CuB binuclear complex in preparation for their transfer to the oxygen. The alterations in barrier size and region occupancy could be due to protonation/deprotonation of 1 or more amino acid residues in the pocket or channel to the protein exterior. Doster et al. [10] observed pH-dependent CO binding to myoglobin and the beta-chain of hemoglobin, attributed to a single titratable residue with a pK of 5.7, likely the distal histidine. That the plots between pH 6 and 8.5 are not different than that observed at pH 7.4 (in agreement with studies by Wohlrab and Ogunmola [11] suggests that the change at pH 9 may be due to deprotonation of a tyrosine or cysteine residue and the change observed at pH 5.5 may be due to deprotonation of a histidine residue. Fabian and Malmstrom [1] and Papadopoulos et al. [12] have suggested that changes in Soret absorbance maxima may be due to protons associating with the histidine of cytochrome a_3.

Oliveberg et al [13] reported that the effects of pH on intra-oxidase electron transfer in mixed valence isolated oxidase were not due to pH dependence on the formation of the 'oxy' form, although the authors concede a large experimental uncertainty in their measurement of rates from room temperature flow-flash experiments. The stimulation of both CO and oxygen binding to reduced pigeon heart mitochondrial oxidase following 'energization' by ATP hydrolysis suggests that oxygen binding closely parallels CO binding [7,14]; we thus expect that the formation of the oxygenated ferrous intermediate compound A [15] would also show pH-dependent rates of formation at low temperatures; those experiments are being performed.

The overall rates of ligand binding at room temperature are reported to be pH-insensitive [13];

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INHIBITION OF CYTOCHROME OXIDASE BY DIBUCAINE

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Abstract—Dibucaine–HCl inhibited mitochondrial cytochrome *c* oxidase activity in intact mitochondria with 50% inhibition occurring at 1.1 mM dibucaine–HCl. Dibucaine–HCl did not prevent the reduction of cytochrome oxidase by ascorbate plus *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) when measured at 604 nm but prevented 50% of the absorbance change at 445 nm; dithionite reduced the oxidase completely. Dibucaine prevented binding of CO to oxidase reduced with ascorbate plus TMPD by preventing the reduction of cytochrome a_3. The midpotentials of cytochrome *c* and cytochrome oxidase, the visible absorbance wavelength maxima, and the position and intensity of the signals of the EPR spectrum of the oxidase were not affected. Dibucaine–HCl prevented ascorbate plus TMPD-driven reduction of the near infra-red detectable copper center associated with cytochrome *a*; dithionite subsequently reduced this center. Dibucaine–HCl inhibited cytochrome oxidase activity by interacting between cytochrome *a* and its associated copper. Since respiration was 8-fold less sensitive in submitochondrial particles, this site of inhibition is on the cytoplasmic side of the membrane.

Local anesthetics such as lidocaine, tetracaine, and dibucaine are used for anesthesia as well as in the treatment of cardiac arrhythmias. In the micromolar concentration range, the local anesthetics depress nerve action and cardiac signal conduction by blocking ion (sodium, potassium, calcium) channels in the membrane, either by interaction/perturbation with the ion channels themselves and/or the surrounding membrane lipids. Local anesthetics are known to interact with membrane lipids [1–3], but protein perturbation by, and interaction with, local anesthetics is also reported [4–7].

To increase our understanding of anesthetic–protein interactions, we have investigated the effects of dibucaine on the enzymatic activity and physicochemical characteristics of an integral membrane protein complex, cytochrome oxidase. While these studies do not attempt to explain the basis of anesthesia or antiarrhythmic action by interaction with specific ion channels, they may help explain the effects of general narcosis and mycocardial depression associated with administration of these drugs.

Inhibition of cytochrome *c* oxidase activity by local anesthetics has been described previously by others [8–12] as has the concentration dependence of the inhibition [8, 13]. Singer [8] and Casanovas *et al.* [11, 12] report that the molecule can interact electrostatically with the oxidase, possibly competing for the charged cytochrome *c* binding site to increase K_m. Because of a strong correlation between the concentration dependence and the octanol/water coefficient for many anesthetics including dibucaine, Singer [8, 9] and Casanovas *et al.* [11] suggested a hydrophobic interaction, most likely with the lipid associated with the oxidase [13]. Chazotte and Vanderkooi [14] and Vanderkooi and Chazotte [15] demonstrated that the concentration of dibucaine needed to inhibit cytochrome oxidase in rat liver submitochondrial membranes is inversely related to temperature, further suggesting a hydrophobic interaction. They suggested a reversible perturbation of cytochrome oxidase protein conformation but could not determine if the anesthetics react with the protein directly (as suggested by their observation of inhibition of lipid-free isolated mitochondrial ATPase [16–19]) or with its "boundary lipid" [13].

Despite these extensive studies on the mechanism and type of inhibition, the site of action of dibucaine and other anesthetics in cytochrome oxidase has not been identified. In this report, we define the functional site of dibucaine inhibition in oxidation and reduction of cytochrome oxidase and suggest its topographical location in the membrane.

MATERIALS AND METHODS

Mitochondria containing endogenous cytochrome *c* were isolated from fresh beef heart by the method of Crane *et al.* [20]. Cytochrome *c*-depleted beef heart mitochondria were isolated as described by Harmon and Crane [21]. Exogenous cytochrome *c* (Sigma, Type VI) was added to the mitochondria (100 μg cytochrome *c*/mg mitochondrial protein) prior to recording spectra. Differences in spectra or kinetic activity were not observed between these two mitochondrial preparations.

