DIFFUSION IN THE MITOCHONDRIAL INNER MEMBRANE: IMPLICATIONS FOR THE MECHANISM OF ELECTRON TRANSFER AND HEXAAMMINERUTHENIUM, AN EFFECTIVE ELECTRON DONOR TO CYTOCHROME OXIDASE

By

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A DISSERTATION

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1984

This dissertation is dedicated to my mother and father who have given me love, encouragement, and every opportunity to learn.

Acknowledgements

One of the pleasures associated with completing this dissertation is having an opportunity to express in writing my appreciation to all the people who helped make my graduate career a rewarding and enjoyable experience. First and foremost, I would like to thank Martha Purdon for giving me help and support and making me smile during the difficult times.

I am greatly indebted to Dr. Shelagh Ferguson-Miller who I consider to be a role model for both my scientific and personal development. It would be futile to attempt to list the attributes that make Dr. Ferguson-Miller such a good mentor, therefore, I will leave it to say that working for her has been both highly instructive and enjoyable. These feelings have been further enhanced through interactions with my co-workers Debra Thompson and Maria Suarez. "You both are marvelous!"

I am also grateful to Dr. Melvin Schindler for giving me the opportunity to collaborate with him on the photobleaching experiments as well as for his inciteful suggestions and contagious enthusiasm. I would also like to thank Dr. John Wang, Dr. Jack Holland, Tom Metcalf, Mark Swaisgood, and members of the M.S.U. mass spectroscopy facility for their discussions and assistance during the FRAP studies.

Finally I would like to acknowledge some of the people who, in many ways positively influenced my research: Tom Carlson, Jeff Nickerson, Steve Brooks, Terry and Sue Van Aken, Joyce Robinson, Theresa Fillwock, and Betty Brazier.

ABSTRACT

DIFFUSION IN THE MITOCHONDRIAL INNER MEMBRANE:
IMPLICATIONS FOR THE MECHANISM OF ELECTRON TRANSFER

AND

HEXAAMMINERUTHENIUM, AN EFFECTIVE ELECTRON DONOR
TO CYTOCHROME OXIDASE

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Chapter I. Chromatography of the calcium transport inhibitor, ruthenium red, on a cation exchange resin yields a colorless component capable of donating electrons to cytochrome oxidase. On the basis of spectral, physical and activity studies the compound was identified as $Ru(NH_3)_6^{2+/3+}$. $Ru(NH_3)_6^{2+}$ is an efficient electron donor to cytochrome oxidase, and exhibits biphasic steady state kinetics qualitatively similar to cytochrome c, but with lower affinity (K_{m_1} = 8 μM, K_{m_2} = 88 μM). Under conditions that favor tight binding to cytochrome oxidase, cytochrome c acts as a competitive inhibitor of $Ru(NH_3)_6^{2+}$, indicating that the two electron donors interact at the same site(s). The ability of $Ru(NH_3)_6^{2+}$ to efficiently donate electrons to cytochrome oxidase in a manner similar to cytochrome c make it a potentially valuable tool for studying energy conservation by cytochrome oxidase.

Chapter II. The role of diffusion in mitochondrial electron transfer is the subject of a current controversy. In general, two limiting cases have been proposed: (i) electron transfer occurs through a unit electron transfer chain in which stable associations exist between the redox components or (ii) electrons are transferred by random collisions between freely diffusing

redox partners involving only transitory associations between the components. To elucidate this problem we have measured the mobilities of phospholipid, ubiquinone, cytochrome c, and cytochrome oxidase in inner membranes of giant mitochondria from cuprizone fed mice. Using affinity purified morpholinorhodamine labelled antibodies against cytochrome oxidase the diffusion coefficient for this component was found to be 1.5×10^{-10} cm^2/sec. The diffusion coefficients for singly modified derivatives of cytochrome c which had native-like activity were found to be $1.6\text{-}3.6 \times 10^{-10}$ cm^2/sec in 42 mM mannitol, 8 mM Hepes and 7×10^{-10} cm^2/sec in 25 mM (Tris) cacodylate. These values are too low to be consistent with a random diffusion mechanism of electron transfer in this segment of the respiratory chain. The diffusion coefficient for fluorescently labelled ubiquinone (8.5×10^{-9} cm^2/sec) is similar to the value obtained for the phospholipid, NBD-phosphatidylethanolamine (6.0×10^{-9} cm^2/sec) and is consistent with ubiquinone functioning as a mobile electron carrier. However, when digitonin is incorporated into the mitoplasts decreased rates of ubiquinone diffusion and increased rates of electron transfer are observed. A model is presented to explain these results in which an equilibrium exists between freely diffusing and associated electron transfer components.

Some of the work presented in Chapter II has previously been reported and is reprinted by permission of the publishers from "Dynamics of Electron Transfer in Mitochondrial Membranes" by Hochman, J.H., Schindler, M., Lee, J.G. and Ferguson-Miller, S. (eds. F.W. Stratman, D.L.F. Lennon and R.L. Zalton), in Biochemistry of Metabolic Processes, pp. 441-450. Copyright 1982 by Elsevier Science Publishing Co., Inc.

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One and Three Letter Codes for Amino Acids

AMINO ACID		
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Å	Angstrom (10^{-8} cm)	
ADP	adenosine 5'-diphosphate	
ATP	adenosine 5'-triphospate	

CM	carboxymethyl
FRAP	fluorescence redistribution after photobleaching
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane-sulfonic acid
LR	lissamine rhodamine
MR	morpholinorhodamine
NBD	N-4-nitrobenz-2-oxa-1,3 diazole-L-x-
NMR	nuclear magnetic resonance
$Ru(NH_3)_6^{2+/3+}$	hexaammineruthenium
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine
TMR	tetramethylrhodamine

MITOCHONDRIAL ELECTRON TRANSFER: AN OVERVIEW

Oxidative phosphorylation constitutes the predominant mechanism for conservation of energy in biological systems. The process, which is obligatory to all aerobic life, occurs in the mitochondria of eucaryotes and the plasma membrane of procaryotes. Being responsibile for over ninety percent of the energy conserved in aerobic metabolism, oxidative phosphorylation represents one of the most important adaptions in evolution.

The concept of the respiratory chain was first reported by Keilin in 1925 (Keilin 1925, 1966) who demonstrated the existence and the ubiquitous nature of the cytochromes a,b and c in aerobic life forms. Originally the respiratory chain was thought of only as a pathway in which the oxidation of metabolites was linked to the reduction of oxygen. However by the end of the 1940's numerous reports appeared which associated this activity with the phosphorylation of ADP (Lipmann, 1941, 1946; Ochoa, 1940; Freidkin and Lehninger 1948). As a result of these studies considerable attention was directed toward elucidating the mechanism by which this oxidative phosphorylation occurs.

Initial attempts to explain this process were largely inspired by knowledge

of the chemically linked, ATP forming reactions in glycolysis. As a result of this influence, models were proposed in which phosphorylated (Lipmann, 1946) and nonphosphorylated (Slater, 1953; Lehninger, 1955) high-energy intermediates were involved in phosphorylation of ADP. However, in spite of numerous attempts to isolate the energy-rich intermediates, no compounds were found with this activity. Moreover, the ability of structurally diverse uncouplers to inhibit phosphorylation of ADP while stimulating electron transfer activity and ATP hydrolysis was difficult to reconcile with a chemical coupling mechanism. Recognizing these discrepancies and the significance of the membrane localization of the respiratory chain, Peter Mitchell (1961) introduced the chemiosmotic hypothesis. The model, derived from physiological rather than chemical considerations, proposes that an asymmetrically-oriented respiratory chain can catalyze vectorial electron and proton transfer across the membrane such that the free energy from the redox reactions is conserved in the form of a pH and electrochemical gradient. This gradient, later termed the proton motive force, is then used to drive the synthesis of ATP.

Although the chemiosmotic hypothesis initially met with considerable opposition, experimental support for the model gradually accumulated. Most notably, experiments with chloroplasts (Jagendorf and Uribe, 1966), and with the purified mitochondrial ATPase reconstituted into liposomes (Kagawa and Racker, 1971; Racker and Stockeneus, 1974) showed that ATP synthesis could be reversibly driven by an electrochemical gradient.

At the present time the basic premise of the chemiosmotic hypothesis is widely accepted and the model serves as the central dogma for studying bioenergetics. While this understanding has contributed greatly to our knowledge of oxidative phosphorylation, many questions concerning the details of the process still remain to be answered. One of the most intriguing problems

concerns the molecular mechanisms for generating the proton motive force in mitochondia. The original hypothesis proposed by Mitchell (1961) states that the redox centers alone are responsible for generating the membrane potential. However, this relatively simple model has been challenged by recent reports that some of the respiratory complexes may function as redox-linked proton pumps (Wikstrom, 1977; Wikstrom and Krab, 1980). The implications of the proton pump mechanism is that proton translocation is comformationally linked to the redox reactions, possibly even involving communication between the respiratory complexes (Chance, 1981). In order to resolve this problem it is necessary to consider the structure/function relationships of the respiratory chain components and the membrane in which they reside.

Cytochrome oxidase (Complex IV):

Cytochrome oxidase is the most extensively studied integral complex in the electron transfer chain. Although there are controversies concerning even the most basic properties of the protein, a fairly detailed understanding of its structure and function is beginning to emerge.

Cytochrome oxidase is the terminal electron carrier in the respiratory chain. The enzyme catalyzes the transfer of electrons from cytochrome c through its four metal centers (hemes a and a_3; Cu_a and Cu_{a_3}) to oxygen, yielding the net reaction:

$$1 \text{ cytochrome } c^{2+} + 1\ H^+ + 1/4 O_2 \rightarrow 1 \text{ cytochrome } c^{3+} + 1/2\ H_2O.$$

The details concerning the mechanisms for this reaction are still being elucidated; however it is generally recognized that heme a_3 and Cu_{a_3} are involved in the binding of oxygen and its conversion to water while heme a and Cu_a are the initial electron acceptors from cytochrome c (For review

see chapter 6 of Wikstrom et al. 1981).

The net reaction catalyzed by cytochrome oxidase is very exothermic, releasing approximately 23 kcal per mole of water produced (Wikstrom et al., 1981). This energy is not released as heat but is conserved in the form of a membrane potential (Hinkle et al. 1982, Hinkle 1973). The mechanism by which cytochrome oxidase contributes to proton motive force is currently the subject of considerable controversy. The original model proposed (and still held) by Mitchell (1961, 1966; Moyle and Mitchell, 1978) states that electrons are transferred from cytochrome c on the cytoplasmic side of the membrane to oxygen on the matrix side resulting in the consumption of protons from the matrix. According to this model no protons are ejected onto the cytoplasmic side of the membrane and one charge is translocated per electron. In contrast to this view, Wikstrom (1977; Wikstrom and Saari, 1977; Krab and Wikstrom 1978) has proposed that cytochrome oxidase may also function as a redox-linked proton pump such that protons are taken up in the matrix and ejected on the cytoplasmic side of the membrane. While the majority of researchers in the field have obtained evidence that supports the proton pump model, it is not yet possible to completely rule out that the very small transient acidification observed in this complex system is some kind of artifact.

Analyzing the structural requirements for cytochrome oxidase has been difficult due to the complexity of the enzyme and the problems in purifying an intrinsic membrane protein in a reproducible way. Conventional procedures for preparing cytochrome oxidase utilize differential detergent solubilization of mitochondrial membranes followed by repeated ammonium sulfate fractionation (Yonetani, 1961; Fowler et al. 1962, Kuboyama et al. 1972). More recently milder purification procedures have been developed which utilize ion exchange (Merle and Kadenbach, 1980) and/or cytochrome c affinity

chromatography (Weiss and Kolb, 1979; Bill et al. 1980, 1982, Azzi et al. 1982; Thompson and Ferguson-Miller 1983). Enzyme prepared by these techniques have been reported to consist of anywhere from 6 to 13 subunits (Poyton and Schatz, 1975; Briggs et al. 1975; Downer et al. 1976; Verheul et al. 1979; Merle and Kadenbach, 1980, 1982; Wei and King, 1981; Merle et al. 1981; Kadenbach and Merle, 1981; Bill et al. 1982; Thompson and Ferguson-Miller, 1983; Kadenbach et al. 1983). The number of subunits is usually assessed by the number of major bands revealed by SDS-polyacrylamide gel electrophoresis. Most researchers accept that mammalian cytochrome oxidase is composed of at least seven subunits, and complete sequence analysis of the beef heart oxidase reveals 12 polypeptides in one to one stoichiometry. Kadenbach and co-workers (Merle and Kadenbach, 1980, 1982; Kadenbach and Merle, 1981, Merle et al. 1981, Kadenbach et al. 1983) contend that the mamalian enzyme consists of 13 different polypeptides, arguing that conventional SDS gel electrophoresis is not able to resolve the smaller polypeptides. Whether all these polypeptides represent true subunits or are artifacts resulting from contamination or proteolytic degradation has yet to be determined.

The three largest subunits (I, II and III) are coded for by the mitochondrial DNA (Mason and Schatz, 1983; Rubin and Tzagoloff, 1973; Yatscoff et al. 1977; Bernstein et al. 1978; Rascati and Parsons 1974; Hare et al. 1980). The DNA sequences for these subunits have been determined for yeast (Bonitz et al. 1980), humans (Anderson et al. 1981), mouse (Bibb et al., 1981), rat (Grosskopf and Feldman 1981) and beef (Anderson, 1982) enzyme. A comparison of these sequences shows that they are highly conserved suggesting that these subunits play an important role in the enzyme's function. Indeed, at present these are the only subunits which have been ascribed functions.

The central roles of subunits I and II are suggested from the analysis of

cytochrome oxidase isolated from the procaryote Parracoccus denitrificans. This enzyme has electron transfer activity and spectral properties analogous to the mitochondrial enzyme but consists of only two subunits with molecular weights similar to those for subunits I and II from eucaryotic sources (Ludwig and Schatz 1980). Subunit I from this enzyme demonstrates abnormal behavior on SDS polyacrylamide gels characteristic of the hydrophobic subunit I from eucaryotic sources (Ludwig and Schatz 1980, Sone 1981). Moreover, subunit II from Parracoccus is immunologically cross reactive with that from mitochondia (Ludwig 1981). However it should be noted that antibodies to Parracoccus subunit II not only react with subunit II from other sources but also cross react with some of the smaller polypeptides. Consequently, while these results are suggestive of subunits I and II from mitochondria being intimately involved in the electron transfer reactions, some involvement of the smaller subunits can not be ruled out.