Cytochrome *c* oxidase and succinate oxidase activities were measured polarigraphically at 25° in a 1.7-mL volume water-jacketed glass chamber fitted with a Clark oxygen electrode in medium containing 83 mM sodium phosphate buffer (pH 7.4) using 100 μg mitochondrial protein and 200 μg cytochrome *c* (Sigma type VI) in each assay. Sodium ascorbate (9 mM) and 28.1 μM (final concentrations) *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD†) were used as substrate to

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† Abbreviations: TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride; CuA, infra-red detectable copper center associated with cytochrome *a*; PMS, phenazine methosulfate; and DAD, diaminodurene; 2,3,5,6-tetramethyl-*p*-phenylenediamine.

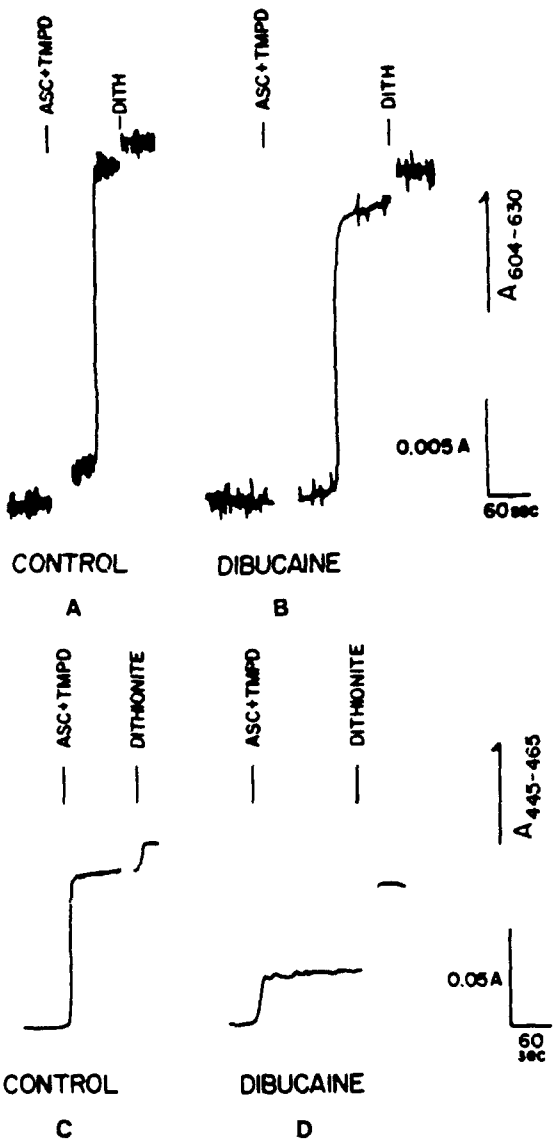

Fig. 2. Reduction of cytochrome oxidase as measured using the 604 *minus* 630 nm pair in the absence (A) and presence (B) of 1.5 mM dibucaine–HCl at room temperature and the reduction of cytochrome oxidase as measured using the 445 *minus* 465 nm pair in the absence (C) and presence (D) of 1.5 mM dibucaine–HCl. Cytochrome *c*-depleted mitochondria were suspended at 2 mg/mL in 0.25 M sucrose–50 mM sodium phosphate buffer (pH 7.4). Ascorbate (5 mM) and TMPD (28.1 μM) with 200 μg cytochrome *c* (Sigma, type VI) were used as reductants.

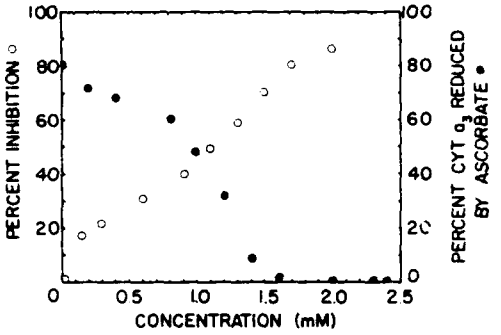

Fig. 3. Effect of dibucaine concentration on the reduction of cytochrome a_3 following addition of ascorbate plus TMPD. Conditions were as described in the legend of Fig. 2. Total reduction of cytochrome oxidase and maximal absorbance increase was measured after the addition of dithionite. Control (uninhibited) activity: 592 ng-atom O/min/mg protein. Full scale absorbance change was 0.05 A, corresponding to 2.1×10^{-9} moles cytochrome *a*.

chrome a_3; both cytochromes absorb light equally at 445 nm [25]. The data in Figs. 2 and 3 are consistent with this model. Over 80% of the 604 nm and approximately 50% of the 445 nm absorbance were recorded in the presence of the anesthetic when reduced with ascorbate plus TMPD. Addition of dithionite caused only a slight increase in 445 nm absorbance. In the absence of the anesthetic, essentially complete reduction of both cytochromes was observed. Thus, dibucaine–HCl inhibited electron transport between cytochromes *a* and a_3, allowing the reduction of cytochrome *a* (80% of 604 nm and 50% of 445 nm absorbance) but not cytochrome a_3.

The presence of dibucaine did not alter the midpotential of cytochrome *c* (Harmon HJ, unpublished results). The midpotential of cytochrome oxidase was increased from 234 ± 4 to 255 ± 4 mV in intact mitochondria in the presence of 1.5 mM dibucaine–HCl.

In the presence of up to 10 mM dibucaine–HCl, neither the position nor the intensity of the g = 3 EPR signal due to ferric cytochrome *a* or the g = 2 signal due to CuA was altered. The field position of the signal in the presence of dibucaine was within 5 G that of the untreated enzyme while the half-bandwidth of the g = 3 signal in both cases was 53 G.