In the eucaryotic enzyme, radiation inactivation studies indicate that the molecular weight for the functional unit necessary for electron transfer is between 70 (Kagawa, 1967; Thompson et al., 1982) and 110 kd (Suarez et al., 1984) similar to the sum of the molecular weights of subunits I and II. In agreement with this are the results of studies in which mild treatments were used to dissociate the subunits of cytochrome oxidase. In these studies subunits I and II were found to contain all the heme A (Winter et al. 1980), and the copper was exclusively associated with subunit II (MacLennan and Tzagoloff 1965; Winter et al. 1980). Chemical modifications (Millett et al. 1983) and cross-linking studies (Millett 1982; Briggs and Capaldi 1978; Bisson et al. 1977, 1975, 1980) also identify subunit II as the high affinity binding site for cytochrome c. It thus seems likely that subunits I and II have all the properties necessary to account for the electron transfer reactions in

cytochrome oxidase.

Subunit III has been reported to play an integral role in the apparent proton pump activity of cytochrome oxidase. The first evidence of involvement of subunit III in this activity was reported by Casey et al. (1979) who showed that the hydrophobic carboxyl-modifying reagent, DCCD [a known inhibitor of proton translocating ATPases (Beechley et al. 1967; McCarty and Racker 1967)], inhibits proton ejection activity but not electron transfer of cytochrome oxidase. Analysis of the DCCD treated enzyme showed that the compound binds to subunit III (Casey et al. 1980, 1981); Prochaska et al. 1981) and is attached to a glutamic acid residue which is part of a sequence similar to the DCCD binding region of the ATP synthetase (Prochaska et al. 1981). Cytochrome oxidase preparations depleted of subunit III do not appear to exhibit proton pumping when reconstituted into phospholipid vesicles (Pentilla et al. 1979). However subunit III depleted enzyme does demonstrate respiratory control (Carroll and Racker 1977; Pentilla and Wikstrom 1981; Wikstrom 1981; Wikstrom et al. 1981; Saraste et al. 1981; Thompson and Ferguson-Miller 1983) and exhibits normal electron transfer activity (Thompson and Ferguson-Miller 1983). These results suggest that proton translocation by subunit III is only loosely coupled to electron transfer and that in the absence of proton pump acitivity electron transfer still proceeds with the formation of an electrochemical gradient.

It should be emphasized that the proton pump activity of cytochrome oxidase and the role of subunit III in this process are quite controversial. Using the purified two subunit Parracoccus denitrificans enzyme Ludwig (1981) observed proton pump activity in reconstituted liposomes suggesting that subunit III is not necessary for proton pumping. Moreover the possibility that structural damage may have been imposed on cyctochrome oxidase upon DCCD treatment

or subunit III removal can not be ignored.

The role of the smaller cytoplasmically derived subunits is still largely unknown. Although some reports have appeared indicating subunit V contains heme A (Yu et al. 1977a) and that cytochrome c cross-links to subunits V and VI (Erecinska 1977) these results have not been further substantiated.

The structure of cytochrome oxidase has been studied using several techniques. A low resolution (12 Å) image of the enzyme has been obtained from 2 dimensional ordered arrays produced in detergent-extracted membranes (Vanderkooi et al. 1972; Wakabayashi et al. 1972; Vail and Riley 1974; Henderson et al. 1977 Seki et al. 1970; Fuller et al. 1979) using electron microscopy with image reconstruction. The shape of the enzyme is similar to a slanted Y in which the forks extend into the matrix approximately 15 Å and are separated by 15 Å, and the tail extends into the intermembrane space 60 Å. The orientation of the subunits within this structure, has been studied using crosslinking (Briggs and Capaldi 1977, 1978; Bisson et al. 1978, 1980), susceptibility to labelling by hydrophobic and hydrophilic reagents (Eytan et al. 1975; Chan and Tracy 1978; Eytan and Broza 1978; Bisson et al. 1979; Ludwig et al. 1979; Prochaska et al. 1980; Cerfetti and Schatz 1979; Georgevich and Capaldi 1982) and accessibility to subunit specific antibodies (Frey et al. 1978; Chan and Tracy 1978). The results of these studies aresummarized in Figure 1.

The enzyme has been isolated in active monomeric (MW = 130-200 Kd) and dimeric (MW = 260-400 kd) forms (Robinson and Capaldi 1977; Bisson et al. 1980; Ferguson-Miller et al. 1982; Thompson et al. 1982; Thompson and Ferguson-Miller 1983; Nalecz et al. 1983; Georgevich et al. 1983; Suarez et al. 1984a). The distribution of cytochrome oxidase in these two forms appears to be dependent on both the pH and ionic strength of the aqueous environment

Figure 1. Structure of and arrangement of subunits in cytochrome oxidase summarizing data from electron microscopy with image reconstruction, cross-linking, chemical labeling and antibody accessibility studies. Reproduced with permission from Thompson (1984).

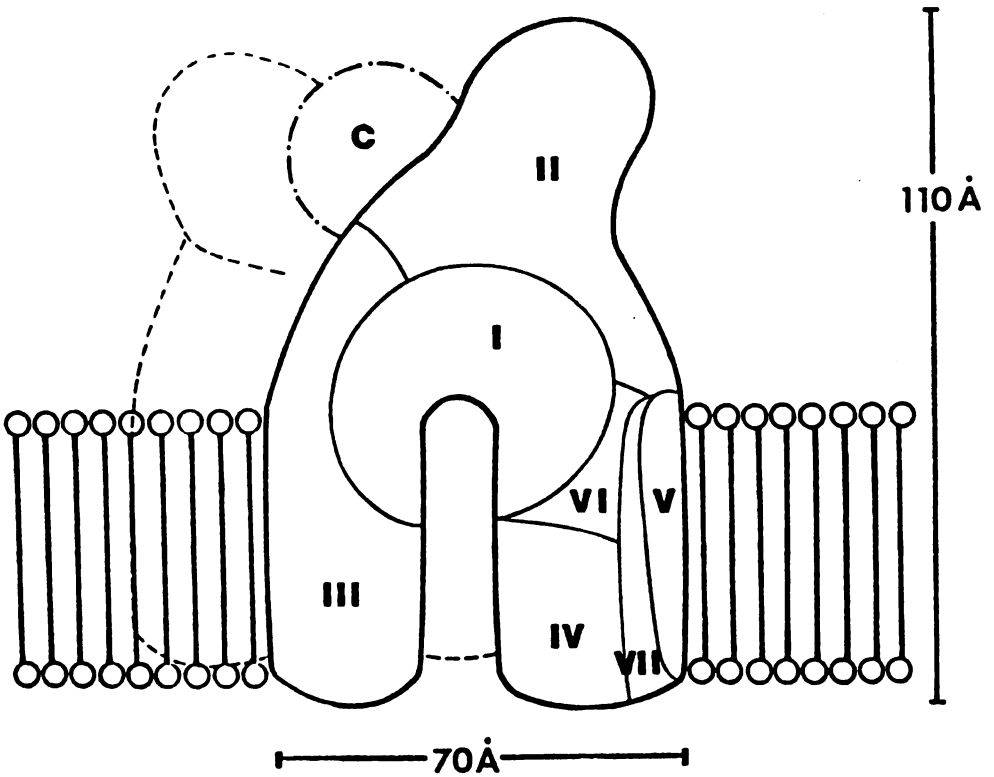


Figure 1

(Nalecz et al., 1983; Georgevich et al. 1983; Suarez et al. 1984b). Whether the dimer represents a physiologically important form or just a feature of the purified, detergent-solubilized enzyme remains to be determined. Regardless, further analysis of the proton pumping activity and the pre-steady state kinetics of the two forms may provide revealing clues toward elucidating the enzymes mechanism.

Cytochrome c:

Cytochrome c is the only small water soluble component in the electron transfer chain. The protein resides on the cytoplasmic side of the inner mitochondrial membrane and catalyzes one electron transfers from cytochrome bc_1 to cytochrome oxidase. The molecule consists of a single polypeptide chain slightly over 100 residues long with one heme c, and has a molecular weight of about 12,400 daltons. Having a relatively large number of lysine residues, cytochrome c has a large net positive charge with an isoelectric point of 10 (Keilen and Hartree, 1945; Barlow and Margoliash 1966). As a result of these features cytochrome c has readily lent itself to purification and to various studies resulting in a detailed understanding of the molecule's evolutionary, structural and kinetic properties.

The tertiary structures of cytochrome c from horse, bonito and tuna have been determined to 2 Å resolution (Dickerson et al. 1971, 1972; Takano et al. 1973; Ashida et al. 1973; Tsukihara et al. 1973; Tanaka et al.; 1975; Swanson et al. 1977). In spite of the evolutionary distance between the three species, the folding of the polypeptide backbone in the three cytochromes is identical (Ferguson-Miller et al. 1979). Basically, the structure as shown in Figure 2 is that of a typical water soluble globular protein with virtually all the hydrophobic side chains in the interior and the hydrophilic residues on the outside. The protein contains five α-helical regions, a tight hydrogen

Figure 2. Front view of Tuna Cytochrome c. The structure is deduced from x-ray diffraction data at 2 Å resolution. The amino acid sequence is:

*G-D-V-A-K-G-K-K-T-F-V-Q-K-C-A-Q-C-H-T-V-E-N-G-G-
H-K-V-G-P-N-L-W-G-L-F-G-R-K-T-G-Q-A-E-G-Y-S-Y-T-
T-D-A-N-K-S-K-G-I-V-W-N-N-D-T-L-M-E-Y-L-E-N-P-K-K-
Y-I-P-G-T-K-M-I-F-A-G-I-K-K-K-G-E-R-Q-D-L-V-A-Y-L-K-
S-A-T-S-O.

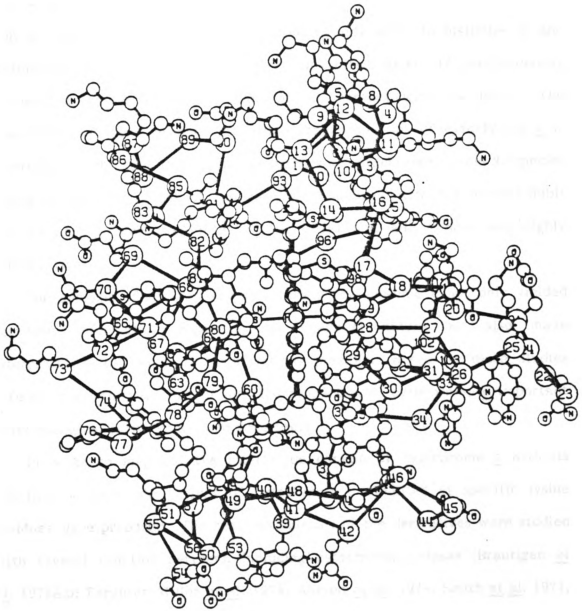


Figure 2

bonded structure on the right, a loose structure on the left and a hydrophobic crevice in which the heme is held.

The heme is buried within the protein only exposed to the aqueous environment at the outer edges of pyrrole rings II and III. It is covalently attached to the polypeptide chain via thioether bonds between the vinyl side chains of pyrrole rings I and II and cysteinyl residues 14 and 17. The heme iron is hexacoordinate with the two axial ligands being to histidine 18 and methionine 80. In addition to the residues cited above, 16 predominantly hydrophobic amino acid side chains are in contact with the heme. The importance of these residues in defining the properties of cytochrome c is illustrated by the fact that sequence analysis of cytochrome c from 80 species indicates that only eight of the residues associated with the heme exhibit any variability (Ferguson-Miller et al. 1979) and all changes are highly conservative.

The analysis of the tertiary structure of cytochrome c not only yielded pertinent information regarding its chemical nature but also made interpretation of chemical modifications possible. Based on these studies a fairly comprehensive insight into the properties of cytochrome c important in its biological activity has been developed.

In order to map out the interaction domain of cytochrome c with its reaction partners, derivatives of cytochrome c modified at specific lysine residues were prepared. The reaction kinetics of the derivatives were studied with several reaction partners, including cytochrome oxidase (Brautigan et al. 1978a,b; Ferguson-Miller et al. 1978; Ahmed et al. 1978; Smith et al. 1977, 1980; Osheroff et al. 1980), cytochrome bc_1 (Speck et al. 1979), purified cytochrome c_1 (Konig et al. 1980), cytochrome b_5 (Smith et al. 1980) and sulfite oxidase (Speck et al. 1981). At about the same time other groups

approached the problem by looking at the susceptibility of lysine residues to chemical modification when cytochrome c is bound to its redox partners relative to when the protein is free in solution (Rieder and Bosshard, 1978a,b, 1980). The results of the two approaches were in good agreement and showed that the same interaction domain on cytochrome c is involved in binding to all of the redox partners tested (Margoliash and Bosshard, 1983). This domain contains lysine residues 13, 27, 72, 86 and 87 which encircle an area of the protein that includes the exposed heme edge and several hydrophobic and hydrophilic residues of low evolutionary variability.

In addition to the large decrease in activity observed for the modifications within the binding site ($\geq$ 90% inhibited), derivatives outside of this region also showed some degree of inhibitions. This phenomenon was explained in terms of the influence of the chemical modification on the dipole moment of the molecule, which has a high concentration of positively charged residues oriented toward the front and essentially all the negatively charged residues on the back (Koppenol and Margoliash, 1982). These results have been summarized in a model where cytochrome c associates with its redox partners at the interaction domain and transfers electrons via the exposed heme edge, while the probability of such interactions occurring is optimized by the large dipole moment of the molecule (Koppenol and Margoliash, 1982; Margoliash and Bosshard, 1983).

The reaction of cytochrome c with cytochrome oxidase is very complex, exhibiting biphasic (Nicholls 1964, 1965; Ferguson-Miller et al. 1976, Errede and Kamen 1978) and even triphasic (Ferguson-Miller et al., 1978; Thompson et al. 1982) kinetics. The original model proposed to explain these kinetics involved two (or three) separate kinetically active binding sites on cytochrome oxidase (Ferguson-Miller, 1978). Alternatively, the triphasic kinetics observed

under some conditions (Thompson et al., 1982) could be ascribed to inhomogeneity in the cytochrome oxidase population, due to different states of aggregation of the enzyme. Recently an alternative model has been introduced where cytochrome c interacts with a single catalytic site within a negatively-charged domain. Binding of the first cytochrome c molecule will be strongest and will decrease the net electrostatic field, thus reducing the affinity for a second (or a third) molecule. Similarly, binding of the second cytochrome c results in sufficient perturbation of the electrostatic field to lower the affinity of the first molecule (Margoliash and Bosshard, 1983). It is worth noting that these mechanisms may not be necessary to understand the activity of cytochrome c in repleting the electron transfer chain (Nicholls 1974, 1976) where monophasic high affinity binding, similar to the initial phase for cytochrome oxidase, is observed.

Cytochrome bc_1 (Complex III):

Cytochrome bc_1, also termed ubiquinol-cytochrome c reductase, is a structurally intricate transmembrane complex which communicates electrons between ubiquinone and cytochrome c and accomplishes proton translocation (for review see Bowyer and Trumpower, 1981; Nelson, 1981). The enzyme has been isolated from several sources (Hatefi et al., 1962; Rieske et al., 1964a; Yu et al., 1978; Weiss and Kolb, 1979; Gellerfors et al., 1981) and generally exhibits eight bands on SDS electrophoretic gels. However, as is the case with cytochrome oxidase the number of subunits observed, and their apparent molecular weights vary under different electrophoretic conditions (Marres and Slater, 1977; Capaldi et al., 1977) and as many as 10 polypeptides have been observed (Marres and Slater 1977).

Subunits I and II [MW = 50 and 45 kd respectively (see Nelson 1981)] are termed the core proteins. Although no redox centers have been associated

with these polypeptides, alkylation of core protein I leads to partial inactivation of the enzyme suggesting that this peptide plays a catalytic or structural role in the holoprotein (Gellerfors et al. 1976). Attempts to deduce the topology of these components in the native membrane has largely led to conflicting results. In ^{35}S-DABS labelling studies using beef heart mitochondria core protein I has been localized exclusively on the matrix side of the membrane in some studies (Mendel-Hartvig and Nelson 1978) and only on the cytoplasmic side in others (Bell et al. 1979). Similarly core protein II has been shown to label solely on the matrix side (Mendel-Hartvig and Nelson 1978) and on both sides of the membrane (Bell et al. 1979). In Neurospora crassa both proteins have been proposed to be water soluble components located on the matrix surface (Weiss et al. 1979; Hovmoller et al. 1981).

The peptides containing metal centers (cytochrome b-566 and b-562, cytochrome c_1, and the Reiske iron sulfur cluster) have been identified as subunits III, IV and V. These subunits are recognized to be in the complex in a molar ratio of 2:1:1 (cytochrome b:cytochrome c_1: iron sulfur cluster). Their stoichiometries with respect to the other components is not yet resolved (Gellerfors and Nelson, 1975; Marres and Slater, 1977; Yu et al., 1977b; Weiss and Juchs, 1978; Mendel-Hartvig and Nelson 1981).

Subunit III has been identified as cytochrome b (Weiss and Ziganke, 1974; Weiss, 1976; Marres and Slater, 1977; Lin and Beattie, 1978). The molecular weight of the heme-free polypeptide chain has been calculated from its DNA sequence to be 42,505 daltons (Nobrega and Tzagoloff, 1980; Anderson et al., 1981); however due to the high concentration of hydrophobic residues in the chain the subunit migrates with an apparent molecular weight of only 27,000-33,000 daltons on SDS polyacrylamide gels. Since both b hemes appear to reside in identical polypeptides the differences in the properties of b-562

and b-566 are hard to explain. Although some reports indicate that a second small molecular weight heme b containing peptide may exist (Gellerfors and Nelson, 1975; Das Gupta and Rieske, 1973; Baum et al., 1967; Yu et al., 1975; King, 1981) the results of Marres and Slater (1977) suggest that this is due to non-specific absorption of dissociated heme. Alternatively, Slater (1981) ascribes the discrepancies between the hemes properties to slight conformational differences.

Cytochrome c_1 has been identified as subunit IV and is reported to have a molecular weight of 29,000-31,000 (Yu et al., 1972; Trumpower and Katki, 1975; Konig et al., 1980; Robinson and Talbert, 1980). Recently the complete amino acid sequence has been determined from the beef heart protein (Wakabayashi et al., 1980). Similar to cytochrome c, the heme in cytochome c_1 is covalently attached to the polypeptide chain via two cysteine residues located close to the amino terminal (King, 1981). The last 42 residues toward the carboxyl terminal have a high concentration of hydrophobic and basic amino acids which have been implicated in anchoring the protein into the membrane (Wakabayashi et al., 1980; Li et al., 1981). There are two highly acid regions extending from residues 58 to 79 and 167 to 173 which may be involved in cytochrome c binding (Wakabayashi et al., 1980). There are also several unique sequences including 1 pro-x-pro-x-pro-x-pro sequence and 5 pro-pro doublets which have not been assigned functions (Wakabayashi et al., 1980).

The fifth subunit has been identified as the Rieske iron-sulfur protein (Trumpower and Edwards, 1979). The purified protein contains a single 2S:2 Fe center (Rieske et al., 1964b; Siedow et al., 1978) and exhibits an apparent molecular weight of 25 kd on SDS-polyacrylamide gels. Both the iron-sulfur protein and cytochrome bc_1 depleted of this protein have recently been prepared in reconstituted active forms (Trumpower and Edwards, 1979;

Trumpower et al., 1980; Trumpower, 1981a). During intermediate steps in its isolation, the iron-sulfur protein is tightly associated with cytochrome c_1 and a smaller subunit suggesting that a functional association may exist between these components in the native complex (Trumpower and Edwards, 1979; Trumpower, 1981a). Indeed, studies on this system suggest that the iron sulfur center directly precedes cytochrome c_1 in the electron transfer sequence (Trumpower, 1976, 1981a,; Edwards and Bowyer, 1981). Further studies revealed that the iron sulfur protein is required for the reduction of cytochrome b in the presence, but not in the absence of antimycin (Edwards and Bowyer, 1981). These results indicate that a linear electron flow through the bc_1 complex is not tenable. Instead, branched cyclic electron flow mechanisms have been proposed (Mitchell, 1976; Wikstrom and Krab, 1980).

In addition to the four metal centers, two ubiquinone binding proteins have been reported. One of these proteins (QPs) has been isolated and is implicated in the ability of cytochrome bc_1 to reconstitute with succinate dehydrogenase (Yu et al., 1977c). The second bound ubiquinone has been detected in the purified enzyme by the appearance of a stable ubisemiquinone in the presence of catalytic amounts of succinate dehydrogenase (Yu et al., 1978, 1980; Nagoaka et al., 1981). Using an arylazido-ubiquinone analogue Yu and Yu (1980) have identified these two proteins as 15-17 kd and 37 kd molecular weight components.

The structure of cytochrome bc_1 in the membrane is still largely unresolved. The complex has been isolated as a dimer in triton X-100 (von-Jagow et al., 1977), however active monomeric preparations have recently been reported (Nalecz and Azzi, 1984). Membranous 2-dimensional crystals have been prepared of the complex from Neurospora crassa and a low resolution (25 Å) image was obtained (Weiss et al., 1979; Wingfield et al., 1979), and

more recently to 10 Å (Perkins and Weiss, 1983). The molecule appears to have cylindrical shape 150 Å long by 70 Å across and spans the membrane extending 70 Å from one side of the membrane (presumably the matrix side) and 30 Å from the other.

Ubiquinone:

Ubiquinones are a famiy of lipid soluble molecules which function in mitochondria as electron carriers between the dehydrogenases and cytochrome $\underline{bc_1}$. The structure of ubiquinone-10, the form most predominant in mammalian mitochondria, is shown in Figure 3. The molecule consists of a 2,3-dimethoxy-5—methyl-1,4-benzoquinone group attached to a 50 carbon (10 unit) isoprenoid chain. The vicinyl methyl moieties on the isoprenoid side chain impose steric hindrance to rotation around the single bonds while the extensive unsaturation introduces periodic rigidity (Trumpower, 1981b). As a result of these factors the molecule is conformationally restrained.

The redox properties of ubiquinone are due to the substituted 1,4 benzoquinone which can reversibly accept two electrons in a manner that is coupled to the uptake and release of protons. The methoxy and alkyl ring substituents in part regulate the redox potential of this group, lowering its midpoint potential by 100 mV from that of unsubstituted 1,4-benzoquinone (Morton, 1965). The substitutions on all four positions also prevent the benzoquinone ring from irreversibly reacting with protein sulfhydryl groups which otherwise could form thiol-ether adducts with the quinone (Snell and Wiessberg, 1939; Redfearn and Whittaker, 1962; Trumpower 1981b).

The redox-linked uptake and release of protons by ubiquinone is particularly interesting because of its apparent involvement in the generation of a membrane potential (Mitchell, 1966, 1976). If the oxidation and reduction reactions occur asymmetrically on opposite sides of the membrane, then

Figure 3. Structure of ubiquinone-10 and its oxidation states: ubiquinone (top) semiquinone (center) and ubiquinol (bottom).

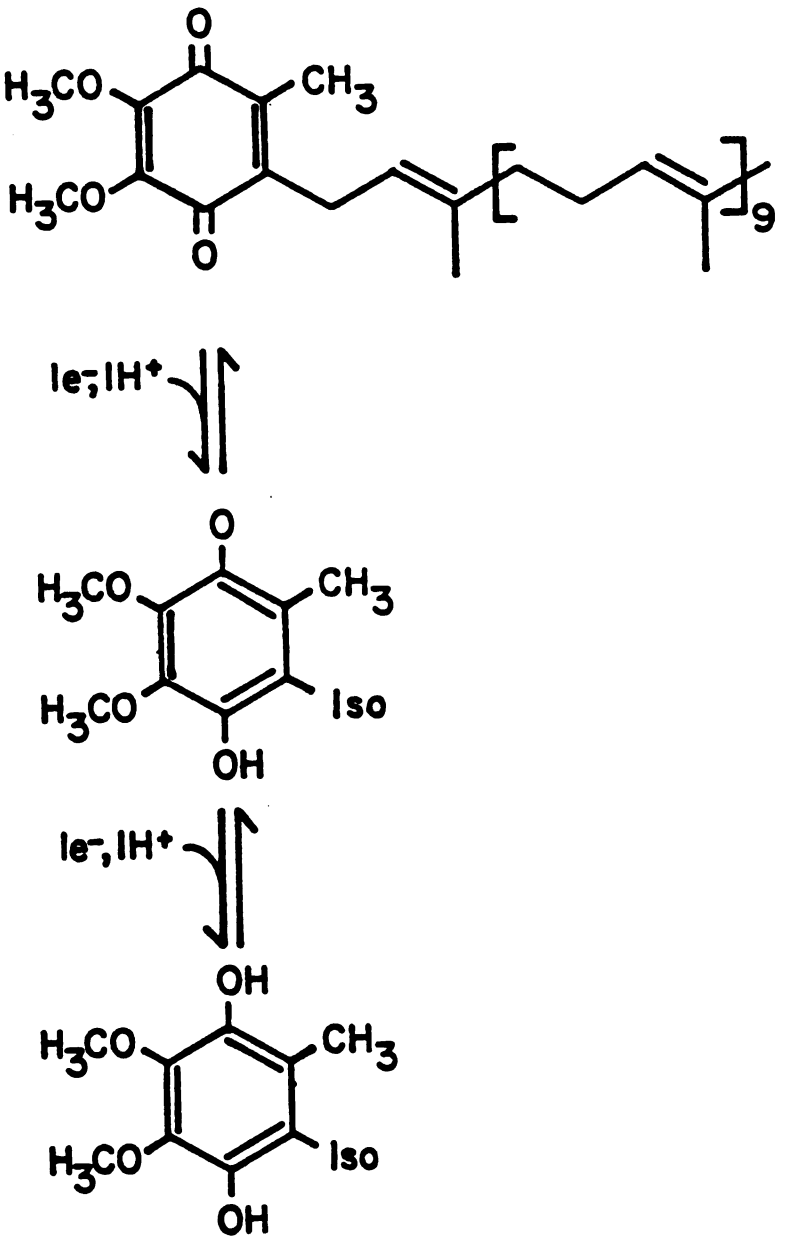

O
H3CO
CH3
H3CO
O
9
1e-;1H+
O
H3CO
CH3
H3CO
Iso
OH
1e-,1H+
OH
H3CO
CH3
H3CO
Iso
OH

Figure 3

ubiquinone can directly participate in the generation of the proton motive force. Studies designed to determine the rate that ubiquinone flip-flops (Kingsley and Feigenson, 1981) and transports reducing equivalents (Hauska, 1977a,b, Futami et al., 1979) across the membranes of phospholipid vesicles indicate that the processes ocur at rates more than sufficient to account for such a mechanism.

The orientation of ubiquinone within the membrane is largely unresolved. The length of ubiquinone-10 is 56 Å (Trumpower, 1981) suggesting that the molecule would span the entire width of the lipid bilayer. However, NMR (Kingsley and Feigenson, 1981) and fluorescence quenching measurements (Chance, 1972) place the benzoquinone head group predominantly in a hydrophobic region of the membrane. In order to accommodate the whole molecule in the hydrophobic region of the membrane, Trumpower (1981b) has proposed that a bend may exist in the isoprene chain. Alternatively, it has been suggested that ubiquinone may predominantly reside in the center of the bilayer with the side chains lying parallel to the plane of the membrane (Quinn and Esfahani, 1980; Katsikas and Quinn, 1982). Taking into account the rapid flip-flop rate for ubiquinone ($\geq$ 23 sec^{-1}) (Kingsley and Feigenson, 1981) both perpendicular and parallel orientations of ubiquinone must exist to some extent. The position of ubiquinone also appears to be dependent on its redox state in as much as the time averaged location of the reduced quinol ring is closer to the membrane surface than that of the quinone form (Kingsley and Feigenson, 1981). Since these studies have been performed for the most part on protein-free liposomes it is not known to what extent the findings can be applied to the protein rich mitochondrial membrane.

The Dehydrogenases (Complex I and Complex II):

The initial electron acceptors in the respiratory chain are the flavin-linked dehydrogenases, succinate ubiquinone reductase (Complex II) and NADH

dehydrogenase (Complex I). These complexes oxidize substrates (succinate and NADH respectively) on the matrix side of the mitochondrial membrane and transfer electrons to ubiquinone. In the case of NADH dehydrogenase this is accompanied by the translocation of protons from the matrix to the inter-membrane space.

These proteins are structurally and functionally complex and at present relatively little is known about them. Succinate ubiquinone reductase consists of five polypeptides (two of which reside on the matrix surface of the membrane and constitute succinate dehydrogenase) and contains three iron sulfur centers, one cytochrome b, one FAD, and two bound ubiquinones (for review see Ohnishi, 1981). NADH dehydrogenase is considerably more complex and has been reported to be comprised of 26 non-identical subunits (Heron et al., 1979), 5-6 iron sulfur centers, one FAD (Ragan et al., 1981; Singer et al., 1981; Ohnishi, 1981) and two bound ubiquinone molecules (Suzuki, 1983).

Structure of the Inner Mitochondrial Membrane:

From the previous discussion it is apparent that the components of the respiratory chain are very intricate both with regard to their structure and the mechanisms by which they fulfill their biological roles. In agreement with the structural requirements predicted by the chemiosmotic hypothesis the components appear to span the lipid bilayer in a manner that allows asymmetric translocation of electrons and protons. Based on the total content of flavin, hemes and ubiquinone in the mitochondrial inner membrane (Vinogradov and King, 1979; Azzone et al., 1979) the stoichiometry of the electron transfer components is approximately 1.5:10:1:2:2 for complexes I plus II: Q: cytochrome bc_1: cytochrome c: cytochrome oxidase. In addition to these complexes, cytochrome b_5 in the outer membrane (Sottocasa et al., 1967) and sulfite oxidase in the intermembrane space (Cohen et al., 1972)

are present in much smaller amounts and are capable of feeding electrons to cytochrome c.