As shown in Fig. 4, ascorbate plus TMPD was capable of reducing the "visible" CuA moiety in the absence but not the presence of either 1.5 or 2 mM dibucaine–HCl. Reduction of the CuA center is shown by a decrease in absorbance in the 840–860 nm region of a reduced minus oxidized difference spectrum [26, 27]. In the presence of dibucaine, addition of dithionite caused a decrease in near infra-red absorbance, indicating that the CuA center is capable of being reduced by a strong reductant. Similar results have been obtained using either isolated oxidase or mitochondria washed with bathophenanthroline sulfate and bathocuproine sulfonate to remove adventitious copper [26–29].

Reduced cytochrome oxidase exhibited a characteristic alpha band at 604 nm; addition of CO resulted in an increase in 590 nm absorbance due to formation of ferrous carboxy-cytochrome a_3. In the presence of dibucaine, the alpha band at 604 nm was still observed with ascorbate plus TMPD as reductant but the absorbance increase at 590 nm following CO addition was not observed; the 590 nm carboxy-oxidase band was observed when dithionite was added to the sample, however (data not shown). This is to be expected since dibucaine prevented reduction of cytochrome a_3 by ascorbate plus TMPD.

DISCUSSION

Chazotte and Vanderkooi [14] reported LD_{50}

by the University Center for Water Research at Oklahoma State University.

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Site of Inhibition and Additive Effects of Benzene, Naphthalene, Acenaphthene, and 1–Chloronaphthalene on Mitochondrial Respiration In Vitro

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Abstract

NADH oxidase activity in intact mitochondria is inhibited 50% by 525 ppm (6.7 mM) benzene, 15 ppm (117 μM) naphthalene, 3.9 ppm (25.5 μM) acenaphthene, or 3.8 ppm (23.4 μM) 1–chloronaphthalene. NADH oxidase, NADH–>Q_{10} reductase, and QH_2–>O_2 activities are inhibited by all four compounds while succinate–, cytochrome c–, and duroquinol oxidase activities are not inhibited. Inhibition of mitochondrial respiration occurs at the level of coenzyme Q for all four aromatic hydrocarbons. The ultraviolet absorbance spectrum of coenzyme Q is altered in the presence of naphthalene, acenaphthene, or 1–chloronaphthalene. Inhibition by a mixture of 2, 3, or 4 of the compounds tested is additive, reflecting the sum effect of each compound present in the mixture. This additive nature in vitro is consistent with previously reported effects in vivo and with compounds having similar modes (sites) of action. The similar mode of action is via a specific interaction with coenzyme Q_{10} and not a generalized membrane perturbation.

INTRODUCTION

Aquatic toxicity testing generally focuses on results obtained by exposing organisms to individual compounds. Only rather recently have a limited number of in vivo studies of the toxicity of mixtures of toxicants appeared in the literature. Many of the United States Environmental Protection Agency (EPA) water quality criteria, as mandated through the Clean Water Act of 1981, the Resources Conservation and Recovery Act of 1980, and the Federal Water Pollution and Control Act Amendment of 1982, have been established by testing individual compounds (1). However, the utility of these criteria is limited by the fact that aquatic organisms are much more likely to encounter mixtures of toxicants rather than single compounds (1,2,3). Toxicant interactions as well as physiochemical variables such as salinity or pH may also affect the toxicity of a mixture of compounds (1,4).

Surface waters, ground waters, leachates, and effluents often contain numerous compounds. However, relatively little is known regarding the effects of these mixtures to aquatic life or humans. Most of the recent studies regarding mixtures have focused on metals or low molecular weight, hydrophobic, aliphatic, and aromatic hydrocarbons grouped into a class of compounds usually considered to cause a generalized "narcosis" via a nonspecific membrane perturbation (2,3,5). The current consensus regarding the toxicity and interaction of multiple "narcotizing agents" has been formed from in vivo studies using either fathead minnows or cladocerans (2,3,5–7). These compounds have been found to act additively in mixtures (no evidence of synergism or antagonism) and are assumed to produce this "additive effect" via the common, nonspecific interaction mentioned above.

An in vitro study elucidating the interactions of multiple compounds is the focus of this study. We report the effects of the individual compounds benzene, naphthalene, acenaphthene, and 1–chloronaphthalene and their mixtures on mitochondrial respiration to determine which mitochondrial electron carriers are affected by the compounds. Mixtures of 2, 3, or 4 compounds were tested to determine if, in vitro, the compounds acted additively in a mixture as was previously reported in vivo (2,3,5–7).

EXPERIMENTAL PROCEDURES

Intact bovine heart mitochondria were prepared from fresh heart as described previously (8). Protein concentration of the prepared mitochondria was determined by the double Biuret method using bovine serum albumin as a standard (9).

NADH oxidase, succinate oxidase, duroquinol oxidase, and cytochrome c oxidase [using ascorbate plus N,N,N',N'–tetramethyl–p–phenylenediamine (TMPD) as electron donors] activities were determined by measuring oxygen consumption at 25 degrees C using a 1.95 ml volume glass water–jacketed chamber fitted with a Clark oxygen electrode (10).

Ubiquinol–50 oxidase was measured at 25 C in 1.95 ml volume using 53.3 μM ubiquinone–50 and 1.25 mM dithiothreitol (Cleland's Reagent) as an electron source. Antimycin–insensitive activity was measured after the addition of 0.1 μg antimycin A and subtracted from the total activity to yield the antimycin–sensitive respiratory chain–dependent activity as described previously (10). Enzymatic reduction of ubiquinone–50 was measured spectrophotometrically at 25 C by following the disappearance of NADH at 340 nm as previously described (10) using either a Gilford Model 252 single beam spectrophotometer or a Varian DMS–100S double (split) beam spectrophotometer.