The inner mitochondrial membrane consists of approximately 25% lipid by weight (Fleischer et al., 1961, Collbeau et al., 1971; Vinogradov and King, 1979; Krebs et al., 1979). The lipid composition is almost exclusively phospholipid, predominantly in the form of phosphatidylcholine, phosphatidylethanolamine and cardiolipin (Fleischer et al., 1961, Vinogradov and King, 1979; Krebs et al., 1979). These phospholids appear to be asymmetrically oriented in the membrane (Nilsson and Dallner, 1976; Krebs et al., 1979) with 38% of the phosphatidylethanolamine, 74% of the phosphatidylcholine, and 25% of the cardiolipin on the cytoplasmic side of the membrane (Krebs et al., 1979). The inner membrane contains almost no cholesterol (Madden et al., 1980) and has a high unsaturated fatty acid content which may confer a high degree of fluidity to the membrane (Fleischer et al., 1961; Colbeau et al., 1971).

The remaining 75% of the membrane mass is protein. Although this high protein content led to a popular conception that the proteins may exist in a densely-packed protein lattice, displacement of the membrane protein complexes by inducing lipid patches at low temperatures (Hackenbrock et al., 1976; Hochli and Hackenbrock, 1976, 1977) or by electrophoretic procedures (Sowers and Hackenbrock, 1981) indicate that only one third to one half of the membrane surface area is occupied by protein, and several lines of evidence indicate that free independent diffusion of the integral electron transfer components can occur (Sowers and Hackenbrock, 1981, Hochli et al., 1981; Kawato et al., 1980). However, the question still remains as to what degree association between the electron transfer components is important in efficient electron transfer and energy coupling. In general, two limiting cases can

be proposed: stable associations of the components in a solid state electron transfer unit (Lehninger, 1959; Blair et al., 1963) or random collisional encounters between free diffusing components involving only transitory interactions for electron transfer to occur (Hackenbrock, 1981).

In the work presented in Chapter 2 of this thesis, I have addressed this problem using the technique of fluorescence redistribution after photobleaching. This approach has many advantages which make it attractive. In particular, considerable information can be obtained regarding the structure of the inner mitochondrial membrane. Moreover, by obtaining direct measurements of the diffusion rates for the various components it is possible to make a quantitative assessment of the feasibility of electron transfer occurring by a random diffusion mechanism. The work presented in Chapter 1 discusses the purification and characterization of a contaminant in ruthenium red which is a potent electron donor to cytochrome oxidase. This contaminant mimics cytochrome c in its interaction with cytochrome oxidase but structurally is considerably less complex. Since there are few effective electron donors directly to cytochrome oxidase, the compound may prove to be a useful tool for studying cytochrome oxidase and determining the energy conserving mechanism of this complex.

Chapter 1

Hexaammineruthenium, An Effective Electron Donor To Cytochrome Oxidase

Introduction

Ruthenium red ($[NH_3)_5$-Ru-O-Ru$(NH_3)_4$-O-Ru$(NH_3)_5]^{+6}$) (Fletcher et al. 1961; Carrondo et al., 1979) is a potent inhibitor of mitochondrial calcium transport (Moore 1981; Vasington et al. 1971) and has been reported to be a good mediator of electron transfer to cytochrome c (Schwerzmann et al. 1976). Although it was originally used as a stain for cell surface glycoproteins, ruthenium red has also been shown to bind to a wide range of anions, including RNA, DNA, phospholipids, and acidic peptides (Luft, 1971; Freidman et al. 1979).

The inhibitory effect of ruthenium red on mitochondrial calcium transport was first demonstrated by Moore (1971). Low levels of ruthenium red (10 nmol/mg mitochondrial protein) completely inhibit calcium uptake by mitochondria (Moore, 1971; Vasington et al., 1971). Higher concentrations of the dye inhibit the low affinity binding of calcium to phospholipids (Vasington et al., 1971; Freidman et al., 1971). The inhibition of ruthenium red has been

shown to be specific for calcium transport coupled to mitochondrial respiration, having no effect on calcium transport in microsomes (Bygrave 1978 a,b) or the plasma membrane (McDonald et al. 1976). Furthermore, calcium efflux from mitochondria is insensitive to ruthenium red (Crompton et al. 1976; Caroni et al. 1978; Haworth et al. 1980).

Schwerzmann et al. (1976) reported that low concentrations of ruthenium red (2 μM) stimulated electron transport in whole mitochondria and inner mitochondrial membranes. This activity was insensitive to antimycin and rotenone but inhibited by KCN, indicating that ruthenium red transfers electrons to the terminal portion of the respiratory chain. On the basis of this data, it was concluded that ruthenium red transfers electrons from ascorbate or NADH to cytochrome c, in a manner similar to, but more efficient than, the electron mediator more commonly used, TMPD.

Further studies, undertaken to characterize the potentially useful ability of ruthenium red to mediate electron transfer, showed that the activity of the dye was inversely related to its purity (Ferguson-Miller, personal communication). Resonance raman studies also revealed that highly purified ruthenium red neither binds to nor reduces oxidized cytochrome c (Freidman et al. 1979). This study reports the isolation and identification of a contaminant of ruthenium red that is responsible for the electron mediating activity previously ascribed to the dye. On the basis of physical, spectral, and activity characteristics, we have identified the contaminant to be $Ru(NH_3)_6^{2+/3+}$. Although the electron mediation was originally proposed to occur via cytochrome c (Schwerzmann et al. 1976), we have shown that $Ru(NH_3)_6^{2+}$ is a kinetically efficient electron donor directly to cytochrome oxidase.

Experimental

Material. Cholic acid, deoxycholic acid, rotenone, antimycin A, protamine sulfate, bovine serum albumin, and ruthenium red (40% dye content) were obtained from Sigma Chemical Co. (St. Louis, MO). Prior to use the cholic acid was recrystallized three times from ethanol. $Ru(NH_3)_6^{3+}$ was purchased through Alfa Products (Danvers, MA). TMPD was obtained from Eastman Chemical Co. (Rochester, NY). Lauryl(dodecyl)maltoside was prepared according to Rosevear et al. (1980). All other chemicals used were of the highest grade available.

Preparation of mitochondrial membranes. Beef heart inner mitochondrial membrane particles (Keilin Hartree preparation) were obtained by the method of King (1967) as further detailed by Ferguson-Miller et al. (1976). The isolated particles were stored at -20°C in 0.15 M phosphate-borate buffer, pH 7.8, containing 50% glycerol.

Rat liver mitochondria were isolated from Sprague Dawley rats by the method of Johnson and Lardy (1967). Inner mitochondrial membranes were prepared as intact mitoplasts using the procedure of Sottocasa et al. (1967) as modified by Felgner et al. (1979), and were washed with 0.15 M KCl in 0.45 M sucrose to remove cytochrome c. Whole mitochondria and the inner membranes were resuspended at 30 mg protein/ml in 0.07 M sucrose, 0.25 M mannitol, 10 mM Tris (Cl), pH 7.4 with 20% dimethylsulfoxide for storing at -65°C. Outer mitochondrial membranes were prepared according to Felgner et al.(1979).

Cytochrome c and cytochrome oxidase purification. Horse heart cytochrome c grade VI purchased from Sigma Chemical Co. was purified using the method of Brautigan et al. (1977) and was stored at -20°C. Prior to use, cytochrome c was reduced with dithionite and gel-filtered on Sephadex G-75

superfine (Pharmacia) in 25 mM Tris-cacodylate buffer pH 7.8 in order to remove polymerized cytochrome c.

Cytochrome oxidase was purified with cholate from Keilin-Hartee particles by a modification of the method of Kuboyama et al. (1972). The purified enzyme gave a turnover number of 200-400 sec^{-1} in 50 mM potassium phosphate pH 6.5, 1 mM laurylmaltoside, and had 8 to 10 nmole heme a/mg Lowry protein.

Chromatography of ruthenium red. The cation exchange resins CM cellex (Bio Rad) and CM cellulose-52 (Whatman) were prepared according to the manufacturer's suggested procedures.

To purify the active component in ruthenium red, 1 gm of the commercially prepared dye was applied to a 5 x 75 cm (1472 ml) column of CM-cellex equilibrated with 0.1 M potassium phosphate, pH 7.8. The column was eluted with 4 liters of the same buffer followed by 2 liters of 0.2 M phosphate buffer and 15 ml fractions were collected. The fractions containing the electron transfer activity (as indicated by their ability to stimulate mitochondrial oxygen consumption) were pooled, diluted four times with double distilled water, and applied to a 2.5 x 35 cm (400 ml) CM-cellulose column equilibrated with 25 mM potassium phosphate, pH 7.8. The column was washed with 250 mls of 25 mM phosphate buffer and eluted with 0.1 M potassium phosphate, pH 7.8. After this second chromatography, the fractions containing activity were pooled, diluted as above and concentrated on CM-cellulose in 25 mM phosphate buffer and eluted with 0.5 M potassium phosphate, pH 7.8. The concentrated sample was gel-filtered on a Biogel P-2 column (0.7 x 50) in 50 mM ammonium bicarbonate, pH 8.2, and lyophilized.

Ruthenium red was also chromatographed in ammonium bicarbonate buffer on CM-cellulose. The detailed conditions are described in the legend to Figure 6.

Kinetics of electron mediation by $Ru(NH_3)_6^{2+/3+}$. All the kinetic data were obtained polarographically with a Gilson model K-IC oxygraph equipped with a Yellow Springs instrument electrode using a high sensitivity membrane. The assays were performed in 50 mM Hepes, pH 7.4, with 2.8 mM ascorbate and 1 mM laurylmaltoside. The rate of autooxidation of $Ru(NH_3)_6^{2+}$ was measured in the presence of cytochrome $\underline{aa_3}$ following addition of protamine sulfate to a final concentration 10 μM. This rate was subtracted from the apparent enzymic rate prior to the protamine sulfate addition. Turnover numbers were calculated from the velocities measured in nmol O_2 per second, by multiplying by 4 (to give the nmol of $Ru(NH_3)_6^{2+}$ required to reduce 1 nmole O_2) and dividing by the total nmol cytochrome $\underline{aa_3}$ present in the reaction vessel.

Identification of RX. The infrared spectra on the active contaminant and $Ru(NH_3)_6^{3+}$ were performed on a Perkin-Elmer 167 infrared spectrophotometer. Both samples were chromatographed on CM-cellulose in ammonium bicarbonate, pH 7.0, and lyophilized prior to equilibration in D_2O. Absorption spectra were obtained using an Aminco DW2a dual beam-dual wavelength spectrophotometer or a Beckman DU-8 UV-Vis computing spectrophotometer. Neutron activation analysis of RX was performed at the MSU Nuclear Reactor Facility.

Results

Chromatography of Ruthenium Red. To determine whether the electron mediating activity of ruthenium red was associated with a contaminant, commercial ruthenium red was chromatographed on carboxymethylcellulose ion-exchange resin (CM-Cellex) in potassium phosphate buffer, pH 7.8. The dye fractionated into a number of colored bands and the electron mediating

activity eluted well ahead of the ruthenium red (Figure 4). Ruthenium red itself showed no ability to stimulate respiration in mitochondria, and when added back to the active fractions caused some inhibition. The electron transfer activity co-eluted with a pink component with an absorption maximum at 484 nm (Peak A, Figure 4). Peak B, immediately following, had an absorption maximum of 481 nm. Ruthenium red (absorption maximum 475 nm in the oxidized form) eluted in two peaks corresponding to the reduced form (peak C) and the oxidized form (peak D). When the buffer concentration was raised from 0.1 to 0.2 M, a small amount of remaining ruthenium red was displaced from the resin. Ruthenium violet (Luft, 1971) remained bound to the column even at 2 M potassium phosphate.

Rechromatography of the pooled fractions containing the electron mediating activity, under the same conditions as the first column, resulted in separation of the activity from the remaining colored components. The ability to enhance electron transfer was found in a colorless fraction with absorption in the ultraviolet region.

To ascertain whether the electron mediating activity was in fact a property of the 275 nm-absorbing compound, the material was applied to a column of Biogel P-2 (fractionation range, 100-1800 daltons) equilibrated in 0.05 M NH_4HCO_3, pH 8.2. As shown in Figure 5, the 275 nm absorbance and the activity co-eluted with an approximate molecular weight of 400 to 500 dalton (standardized against cytochrome c and NaCl). This result provided strong evidence that the component absorbing at 275 nm, which will be referred to as RX, was the electron mediating contaminant of ruthenium red.

Since aqueous solutions of ruthenium red are unstable (Luft, 1971) it was of interest to establish purification conditions that would maximize its stability and still achieve the separation of RX and other contaminating species.

Figure 4. Chromatography of commercial ruthenium red on carboxymethylcellulose in potassium phosphate buffer. Ruthenium red (1 gm) in 0.1 M potassium phosphate, pH 7.9, was applied to a 75 x 5 cm column of CM-cellex (BioRad) and chromatographed in the same buffer. Fractions of 15 ml were collected and absrobance was scanned from 350 to 650 nm. The oxidized components, ruthenium red and the 484 nm absorbing species, both absorbed strongly at 475 nm and had minimal absorption at 425 nm. Electron mediating activity (●) was determined by measuring the increase in oxygen consumption produced by the addition of a 50 µl aliquot of a fraction to cytochrome c-depleted mitochondrial particles (0.5 mg per ml) in 25 mM acetate (Tris), pH 7.9, 250 mM sucrose and 2.8 mM ascrobate. The characteristics of peaks A,B,C, and D are discussed in the text.

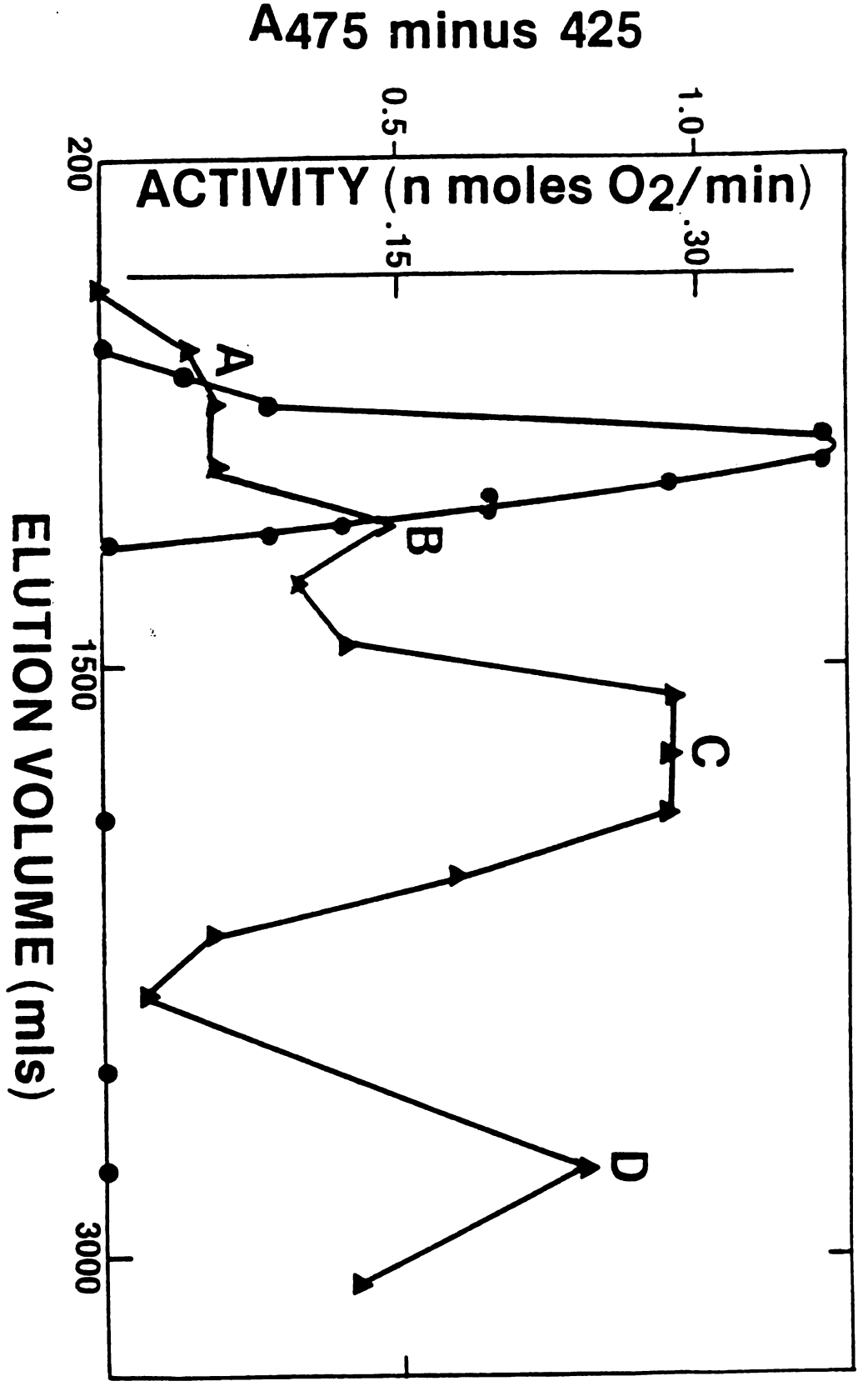

A475 minus 425
0.5
1.0
ACTIVITY (n moles O2/min)
.15
.30
200
1500
3000
ELUTION VOLUME (mls)
A
B
C
D

Figure 4

Figure 5. Gel-filtration on Biogel P-2 of the electron-mediating fraction separated from ruthenium red. An aliquol) of the activity-containing fractio ascroban obtained from the second CM-cellulose chromatography (A_{max} = 275 nm) was applied to a 50 x 0.7 cm column of Biogel P-2 equilibrated in 50 mM ammonium bicarbonate, pH 8.2, and 0.2 ml fractions were collected. Absrobance (●) was monitored at 275 nm and activity (▲) was measured as described in Figure 4.

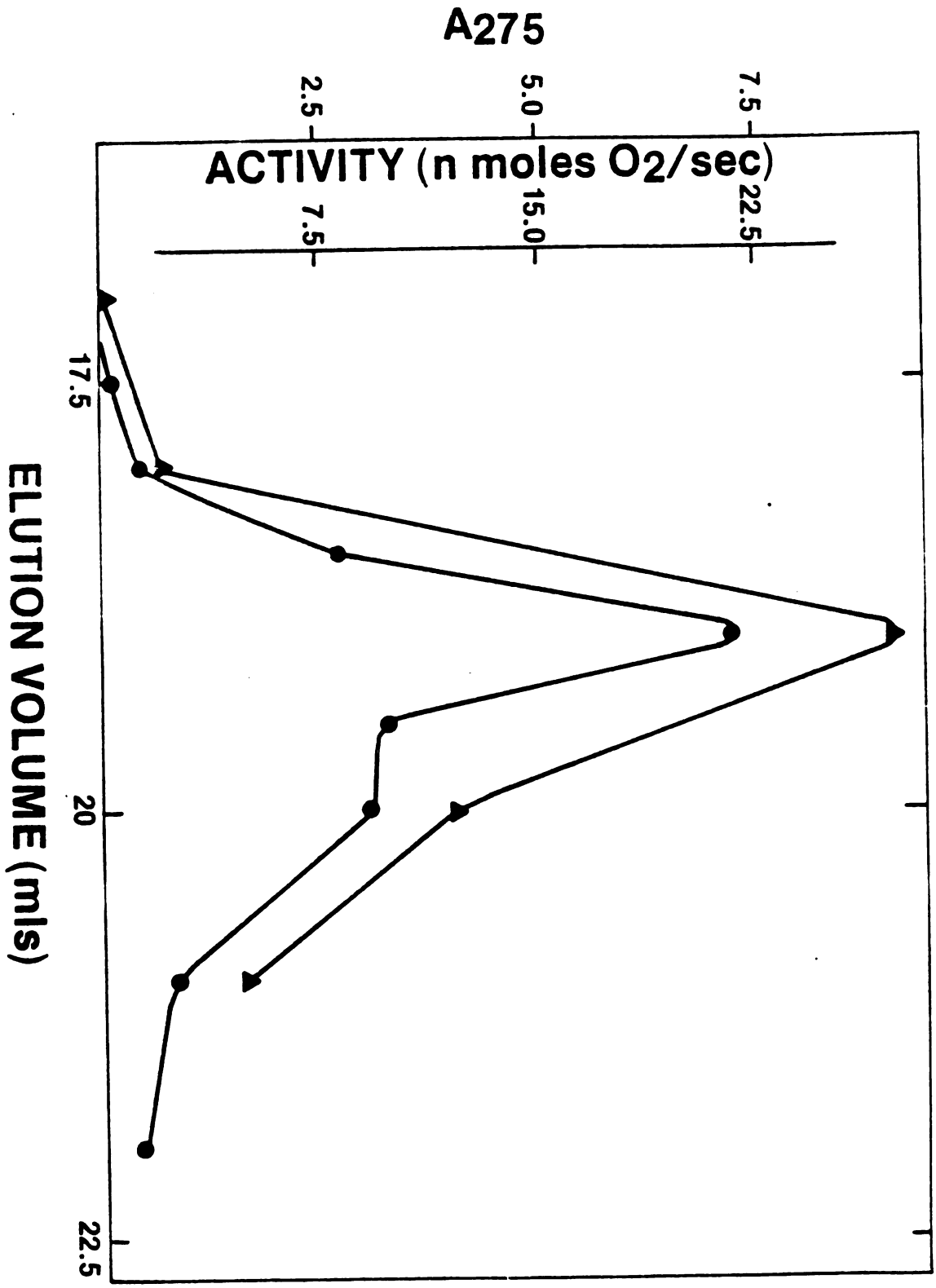

A275
2.5
5.0
7.5
ACTIVITY (n moles O2/sec)
7.5
15.0
22.5
17.5
20
22.5
ELUTION VOLUME (mls)

Figure 5

Ammonium containing buffers at near netural pH have been suggested by Luft (1971) to prevent breakdown of ruthenium red, and use of the volatile buffer ammonium bicarbonate would permit lyophilization of the purified product. Thereafter, ruthenium red was applied to a CM-cellulose column in 0.05 M NH_4HCO_3, pH 7.0, and the column was washed with 0.1 M NH_4HCO_3, resulting in the elution of small amounts of RX and chloropentaammine ruthenium III. Chromatography was continued at 0.5 M NH_4HCO_3, and the elution profile observed at this ionic strength is shown in Figure 6. Ruthenium red eluted in broad band well behind RX and the 484 nm-absorbing component. Though this single column fractionation is not adequate for separating RX from several other contaminants, it provides an effective method for purifying ruthenium red.

Identification of the Electron Mediating Component, RX. Following gel-filtration on Biogel P-2 in NH_4HCO_3 buffer, RX was lyophilized and 130 mg of material were recovered from 1 g commercial ruthenium red. As a first step in identification, the sample was analyzed by neutron activation. Using this technique, it was determined that ruthenium was the major metal species in RX. Smaller amounts of sodium, chlorine, potassium, and a trace of platinum were also found.

To detect the presence of carbon, ^{13}C-NMR was run on a concentrated sample RX (50 mg/ml). No carbon signals were observed.

The results of neutron activation, ^{13}C-NMR, and the small molecular weight estimate obtained by gel filtration, suggested that RX might be a simple ruthenium amine. Figure 7 shows a comparison of the ultraviolet spectra of RX, chloropentaammineruthenium III and hexaammineruthenium III. The spectrum of RX is seen to be essentially identical to that of hexaammineruthenium III, with an absorption maximum of 275 nm, while the

Figure 6. Chromatography of commercial ruthenium red on carboxymethylcellulose in ammonium bicarbonate, pH 7.0. Ruthenium red, 51 mg in 0.05 M NH_4HCO_3 was applied to a column (27.5 x 0.7 cm) of CM-cellulose equilibrated in the same buffer. The column was washed with 320 ml of 0.1 M NH_4HCO_3 and eluted with 0.5 M NH_4HCO_3. Fractions of 3 ml were collected and monitored at 275 nm (▲) 488 nm (■) and 542 (●). Activity (Δ) was measured as described in Figure 4.

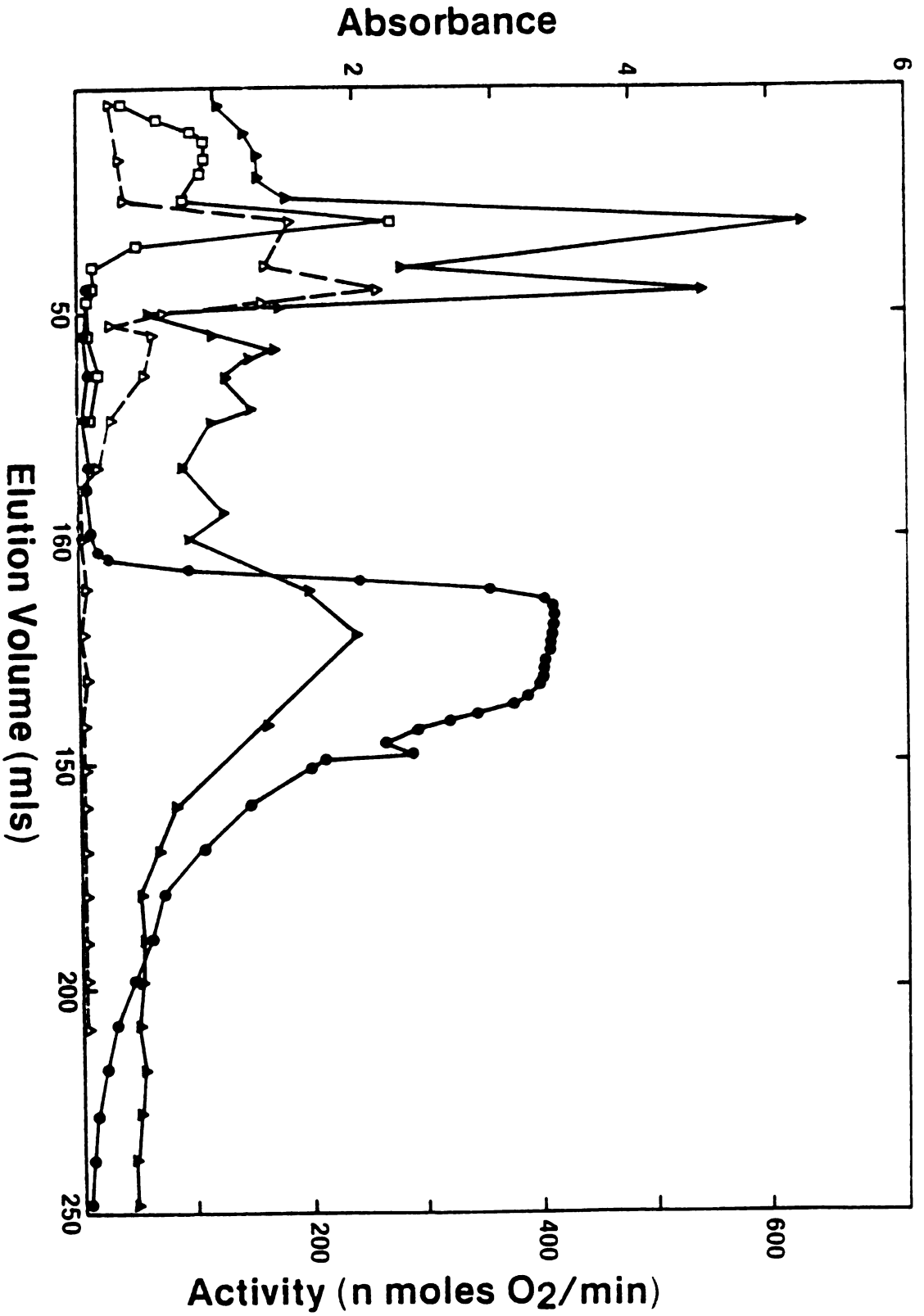

Absorbance
2
4
6
Elution Volume (mls)
50
100
150
200
250
Activity (n moles O2/min)
200
400
600

Figure 6

chloropentaammine has an absorption maximum at 323 nm. Another possible candidate, nitrosylruthenium III chloride, has an absorption maximum at 250 nm (Luft, 1971).

The identity of RX and hexaammineruthenium II/III was further confirmed by equilibrating samples of both compounds in phosphate buffer, and then applying them together to a Biogel P-2 column. They eluted as a single sharp band that contained the electron transfer activity. When applied separately, the two compounds also eluted at the same position.

In Figure 8, the infrared spectra of RX and hexaammineruthenium (III) chloride in deuterated water are compared. Although neither compound is completely pure, they have distinctive bands at 1450, 1085, 985 and 940 cm^{-1} in common.

From the studies discussed above we conclude that $Ru(NH_3)_6^{2+/3+}$ is the electron mediating contaminant in ruthenium red. Further investigation of the activity of $Ru(NH_3)_6^{2+/3+}$ as an electron donor/acceptor in the mitochondrial respiratory system corroborated this identification.

Electron Transfer Activity of $Ru(NH_3)_6^{2+/3+}$. As originally observed by Schwerzmann et al. (1976) ruthenium red mediates electron transfer from NADH or ascorbate to oxygen in the presence of mitochondria, by a pathway that is sensitive to cyanide and insensitive to antimycin and rotenone. These authors concluded that ruthenium red was donating electrons to cytochrome c and thence to cytochrome oxidase. However, the strong positive charge on ruthenium red (+6) made it an unlikely candidate as a kinetically efficient mediator to the positively-charged cytochrome c molecule. This same reasoning applies to $Ru(NH_3)_6^{2+}$, the active species in ruthenium red. Therefore, rat liver inner mitochondrial membranes were depleted of cytochrome c, and unpurified ruthenium red and $Ru(NH_3)_6^{2+}$ were examined with this preparation

Figure 7. Comparison of the ultraviolet absorption spectra of RX (A), $Ru(NH_3)_6^{3+}$ (B) and chloropentaammineruthenium III (C). The spectra were run in 0.5 M ammonium bicarbonate pH 7.0.