The ultraviolet absorbance spectrum of purified ubiquinone–50 (Sigma, St. Louis) was recorded using a Varian DMS–100S spectrophotometer equipped with a DS–15 data station. The instrument was operated with 1 nm half bandwidth. The spectrum of ubiquinone in the presence and absence of benzene, naphthalene, acenaphthene, or 1–chloronaphthalene was recorded at molar ratios (test compound/Q) corresponding to that which would occur at or below the EC_{50}'s determined from the mitochondrial assays as was done in an earlier study (11).

Benzene, naphthalene, acenaphthene, and 1–chloronaphthalene were dissolved in absolute ethanol and prepared fresh daily. These solutions were added to the assay medium containing the mitochondria, buffer, and substrates to yield the desired final concentrations of the compounds. The effect of these compounds was recorded at

concentrations sufficient to yield activity between 10 and 100 percent control activity or at concentrations equal to or less than the water solubility of the compound.

RESULTS

The effects of different concentrations of naphthalene, acenaphthene, 1–chloronaphthalene, and benzene on NADH oxidase activity are shown in Figure 1. Of the compounds tested in this study, 1–chloronaphthalene was the most inhibitory, (EC_{50} = 3.8 ppm, 23.4 μM) followed in order by acenaphthene (EC_{50} = 3.9 ppm, 25.3 μM), naphthalene (EC_{50} = 15 ppm, 117 μM), and benzene (EC_{50} = 525 ppm, 6.7 mM). The acute effect of these compounds follows the trend reported previously of increasing toxicity with increasing hydrophobicity (log P) (3,12) and as shown in Figure 2.

Partial electron transport reactions measuring succinate oxidase (succinate→O_2), duroquinol oxidase (duroquinol→O_2), and cytochrome c oxidase (cytochrome c→O_2) activities were not appreciably inhibited by any of the four test compounds as shown in Table I. NADH–quinone reductase (NADH→coenzyme Q), and quinol oxidase (coenzyme QH_2→O_2), however, were inhibited by all four compounds to an extent similar to that of the NADH–oxidase reactions. This suggests a respiratory inhibition occurring at the level of coenzyme Q for all four aromatic hydrocarbons tested.

The interaction of naphthalene with coenzyme Q_{10} was previously suggested to involve either an interaction with the quinone itself or with a Q–binding protein (11). Naphthalene was subsequently shown to interact specifically with isolated Q_{10}, inducing a split in the 275 nm UV–absorbance maximum of coenzyme Q into maxima at 273 and 279 nm at molar ratios above 175 naphthalene/Q (11). Intact bovine heart mitochondria have been reported to contain coenzyme Q_{10} at a concentration between 2.9 nmoles/mg protein and 3.5 nmoles/mg protein (13,14). Therefore, the 10 ppm (78 μM) EC_{50} previously reported for naphthalene (15) would correspond to a molar ratio (naphthalene/Q) of 379; more than twice the concentration required to perturb the spectrum of ubiquinone in vitro. Concentrations of the other compounds used in this study were chosen to yield a molar

ratio of perturbant compound/coenzyme Q equivalent to or above that given by the ratio of the compound/Q in intact mitochondria at the EC_{50} concentration, and, thus, simulate the concentrations encountered in the respiratory assays.

Acenaphthene affects the UV–spectrum of ubiquinone at ratios above 118 acenaphthene:ubiquinone (Figure 3). The 3.8 ppm (23.5 μM) EC_{50} of acenaphthene required to inhibit respiration corresponds to a molar ratio of at least 123 based on the ubiquinone content in intact mitochondria discussed above. Acenaphthene induces absorbance maxima at 275 nm and 263 nm instead of only at 275 nm in the absence of toxicant.

1–Chloronaphthalene also causes a splitting in the absorbance maxima of ubiquinone. In the presence of 1–chloronaphthalene at a molar ratio greater than 75 1–chloronaphthalene:coenzyme Q, absorbance maxima are observed at 257 nm, 270 nm, and 278.6 nm. Figure 3 shows the spectrum of ubiquinone in the presence of 1–chloronaphthalene at a molar ratio of 95 1–chloronaphthalene to coenzyme Q. The EC_{50} of 1–chloronaphthalene (3.8 ppm) from Figure 1 corresponds to a molar ratio of 90 1–chloronaphthalene to ubiquinone in intact mitochondria.

Benzene, even at molar ratios as high as 55,261 benzene:ubiquinone, failed to cause a significant alteration in the spectrum of ubiquinone (Figure 3) although small shoulders on the spectrum are observed at 250 and 258 nm. The EC_{50} of the benzene mitochondrial assays (525 ppm) corresponds to a ratio of 32,560 benzene to ubiquinone in intact mitochondria.

The effect of mixtures of two, three, and four compounds on NADH oxidase activity was measured. Mixtures where the concentration of acenaphthene was held constant and the naphthalene concentration varied (Table II) showed that the two compounds act in an additive fashion; the total inhibition is equal to the sum of the inhibition expected for each individual compound at its concentration (cf. Fig. 1). Similarly, the effect of the 3 component mixture (benzene and acenaphthene concentration held constant while the

naphthalene concentration was varied) and a 4–component mixture (naphthalene varied, and the other compounds held at constant concentrations) are also additive as shown in Tables III and IV.

DISCUSSION

All four compounds analyzed in this work inhibit NADH–driven mitochondrial respiration yet do not affect succinate–driven respiration. NADH oxidase, NADH–ubiquinone reductase, and quinol oxidase enzyme systems are inhibited while succinate oxidase, cytochrome oxidase, and duroquinol oxidase systems are not (Table I). Since quinol oxidase (QH_2-O_2) is inhibited while duroquinol oxidase is not affected by the compounds, ubiquinone (coenzyme Q) is likely the site of action within the respiratory chain of all four aromatic hydrocarbons; this is the same site reported previously for naphthalene (15).

Both NADH–driven and succinate–driven mitochondrial respiration use ubiquinone as an electron carrier but it is of interest that only NADH–driven activity is inhibited. Short–chain quinone homologs (8 or less isoprenoid units) and long–chain homologs transfer electrons readily in succinate–driven respiration while the short–chain quinones are relatively ineffective in transferring electrons in NADH–driven respiration (16). Inhibition of the NADH oxidase system but not the succinate system is likely the result of 1) the specific requirement of long–chain quinone homologs (like Q_{10}) by NADH dehydrogenase and 2) the interaction of the aromatics with Q_{10}, the naturally occurring quinone. Succinate oxidase activity is unaffected since election transfer occurs directly to the cytochrome chain via short–chain quinone homologs.

Alteration of the spectrum of ubiquinone in the presence of naphthalene was previously speculated to be the result of perturbations either in the pi–electron cloud of the quinone or in its environment (11); a change in the polar environment of a quinone will induce an alteration in its absorbance spectrum (17–19). Acenaphthene and

1–chloronaphthalene, due to their structural similarity to naphthalene, would also be expected to act like naphthalene and alter the electronic configuration of the quinone, affecting its ability to bind a Q–binding protein.

Benzene failed to alter the spectrum of ubiquinone yet affects mitochondrial respiration only at very high concentrations. The single aromatic ring of the benzene molecule may not be capable of altering the ubiquinone due to the lower hydrophobicity of benzene. Alternatively, benzene may be too small to meet some steric requirements necessary for inhibition.

The overall inhibitory effect of mixtures was not different than that expected from the sum of the toxicities of the mixture components (Tables II–IV). The finding of additivity has been observed in vivo with Daphnia magna and Pimephales promelas (2,3,5,6,7) and previously shown to be essentially independent of the concentration of the constituents, such that a compound contributes to toxicity even if it was below its "no effect level" or threshold (2). Since all compounds were tested at concentrations that elicited a small but measurable inhibition, the lowest inhibition observed with the 2, 3, and 4 compound mixtures was 29%, 41%, and 51%, respectively.

Previous studies with the "anesthetic–like" aromatic hydrocarbons assume that these compounds have a similar mode of action (2,3,5,6,7). Previous in vivo studies suggested a nonspecific membrane perturbation as the basis for the toxic effects of the smaller aromatic hydrocarbons (2,3). In the current study, the inhibition of mitochondrial respiration occurs at a specific membrane component. If the inhibition was due to a generalized, nonspecific membrane effect, other enzyme reactions of the respiratory chain would likely be inhibited; this is not observed (Table I). Furthermore, the perturbation of the ultraviolet spectrum of ubiquinone by naphthalene, acenaphthene, and 1–chloronaphthalene (Figure 3) strengthens the concept of a specific interaction of the hydrocarbons with quinone.

These findings do not support the general membrane–perturbing "anesthetic" effect of this class of compound. Evidence from this study shows that the compounds tested have a very specific mode of action in mitochondria. The alteration of mitochondrial function in vitro should not be implied to be the sole mode of action in vivo. Further studies in vivo are necessary to establish if a specific action exists in vivo that may be responsible for the observed toxic effects to the whole organism.

ACKNOWLEDGMENTS

We wish to thank Mr. Bradley K. Stringer for his assistance during the course of this project and Carol Hefler for assistance in manuscript preparation. This research was supported by the Air Force Office of Scientific Research, Air Force Systems Command, USAF, under grant numbers AFOSR 84–0264 and AFOSR 89–0458.

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TABLE I

EFFECT OF NAPHTHALENE, ACENAPHTHENE, BENZENE, AND 1–CHLORONAPHTHALENE ON ELECTRON TRANSPORT REACTIONS

ENZYME ASSAY	TYPICAL CONTROL (nmoles O_2/min /mg/protein)	PERCENT INHIBITION BY NAP.	ACENAP.	BENZ.	1–Cl–NAP
NADH→O_2	600	50	50	50	50
Succinate→O_2	500	0	5	4	9
Cytochrome c→O_2	300	0	3	0	1
NADH–Ubiquinone	250[a]	46	51	49	45
Quinol→O_2	300	50	50	49	53
Duroquinol→O_2	700	0	1	2	0

Naphthalene concentration = 15 ppm (117 μM)

Acenaphthene concentration = 3.9 ppm (25.3 μM)

Benzene concentration = 525 ppm (6.7 mM)

1–chloronaphthalene concentration = 3.8 ppm (23.4 μM)

[a]nmoles acceptor/min/mg protein

TABLE II

COMPARISON OF AN EXPECTED ADDITIVE RESPIRATORY INHIBITION WITH ACTUAL INHIBITION USING A TWO COMPOUND MIXTURE

(HOLDING ACENAPHTHENE CONSTANT AT 5μM AND VARYING NAPHTHALENE)

CONCENTRATION NAPHTHALENE (μM)	EXPECTED INHIBITION IF ADDITIVE (%)	ACTUAL INHIBITION (%)
12	30	29
24	39	33
60	47	47
120	67	65
180	80	76
216	86	82

TABLE III

COMPARISON OF AN EXPECTED ADDITIVE RESPIRATORY INHIBITION WITH ACTUAL INHBITION USING A THREE COMPOUND MIXTURE (HOLDING ACENAPHTHENE AT 5 μM AND BENZENE AT 2.28 mM)

CONCENTRATION NAPHTHALENE (μM)	EXPECTED INHIBITION IF ADDITIVE (%)	ACTUAL INHIBITION (%)
12	39	41
24	48	48
60	56	58
120	76	75
180	88	86
216	95	90