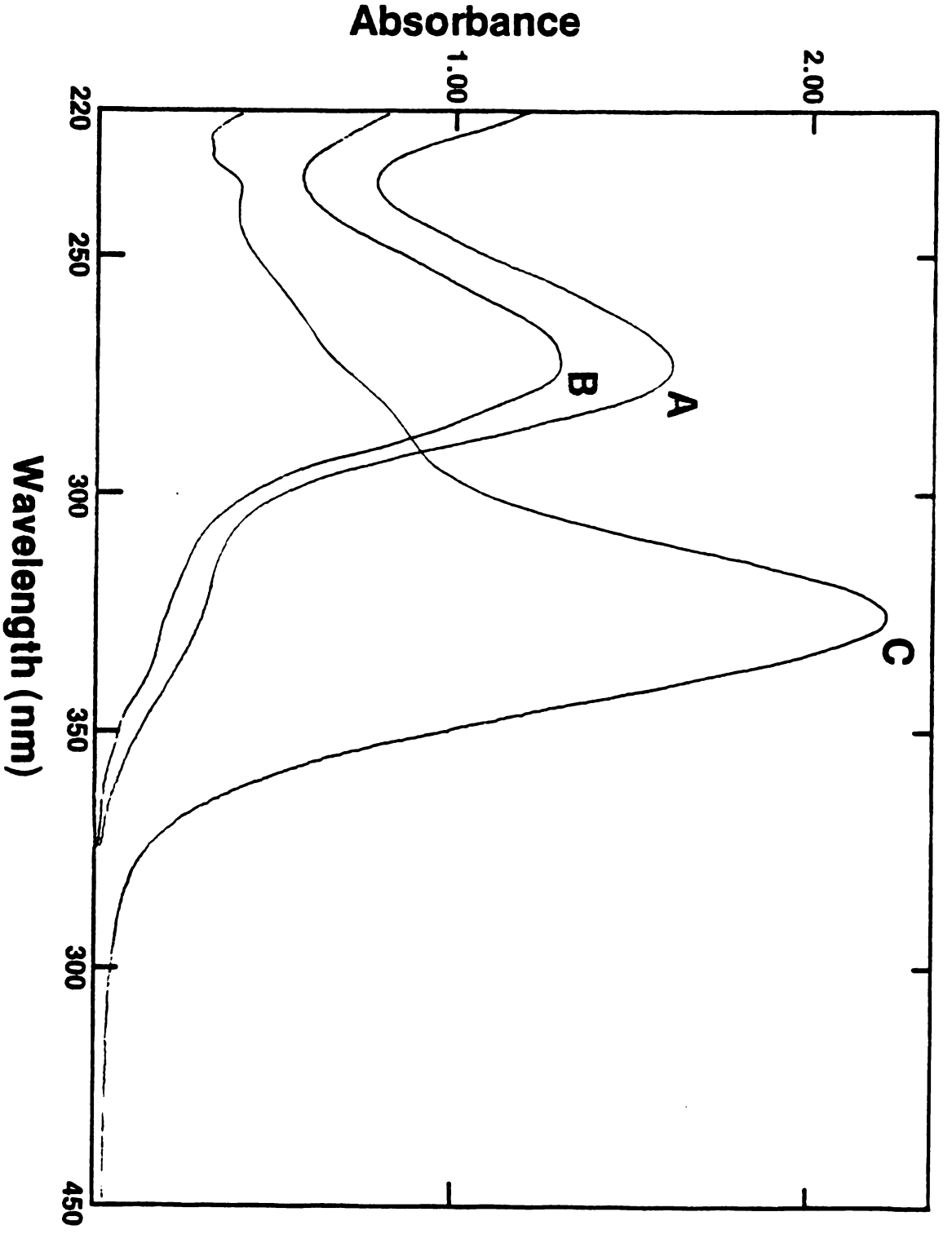


Figure 7

Figure 8. Comparison of the infrared spectra of RX (A) and $Ru(NH_3)_6^{3+}$ (B) in D_2O. The bands at 1450, 1085, 985 and 940 cm^{-1} are characteristic of RX and $Ru(NH_3)_6^{3+}$. Those at 3850, 3450, 2500 and 1200 cm^{-1} are due to D_2O.

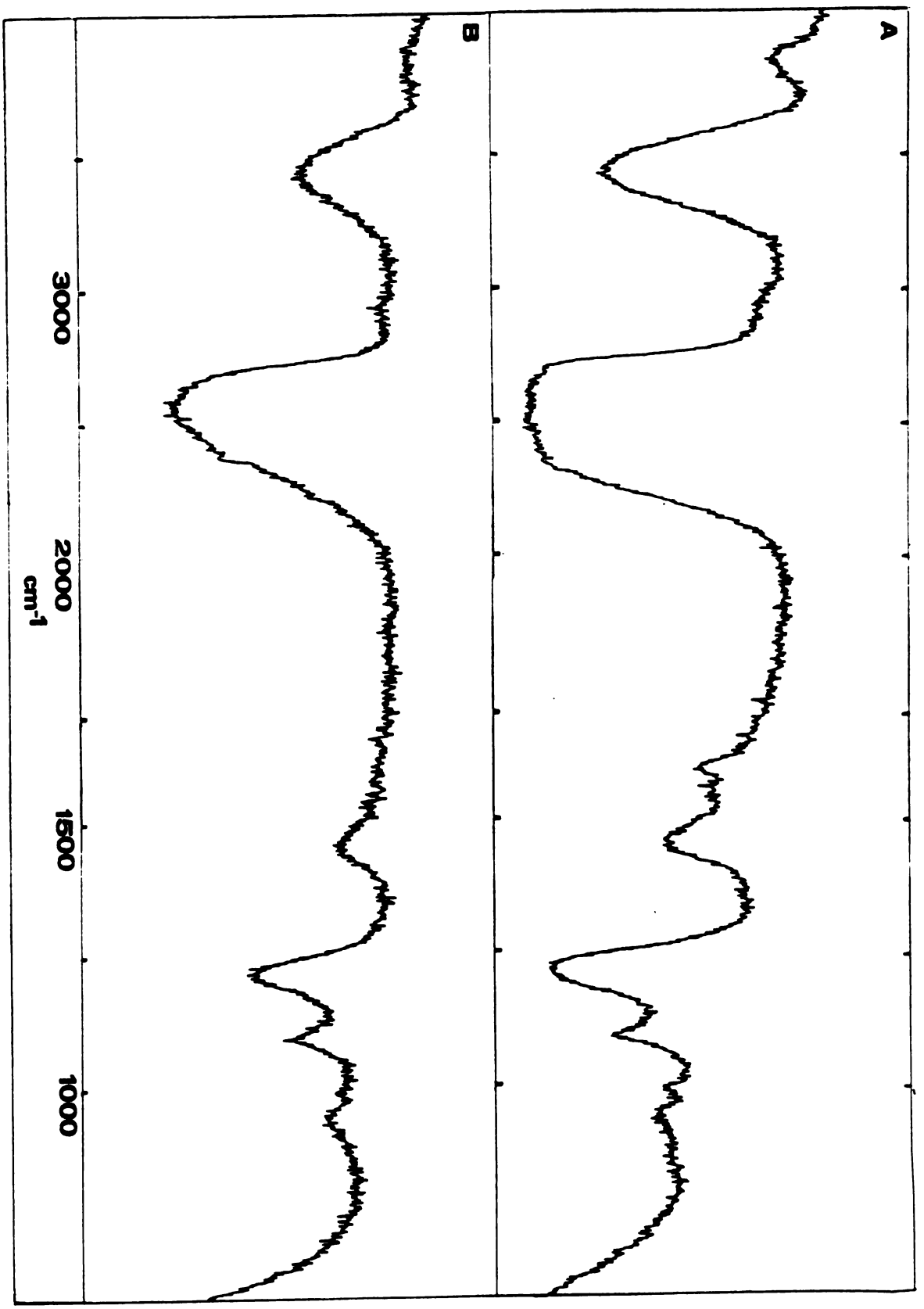


Figure 8

for their ability to stimulate electron transfer from ascorbate. Figure 9A shows that oxygen consumption in the absence of cytochrome c is strongly accelerated by both compounds and is inhibited by protamine sulfate (1 μM), KCN (2mM) or azide (5 mM), all of which inhibit cytochrome oxidase. The results indicate that $Ru(NH_3)_6^{2+}$ is a good donor of electrons directly to cytochrome oxidase. This conclusion is confirmed using purified cytochrome oxidase as shown in Figure 9B. A low rate of autooxidation is observed with ascorbate and the ruthenium compounds alone, while addition of cytochrome oxidase results in a high rate of oxygen consumption that is not further enhanced by cytochrome c.

Electron transfer from succinate to oxygen in the presence of $Ru(NH_3)_6^{3+}$ and cytochrome c-depleted mitochondria was not observed, indicating that none of the respiratory chain components from succinate to cytochrome c_1 are kinetically competent electron donors to $Ru(NH_3)_6^{3+}$.

With whole mitochondria, NADH at μM concentrations serves as an efficient electron donor to the active component in ruthenium red (Schwerzmann et al., 1976). But when the outer membrane is removed, a much higher concentration of NADH (3 mM) is required to support respiratory rates only half those observed with an equal concentration of ascorbate. In Figure 9C, it is seen that purified outer mitochondrial membranes (OMM) added back to an inner membrane preparation, greatly enhance the rate of oxygen consumption in the presence of $Ru(NH_3)_6^{3+}$ and NADH. The rapid rate is sustained until all the NADH is consumed. Outer membranes alone do not give a measurable rate of oxygen consumption with $Ru(NH_3)_6^{3+}$ and NADH. The sharp cut-off respiratory rate when NADH is consumed indicates that a component in the electron transfer sequence has a very high affinity for NADH, and furthermore, it is apparent that this component is associated with

Figure 9. Electron transfer activity of $Ru(NH_3)_6^{2+/3+}$ and ruthenium red. A) Enhancement of electron transfer with cytochrome c depleted rat liver inner mitochondrial membranes. Oxygen consumption was measured polarographically in 0.22 M mannitol, 0.07 M sucrose, 10 mM Tris(Cl), 5 mM phosphate, 2 mM EDTA, 1 mg/ml BSA at pH 7.5, with 2.8 mM ascorbate. Other additions were made as indicated to the final concentrations of: ruthenium red (RR), 70 µg/ml; $Ru(NH_3)_6^{2+}$, 3.5 µg/ml; protamine sulfate (PS), 10 µM. B) Enhancement of electron transfer with purified cytochrome oxidase. Oxygen consumption was measured in 50 mM Hepes, pH 7.4, 1 mM laurylmaltoside. The final concentrations were: cytochrome aa_3, 0.2 µM, cytochrome c, 4.5 µM, KCN, 2 mM. C) Stimulation of $Ru(NH_3)_6^{2+}$ mediated electron transfer from NADH by outer mitochondrial membranes. Conditions were the same as described in 9A. In both traces, cytochrome c-depleted inner mitochondrial membrane particles were present at 0.7 mg protein per ml. Other additions were made as indicated to the final concentrations of: $Ru(NH_3)_6^{2+}$, 6 µg/ml; NADH (first addition), 3.5 mM; outer mitochondrial membranes (OMM), 0.34 mg protein/ml; NADH (second addition) 1.75 mM.