TABLE IV

COMPARISON OF AN EXPECTED ADDITIVE RESPIRATORY INHIBITION WITH ACTUAL INHIBITION USING A FOUR COMPOUND MIXTURE (HOLDING ACENAPHTHENE AT 5 uM, BENZENE AT 2.28 mM, AND 1–CHLORONAPHTHALENE AT 4.67 μM)

CONCENTRATION NAPHTHALENE (μM)	EXPECTED INHIBITION IF ADDITIVE (%)	ACTUAL INHIBITION (%)
6	54	51
12	58	56
18	62	63
24	66	66
30	68	70
60	74	76

Figure Legends

Figure 1. Effect of increasing concentration of benzene, naphthalene, acenaphthene and 1–Cl–naphthalene on the inhibition of NADH oxidase activity. The effects of arenaphthene were not measured at concentrations in excess of 3.85 ppm since the water solubility is 3.93 ppm (25.3 μM).

Figure 2. Correlation between the log of the partition coefficient and the log of the concentration of compound needed to achieve 50% inhibiton of NADH oxidase activity. EC_{50} values obtained from Fig. 1.

Figure 3. Effect of aromatic hydrocarbons on the ultraviolet absorbance spectrum of ubiquinone (Q_{10}). In spectra 2–5, the corresponding amount of perturbant was present in the reference cuvette; thus the absorbance spectrum of the compound is subtracted from the spectrum of Q_{10} in the presence of the compound.

Spectrum 1: Absorbance spectrum of 1.93 μM Q_{10}.

Spectrum 2: Spectrum of 1.93 μM Q_{10} in the presence of 1545 ppm (19.8 mM) benzene, corresponding to a 10,252/1 ratio of benzene/Q.

Spectrum 3: Spectrum of 1.93 μM Q_{10} in the presence of 7726 ppm (99.1 mM) benzene, corresponding to a benzene/Q ratio of 51,260.

Spectrum 4: Spectrum of 1.93 μM Q_{10} in the presence of 35 ppm (227 μM) acenaphthene, corresponding to an acenaphthene/Q ratio of 118.

Spectrum 5: Spectrum of 1.93 μM Q_{10} in the presence of 30 ppm (185 μM) 1–chloronaphthalene, corresponding to a chloronaphthalene/Q ratio of 95.

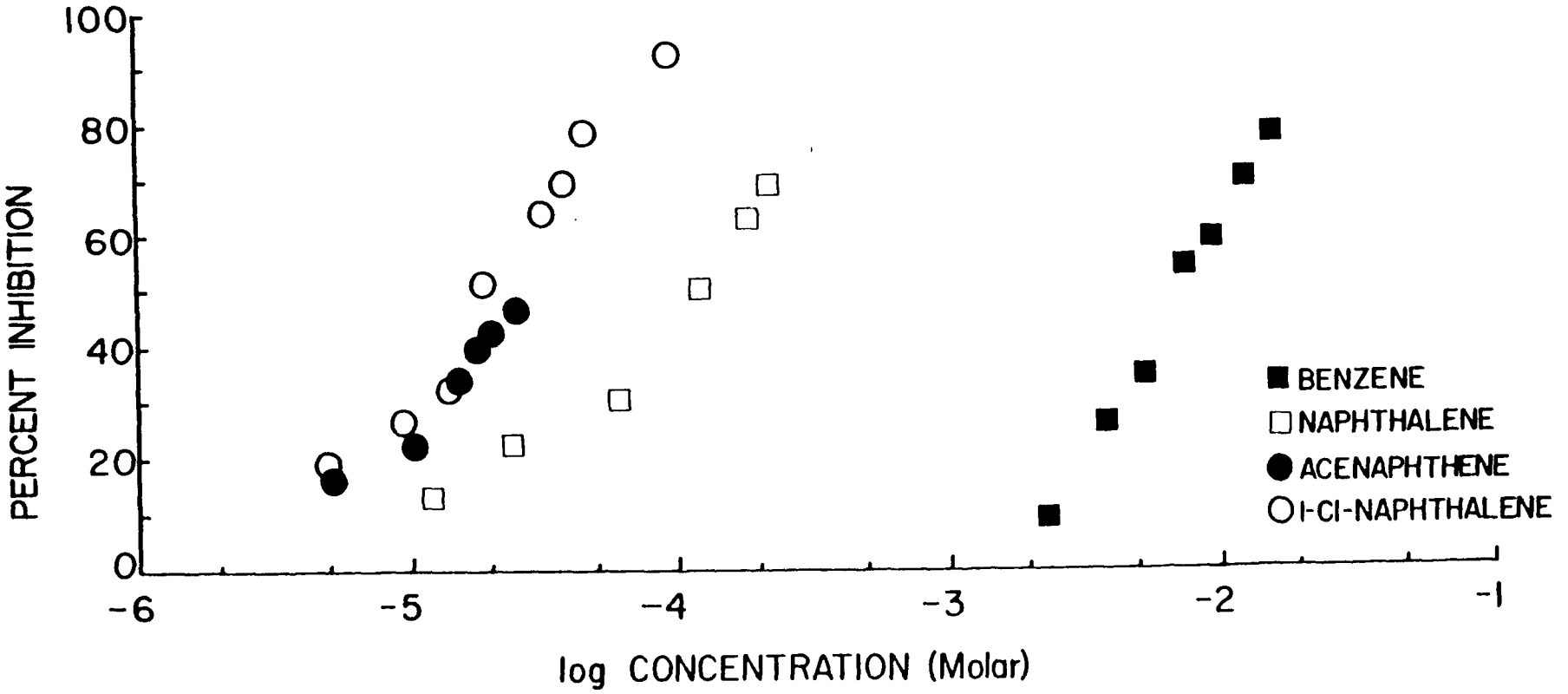
100
80
60
40
20
0
PERCENT INHIBITION
-6
-5
-4
-3
-2
-1
log CONCENTRATION (Molar)
BENZENE
NAPHTHALENE
ACENAPHTHENE
1-Cl-NAPHTHALENE

Figure 1

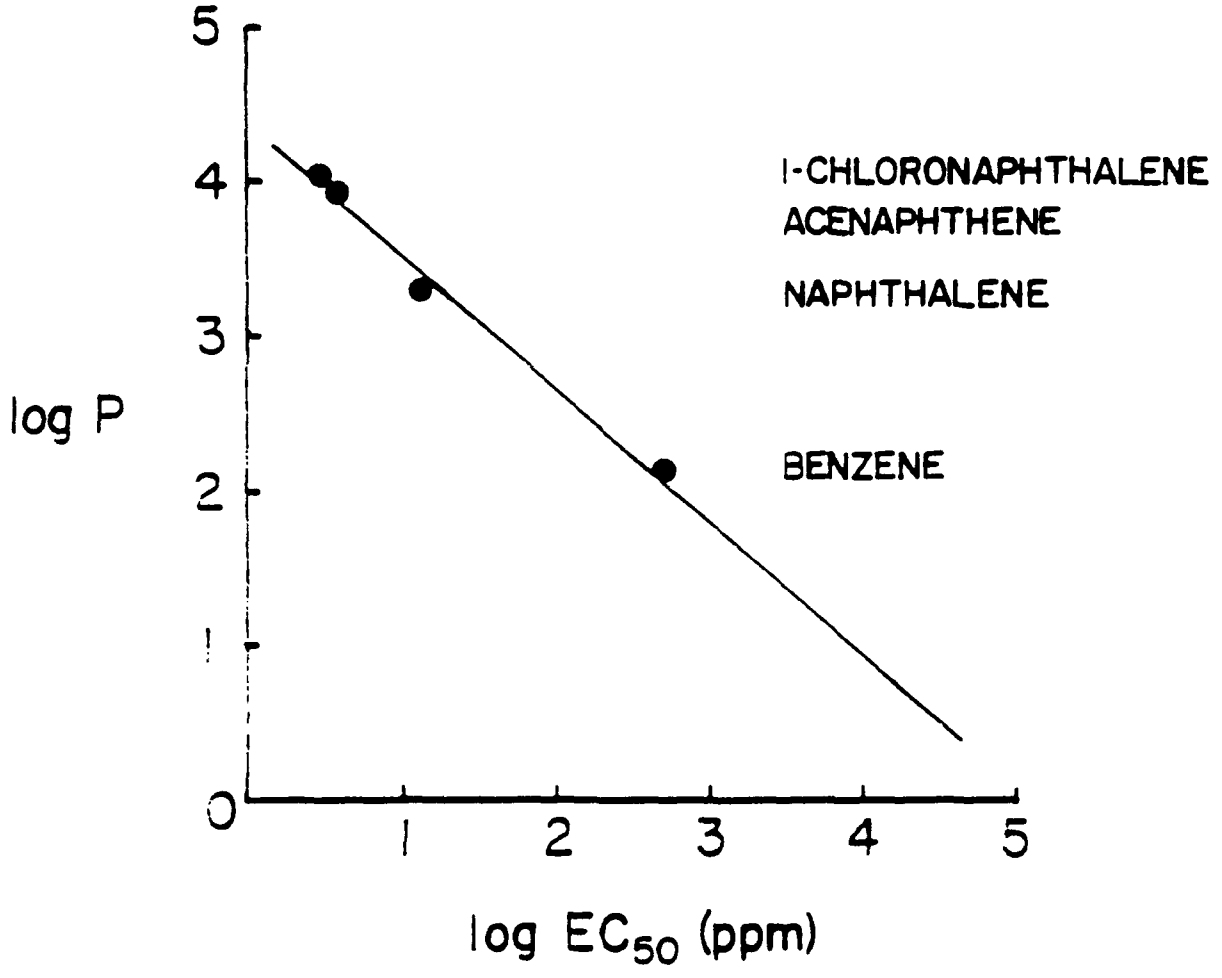
5
4
3
2
1
0
log P
1
2
3
4
5
log EC50 (ppm)
1-CHLORONAPHTHALENE
ACENAPHTHENE
NAPHTHALENE
BENZENE