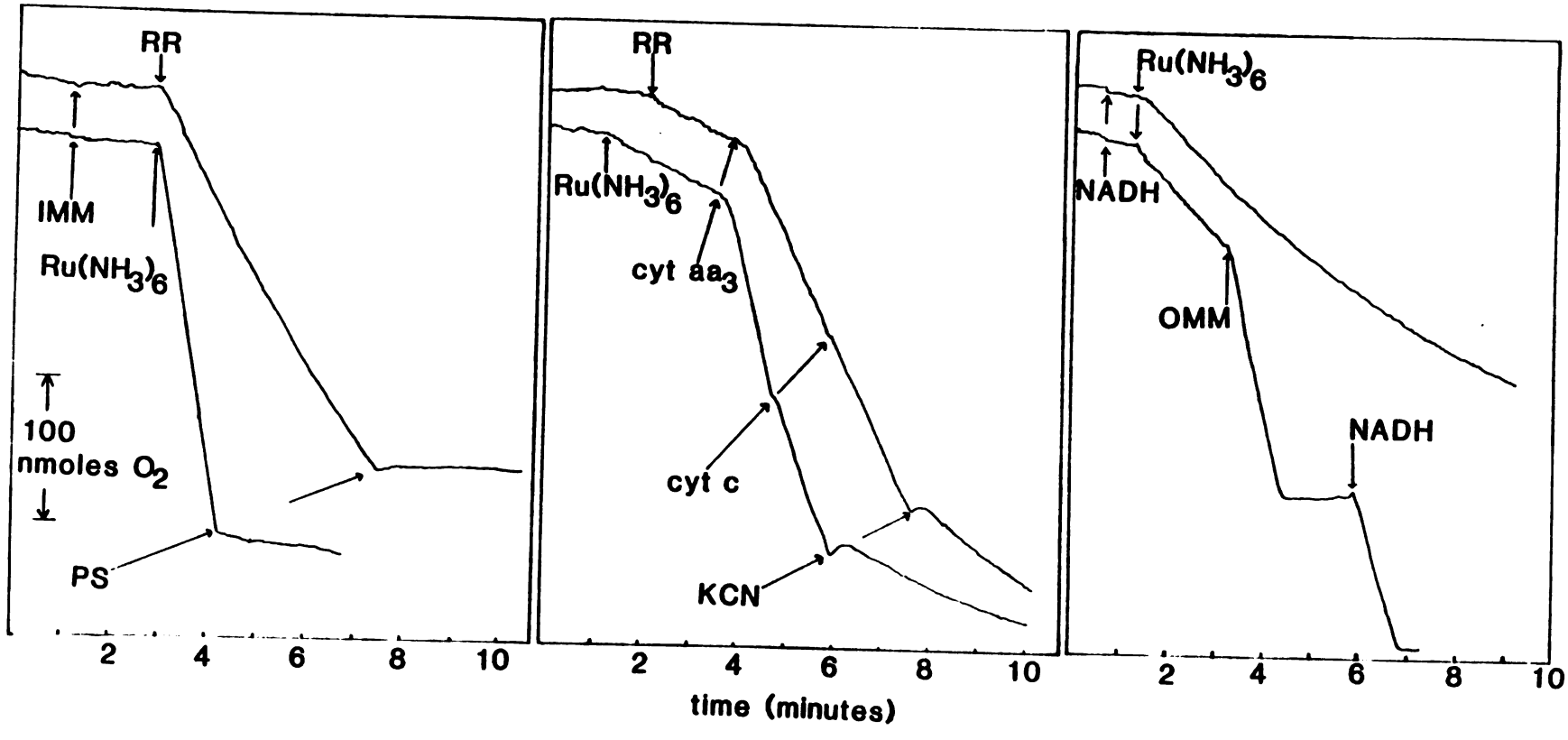
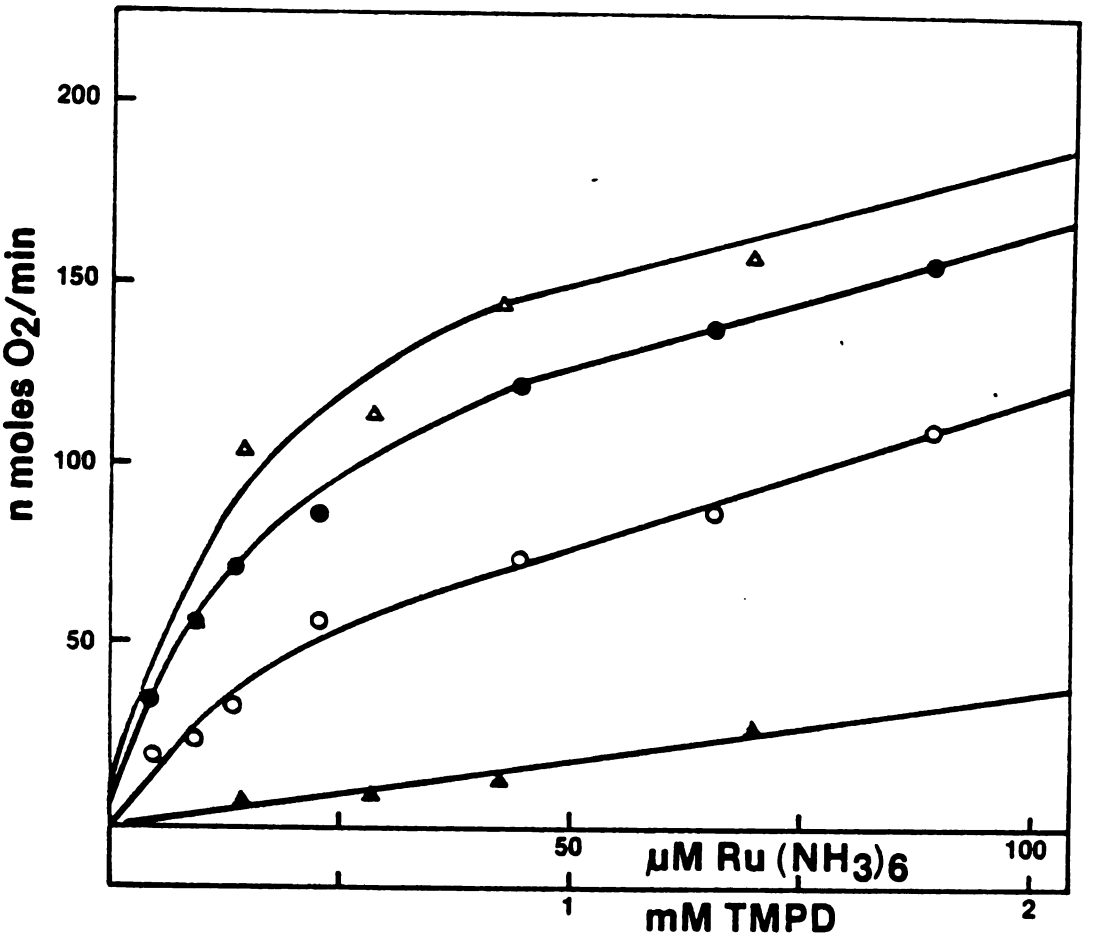

RR
IMM
Ru(NH3)6
100
nmoles O2
PS
2
4
6
8
10
RR
Ru(NH3)6
cyt aa3
cyt c
KCN
2
4
6
8
10
Ru(NH3)6
NADH
OMM
NADH
2
4
6
8
10
time (minutes)

Figure 9

the outer membrane. Similar results are also observed when microsomes are added to inner mitochondrial membranes in the presence of NADH. The most likely explanation of these data is that the NADH-cytochrome b_5 reductase complex in the outer mitochondrial membrane or in microsomes catalyzes the transfer of electrons from NADH to $Ru(NH_3)_6^{3+}$, which subsequently reduces cytochrome oxidase.

Our original interest in the apparent electron mediating capacity of ruthenium red stemmed from its reported superior ability, compared to TMPD, to transfer electrons to cytochrome c. The results of the studies described so far clearly demonstrate that the actual electron mediator, $Ru(NH_3)_6^{2+/3+}$, behaves in a manner quite distinct from TMPD, being a much superior electron donor directly to cytochrome oxidase. Figure 10A shows a comparison of the activities of $Ru(NH_3)_6^{3+}$ and TMPD as electron donors to purified cytochrome oxidase in the presence and absence of cytochrome c. Concentrations of $Ru(NH_3)_6^{3+}$ two orders of magnitude lower than those used of TMPD gave maximal rates of oxygen consumption with cytochrome oxidase alone. Addition of cytochrome c caused inhibition of the $Ru(NH_3)_6^{3+}$-induced oxygen uptake, while the TMPD-induced rates were stimulated by as much as 100-fold under the same conditions. In Figure 10B, the inhibition by cytochrome c is shown to be competitive with respect to $Ru(NH_3)_6^{2+}$. This behavior indicates that $Ru(NH_3)_6^{2+}$ is reacting with oxidase in a manner similar to cytochrome c, possibly at the same negatively-charged site (or sites). As shown in Figure 11, the steady-state kinetics of reaction of $Ru(NH_3)^{2+}$ with cytochrome oxidase in the presence of excess ascorbate are qualitatively similar to those of cytochrome c (Ferguson-Miller et al., 1976). A biphasic pattern is observed in an Eadie-Hofstee plot of the data, from which an approximate K_{m_1} = 8.6 μM and K_{m_2} = 88 μM can be estimated from tangents to the initial and final

Figure 10. Kinetics of the electron transfer reaction of $Ru(NH_3)_6^{2+}$ with purified cytochrome oxidase. A) Comparison of $Ru(NH_3)_6^{2+}$ and TMPD mediated electron transfer. Rates of oxygen consumption by purified cytochrome oxidase (0.1 μM) were measured in 50 mM Hepes, pH 7.4, 1 mM laurylmaltoside, 2.8 mM ascorbate, and various concentrations of: TMPD (▲); TMPD + 1 μM cytochrome c (△); $Ru(NH_3)_6^{2+}$ (●); $Ru(NH_3)_6^{2+}$ + 1 μM cytochrome c (○). B) Lineweaver-Burk plot of oxidase activity from data shown in Figure 7A, comparing the kinetics in the presence of $Ru(NH_3)_6^{2+}$ (●), or $Ru(NH_3)_6^{2+}$ plus 1 μM cytochrome c (○).


200
150
100
50
n moles O2/min
50
μM Ru (NH3)6
100
1
mM TMPD
2

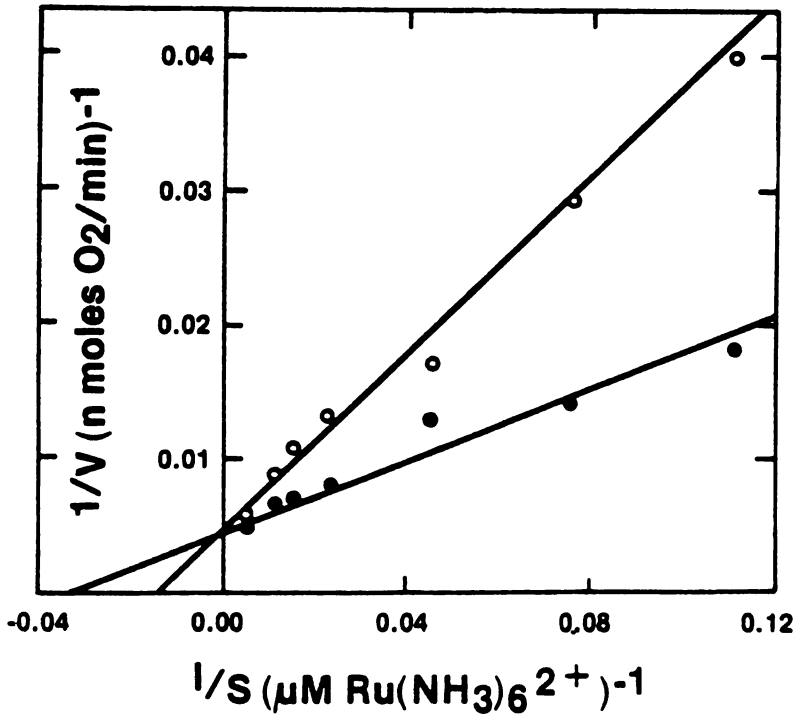

0.04
0.03
0.02
0.01
1/V (n moles O2/min)-1
-0.04
0.00
0.04
0.08
0.12
1/S (μM Ru(NH3)6 2+)-1

Figure 10

Figure 11. Eadie-Hofstee-Scatchard plot of steady state kinetics of the reaction of $Ru(NH_3)_6^{2+}$ with purified cytochrome oxidase. Conditions were the same as described for 10A, except cytochrome $\underline{aa}_3$ was 0.23 μM and the $Ru(NH_3)_6$ concentrations ranged from 0.9 to 223 μM.

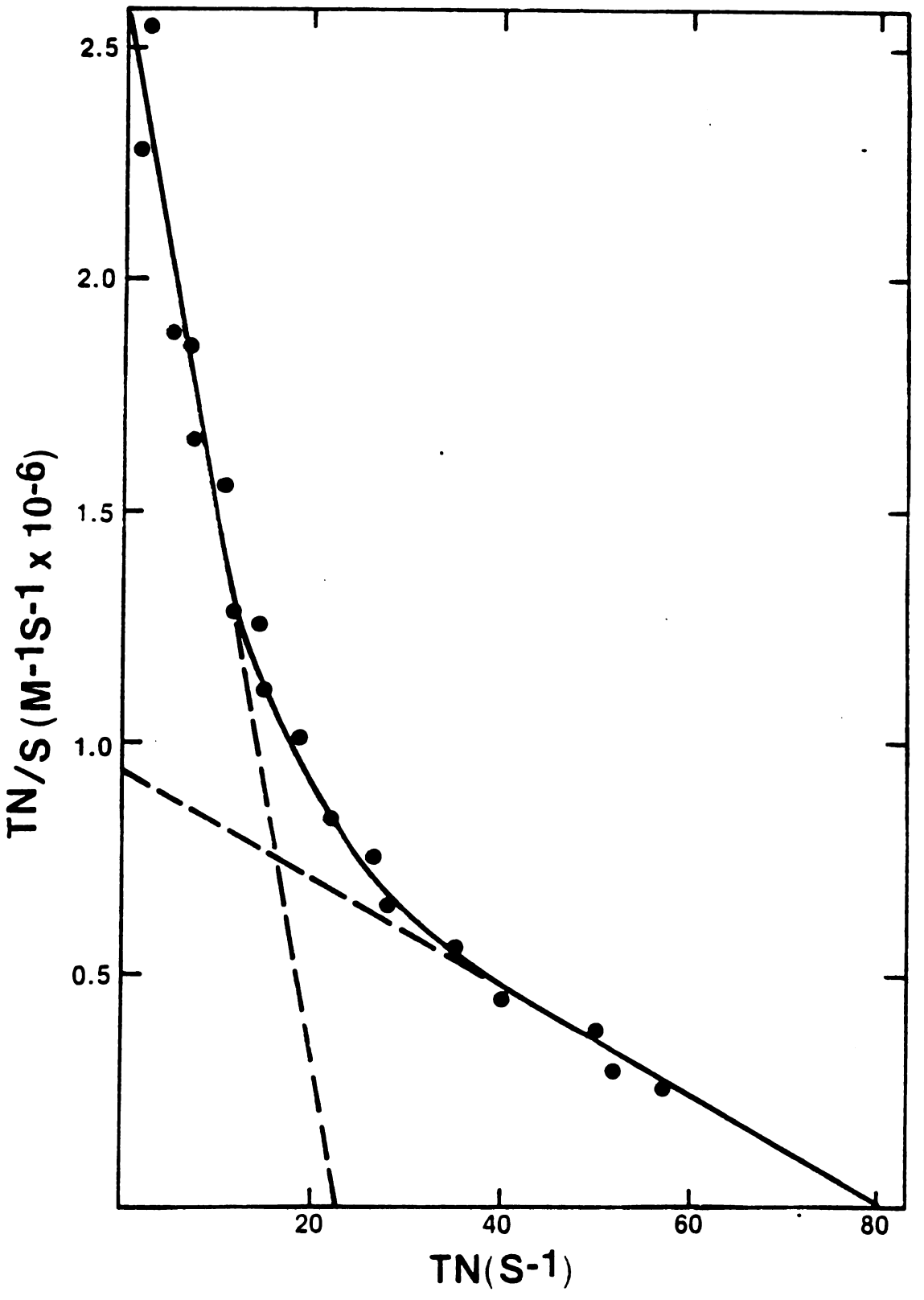


Figure 11

phases of the data.

Discussion

The finding that ruthenium red was an effective mediator of electron transfer in mitochondria (Schwerzmann et al. 1976) as well as a potent inhibitor of calcium transport, warranted further investigation since ambiguities could result from expression of the two activities simultaneously. Furthermore, there are few efficient artificial electron donors available for studying the terminal segment of the mitochondrial respiratory chain, making it a potentially valuable tool.

When commercial ruthenium red was subjected to cation-exchange chromatography it yielded a number of colored components and a colorless fraction absorbing in the ultraviolet region (A_{max} = 275 nm) that contained all the electron mediating activity. Neither the purified ruthenium red nor any of the other colored species were able to stimulate mitochondrial respiratory activity, nor did they enhance the activity of the UV-absorbing component. When the electron mediating fraction was gel-filtered on Biogel P-2, the activity was proportional to, and co-eluted with, the absorbance at 275 nm, at an apparent molecular weight of 400 to 500 daltons. Neutron activation analysis of this substance showed that ruthenium was the predominant metal component, and no carbon was detected by ^{13}C-NMR.

It has previously been suggested by Luft (1971) that a common contaminant of ruthenium red was hexaammineruthenium II/III, since it is an intermediate in the synthesis of ruthenium red from $RuCl_3$ and ammonium hydroxide, and a likely product in its breakdown. Furthermore, $Ru(NH_3)_6^{2+}$ is a known reducing agent for cytochrome c (Ewall and Bennett, 1974; Adegite and Okpanachi, 1979) capable of rapid outer-sphere electron transfer reactions involving little

change in the ruthenium-nitrogen bond distance upon oxidation or reduction (Stynes and Ibers, 1971; Sutin, 1979). A recent paper (Scott and Gray, 1980) has also shown that $Ru(NH_3)_6^{2+}$ reduces cytochrome oxidase.