Figure 2

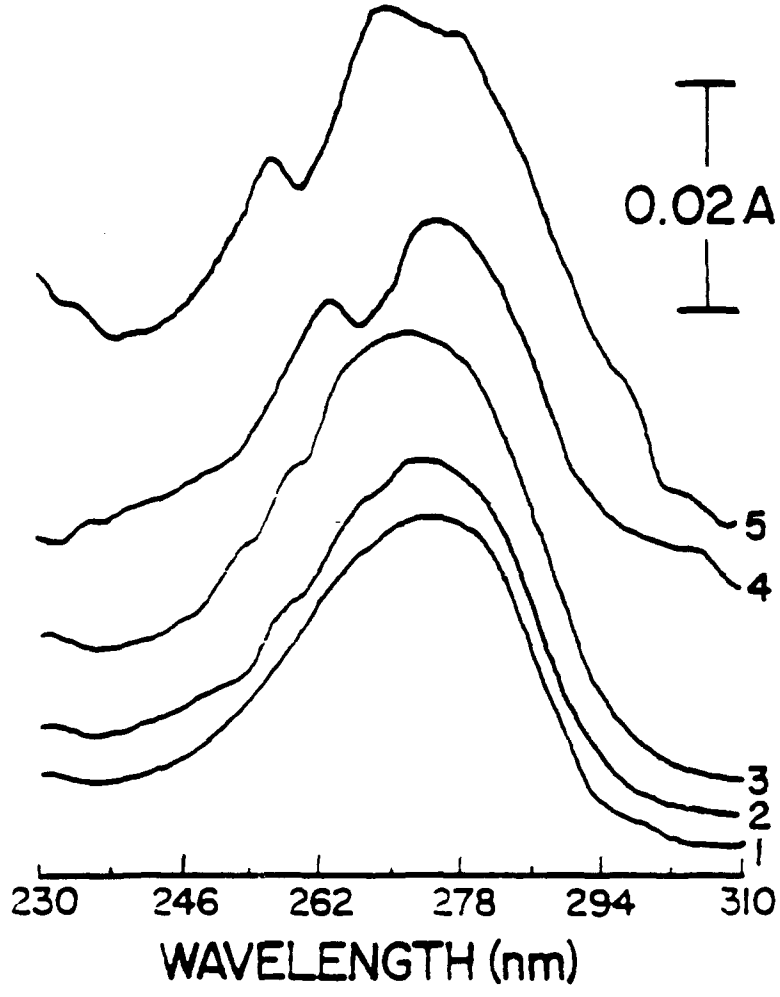

Figure 3

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pH-DEPENDENT CO RECOMBINATION IN CYTOCHROME OXIDASE. H. James Harmon and B.K. Stringer. Oklahoma State Univ., Stillwater, OK 74078.

The rate of recombination of CO with fully reduced cytochrome oxidase in intact beef heart mitochondria was measured at 445nm following flash photolysis at temperatures between 180 K and 230 K. A single Arrhenius slope corresponding to an apparent energy of activation (Ea) of 10.5 kcal/mole is observed at pH 7.4; the rates in the presence of 100% CO are twice those in 1% CO. At pH 5.5, Ea's of 11.3 and 7.1 kcal/mole are observed above and below 210 K with 100% CO, respectively, while Ea's of 7.4 and 11.1 kcal/mole are observed above and below 210 K in 1% CO. At pH 9.0, Ea's of 9.2 (above 210 K), 13.8 (190-210 K), and 2.7 (below 190 K) kcal/mole are observed with 1% CO; Ea's of 10.8, 12.4, and 2.6 kcal/mole are observed in the same temperature ranges with 100% CO present. The findings suggest models where up to 4 energy barriers are encountered in the migration of CO from the suspending medium to the heme iron and where the intermediate regions between the barriers can hold 1 or 2 CO. The size of the barriers and the number of CO involved are pH-dependent. The data suggest a possible mechanism of respiratory control by pH changes induced by proton translocation.

This research is supported by the Air Force Office of Scientific Research, Air Force Systems Command, under grant number AFOSR 89-0458.

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EFFECT OF pH ON CO RECOMBINATION IN CYTOCHROME OXIDASE. BK Stringer and HJ Harmon, Okla. State Univ., Stillwater, OK

Carbon monoxide recombination with cytochrome oxidase in beef heart mitochondria was measured following flash photolysis at low temperatures. At pH 7.4, a monophasic Arrhenius plot is observed in the presence of both 1% and 100% CO with an energy of activation (Ea) of 10.5 kcal/mole. The rate constants with 100% CO are twice those with 1% CO. At pH 5.5 biphasic plots are observed. With 1% CO Ea's of 11.3 and 7.1 kcal/mole are observed below and above 210 K, respectively. In the presence of 100% CO, Ea's of 7.4 and 11.1 kcal/mole are observed below and above 210 K, respectively. The data suggest a model of three intermediate CO holding regions other than the Fe separated by energy barriers. At pH 7.4 all barriers are 10.5 kcal/mole and each region can hold up to 2 CO. At pH 5.5 the barrier between Fe and the innermost region is 7.4 kcal/mole; the other barriers are 11.3 kcal/mole. The innermost and outermost regions can hold 2 CO; the middle region holds only 1 CO.

This research was supported by the Air Force Office of Scientific Research, Air Force Systems Command, USAF, under grant AFOSR 89-0458.

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pH-DEPENDENT AND ENERGY-LINKED CO BINDING TO CYTOCHROME OXIDASE IN INTACT MITOCHONDRIA. H. James Harmon and B. K. Stringer, Department of Microbiology, Oklahoma State University, Stillwater, OK 74078.

The rate of recombination of CO with fully reduced cytochrome oxidase in intact beef heart mitochondria was measured at 445nm following flash photolysis at temperatures between 180 and 230 K. A single Arrhenius slope corresponding to an apparent energy of activation (Ea) of approximately 10.5 kcal/mole is observed at pH 7.4 in the presence/absence of the uncoupler CCCP; in the absence but not the presence of CCCP, recombination rates in the presence of 100% CO are twice those in 1% CO. At pH 5.5, Ea's of 11.3 and 7.1 kcal/mole are observed above and below 210 K in 100% CO, while Ea's of 7.4 and 11.1 kcal/mole are observed above and below 210 K in 1% CO. At pH 9, Ea's of approx. 9.2 and 10.8 kcal/mole are observed in the presence of 1% and 100% CO, respectively; at warmer temperatures the rates in 100% CO are twice the rates in 1% CO. In the presence of CCCP, an Ea of approximately 10.3 kcal/mole is observed with 1% and 100% CO; the rates are the same with 1% and 100% CO. These findings suggest models where up to 3 energy barriers are encountered in the migration of CO from the suspending medium to the heme iron and where the intermediate regions can hold 1 or 2 CO, depending on pH and energy state. The data implicate a role for the undetectable copper center in ligand binding to the oxidase.

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