A comparison of the ultraviolet spectra of hexaammineruthenium (III), chloropentaammineruthenium (III), nitrosylruthenium (III), and the active fraction isolated from ruthenium red, showed that the latter compound has absorption spectrum identical to that of hexaammineruthenium (III). The identity of these two compounds was further confirmed by comparing their infrared spectra, their elution profile by gel filtration, and their activity characteristics with mitochondria, inner mitochondrial membranes and purified oxidase. On the basis of the results of these physical, spectral, and activity studies, we conclude that the ability of impure ruthenium red to stimulate electron transfer from ascorbate or NADH to oxygen in mitochondria, is due to the presence of the contaminant, hexaammineruthenium II/III, and is not a function of ruthenium red itself.

The pathway of electron transfer from NADH and ascorbate catalyzed by $Ru(NH_3)_6^{2+}$, was investigated with whole mitochondria and isolated components. It is evident from the ability of $Ru(NH_3)_6^{2+}$ to stimulate cyanide-sensitive oxygen consumption with cytochrome c-depleted mitochondria and purified cytochrome oxidase, that $Ru(NH_3)_6^{2+}$ transfers electrons directly to cytochrome aa_3, without the intervention of cytochrome c. Although $Ru(NH_3)_6^{2+}$ reduced cytochrome c rapidly at low pH and high ionic strength (1M NaCl) (Ewall and Bennett, 1974), the stopped-flow kinetic data of Scott and Gray (1980) demonstrate that $Ru(NH_3)_6^{2+}$ reduces cytochrome oxidase at rates at least three orders of magnitude greater than those reported for cytochrome c (Adegite and Okpanachi, 1979) under conditions more suitable for enzyme studies, 0.1 M ionic strength. This behavior differs from that of

TMPD, which is a relatively poor mediator of electrons directly to cytochrome oxidase. Under conditions that favor the binding of cytochrome c to cytochrome aa_3 (50 mM Hepes buffer, pH 7.4), electron transfer from TMPD to oxidase is strongly stimulated by addition of cytochrome c, while that from $Ru(NH_3)_6^{2+}$ is competitively inhibited. The competition between cytochrome c and $Ru(NH_3)_6^{2+}$ indicates that the two reducing agents transfer electrons at the same site on the oxidase. In addition, $Ru(NH_3)_6^{2+}$ exhibits biphasic steady-state kinetics with purified cytochrome oxidase, qualitatively similar to those found for cytochrome c (Fergurson-Miller et al. 1976), but with lower affinity in both phases (apparent K_{m_1} = 8.6 μM; apparent K_{m_2} = 88 μM). These results are in agreement with the presteady state kinetic findings of Scott and Gray (1980) which show that $Ru(NH_3)_6^{2+}$, in a manner analogous to cytochrome c, rapidly reduces heme a prior to heme a_3 in cytochrome oxidase.

A further similarity between the behavior of $Ru(NH_3)_6^{2+}$ and cytochrome c was revealed by the requirement for the presence of the outer mitochondrial membrane to obtain efficient electron transfer from NADH. In the absence of outer membrane, millimolar concentrations of NADH were required to support even moderate rates of oxygen consumption with inner membranes and $Ru(NH_3)_6^{2+/3+}$, while in the presence of outer membrane, micromolar concentrations of NADH were sufficient to obtain the same rapid rates observed with ascorbate. These results can be explained in terms of an enzymically mediated reduction of $Ru(NH_3)_6^{3+}$ by NADH via the NADH-cytochrome b_5-reductase complex. The ability of $Ru(NH_3)_6^{3+}$ to act as an electron acceptor from cytochrome b_5 again mimics the activity of cytochrome c (Parsons et al., 1966, Sottocasa et al, 1967 a; Schnaitman and Greenawalt 1968). Since $Ru(NH_3)_6^{2+/3+}$ and cytochrome c are positively-charged molecules, it is not unexpected that they would both transfer electrons with kinetic efficiency

to negatively charged redox partners. On this basis it might also be predicted that $Ru(NH_3)_6^{+2/+3}$ would accept electrons from cytochrome $\underline{c}_1$. However, the reduction potentials of the two compounds [$Ru(NH_3)_6^{2+/3+}$, 0.078 V (Mayer and Traube, 1968) ; cytochrome $\underline{c}_1$, 0.28 V (Leigh and Erecinska, 1975)] are not compatible with $Ru(NH_3)_6^{3+}$ acting as an electron acceptor from cytochrome $\underline{c}_1$ under the conditions used where cytochrome $\underline{c}_1$ would not be maintained highly reduced. No transfer of electrons from succinate to oxidase was observed in cytochrome $\underline{c}$-depleted mitochondria supplemented with $Ru(NH_3)_6^{2+/3+}$. The possibility remains that $Ru(NH_3)_6^{2+}$ may be able to donate electrons efficiently to cytochrome $\underline{c}_1$.

Ruthenium red is used by many investigators in the study of mitochondrial calcium transport because of its potency as an inhibitor of this process. It is recognized that most commercial preparations are impure, but the recrystallization procedures recommended by Luft (1971) and Fletcher (1961) are lengthy and give very low yields. Furthermore, the high rates of electron transfer observed by Schwerzmann *et al.* (1976) were produced by ruthenium red purified according to Luft, indicating that this procedure may not remove $Ru(NH_3)_6^{2+/3+}$, or that this contaminant is re-formed upon storage. Chromatography of ruthenium red on carboxymethylcellulose in ammonium bicarbonate buffer, as described in this paper, provides a more reproducible, high yield method for obtaining pure ruthenium red that can be lyophilized to remove the ammonium bicarbonate and stored in a dry form.

The contaminant of ruthenium red responsible for its electron transferring activity, $Ru(NH_3)_6^{2+/3+}$, is shown by our studies and those of Scott and Gray (1980) to be a kinetically efficient electron donor directly to cytochrome oxidase, with kinetic characteristics qualitatively very similar to cytochrome $\underline{c}$. Therefore, this compound may provide a useful tool for investigating the

kinetic and structural requirements for energy coupled electron transfer in the terminal segment of the respiratory chain. One controversy that may be clarified through the use of the $Ru(NH_3)_6^{2+/3+}$ concerns the function of cytochrome oxidase in proton translocation. Although several researchers in the field have obtained evidence that support the proton pump model, others argue that the small transient acidifications observed are artifacts resulting from the interactions of cytochrome c with the phospholipid vesicles (Moyle and Mitchell, 1978; Papa et al., 1980). Using $Ru(NH_3)_6^{2+/3+}$, it is possible to study this process without cytochrome c, thereby simplifying the system. Indeed, recently Moroney et al. (1984) have used this electron donor to study the proton pump activity of cytochrome oxidase.

CHAPTER II

DIFFUSION IN THE MITOCHONDRIAL INNER MEMBRANE: IMPLICATIONS FOR THE MECHANISM OF ELECTRON TRANSFER

Introduction

Since the discovery that proteins in membranes are capable of long range lateral diffusion (Frye and Edidin, 1970) considerable attention has been focused on characterizing this process and the role that it plays in catalyzing biological reactions. Theoretical considerations suggest that the orientation of enzyme active sites by the membrane (McCloskey and Poo, 1983) and the reduction in dimensionality imposed by limiting a reaction to the membrane surface can result in statistically enhanced reaction rates (Adam and Delbruck, 1968; Berg and Purcell, 1977; Hardt, 1979). However, while it is recognized that 2-dimensional diffusion is a central feature in many membrane-mediated reactions, the precise role of diffusion in specific processes has not been established. In some cases it has been proposed that catalysis occurs by random collisions between reaction partners involving only transitory associations (Leibman and Pugh 1979, 1981; Yee and Leibman, 1978; Hanukoglu and Jefcoate, 1980), while in others, the evidence suggests that the reversible formation of functional aggregates occurs (Haworth et al., 1982; Gut et al., 1983). One system where a collisional mechanism has been implicated is

the mitochondrial electron transfer chain. This system is particularly interesting because of the unique structural features of the inner mitochondrial membrane which is designed to carry out efficient electron transfer between large integral protein complexes in a membrane of unusually high protein concentration, and because of the specialized role of the membrane in the energy conserving process.

Early ideas concerning the structure of the mitochondrial inner membrane were influenced by the observation that the protein to lipid ratio is very high (Colbeau et al., 1971), suggesting that integral membrane proteins might be forced into some kind of organized arrays. The possibility was considered that the electron transport chain might exist as a structural unit (Lehninger, 1959; Blair et al., 1963; Klingenberg, 1968), a concept that has been extended to include direct communication with the ATP synthesizing machinery (Boyer, 1977; Chance, 1977, 1982). These views were further reinforced by findings that unit electron transfer particles could be prepared from mitochondria (Blair et al., 1963) and could be reconstituted from the isolated components (Fowler and Hatefi, 1961; Hatefi et al., 1961). However, the chemiosmotic hypothesis of energy conservation proposed by Mitchell (1961) led to a new perspective on the necessity for physical interactions between the electron transfer and energy coupling processes, since direct chemical or conformational communication was not required for an energy transfer event involving a proton gradient. Experiments designed to investigate the degree of static organization among the inner mitochondrial membrane proteins (Hochli and Hackenbrock, 1976, 1978; Sowers and Hackenbrock 1981) showed that despite the high protein content, large integral proteins are able to diffuse freely and independently over considerable distances and only occupy approximately half the total membrane area (Sowers and Hackenbrock, 1981). In addition,

studies on the rotational mobility of cytochrome oxidase demonstrate that as much as half of the oxidase in native mitochondrial membrane has free rotational mobility (Kawato et al., 1980, 1982). Schneider et al., (1980, 1982 a,b) also showed that artifically induced alterations in the distances between electron transfer components are accompanied by changes in electron transfer rates. When the distances between redox centers are decreased by inducing the formation of lipid-cholesterol patches that excluded proteins, the rate of electron transfer from succinate or NADH to oxygen is increased, while increasing the distances between redox centers by incorporation of phospholipid results in a decrease in the electron transfer rates. On the basis of these findings it was proposed (Hackenbrock, 1981) that mitochondrial electron transfer occurs by a diffusion mediated mechanism in which normal rates of electron transfer are accounted for by rapid diffusion of cytochrome c and ubiquinone.

Cytochrome c has been suggested to communicate electrons between cytochrome bc_1 and aa_3 by rapid diffusion on the negatively charged membrane surface (Chance, 1974; Roberts and Hess, 1977; Margoliash and Bosshard, 1983; Froud and Ragan, 1984) with correct orientation of the mobile carrier maintained by its large dipole moment (Koppenol and Margoliash, 1982). Evidence supporting this idea was obtained from studies on the effects of chemical modification of cytochrome c on its reactivity with redox partners. It was found that the same residues are involved in the binding of cytochrome c to both cytochrome bc_1 and cytochrome aa_3, suggesting that cytochrome c must first dissociate from one complex to react with the other (Brautigan et al., 1978 a,b; Ferguson-Miller et al., 1978; Ahmed et al., 1978; Rieder and Bosshard, 1978, Ferguson-Miller et al., 1979; Speck et al., 1979). In agreement with this is the observation that increasing viscosity of the reaction

medium competitively inhibits the ability of cytochrome c to transfer electrons between cytochrome bc_1 and cytochrome oxidase (Swanson et al., 1982). Nevertheless, studies on the rotational mobility of cytochrome c in rat liver mitochondria indicate that it is similar to that of oxidase, implying their existence as a reasonably long-lived complex in the native membrane (Dixit et al., 1982). Moreover, when cytochrome c is covalently crosslinked to mitochondrial inner membranes, significant rates of electron transfer are retained (Utsumi and Packer, 1967, Erecinska et al., 1975, Waring et al., 1980) suggesting that dissociation of cytochrome c from its redox partners may not be required for its activity.

Ubiquinone, the other potentially fast-diffusing component, participates in the communication of electrons between flavin-linked dehydrogenases and the cytochrome bc_1 complex. Because of the lipid-like nature of the molecule and its presence in excess of its redox partners (Vinogradov and King, 1979), ubiquinone has generally been considered to function as a mobile electron carrier (Green, 1962). By analogy to phospholipids in other membrane systems, this view has been extended to suggest that ubiquinone diffuses at 10^{-8} cm^2/sec, a rate that is compatible with a diffusion mediated role (Hackenbrock, 1981). However, it has been argued that the long rigid isoprenoid tail may impede ubiquinone diffusion (Trumpower and Katki, 1979; Wraight, 1979).

Evidence favoring a role for ubiquinone as a rapidly diffusing electron carrier has been obtained from a number of kinetic studies (Kroger and Klingenberg, 1973a,b; for review see Rich, 1981, 1984, Crofts and Wraight, 1983). Correlating the steady-state levels of reduce ubiquinone and the kinetics of the oxidation and reduction reactions with the overall rates of electron transfer in coupled mitochondria, Kroger and Klingenberg (1973a,b)

demonstrated that 80-90% of the ubiquinone functions kinetically as a homogeneous pool.

However, studies on the ubiquinone binding proteins suggests that ubiquinone may function primarily as a bound prosthetic group (Yu et al., 1974, 1981). Moreover, using the purified components in a reconstituted system, evidence has been obtained for direct interactions of cytochrome bc_1 with soluble succinate dehydrogenase (Yu et al., 1974) and Complex I (Ragan and Heron, 1978; Heron et al., 1978a,b) raising the possibility that electron transfer can proceed via direct association of the complexes rather than exclusively by free diffusion of ubiquinone.

To address the ambiguities regarding the role of diffusion, and the functional significance of physical associations among the electron transfer components, we have measured the rate of diffusion of cytochrome c, cytochrome oxidase, ubiquinone and lipid in the membranes of giant mitochondria from cuprizone-fed mice using the technique of fluorescence redistribution after photobleaching (FRAP). The results provide information necessary to quantitatively assess the feasibility of electron transfer occurring by a random diffusion mechanism. In addition this study demonstrates the feasibility of performing photobleaching measurements on intracellular membranes.

Experimental

Materials

Cuprizone [bis(cyclohexanone)oxaldihydrazone] was purchased from G. Fredrick Smith Chemical Co. (Columbus, OH) or from Aldrich Chemical Co. (Milwaukee, WI) and was recrystallized from boiling 95% ethanol (Tedeschi and Bowman, 1983). CM-cellulose (Whatman, CM-52) was prepared according

to the manufacturer's suggested directions. Cytochrome c (Sigma type VI) was further purified by the procedure detailed by Brautigan et al., 1978). Prior to use in kinetic assays cytochrome c was reduced with dithionite and gel-filtered on Sephadex G-75 superfine (Pharmacia) in 25 mM (Tris)cacodylate pH 7.8 in order to remove polymerized cytochrome c (Brautigan et al., 1978). Tetramethylrhodamine isothiocyanate (Polysciences, Warrington, PA), morpholinorhodamine isothiocyanate (Research Organics, Cleveland, OH), and lissamine rhodamine B sulfonylchloride (Molecular Probes, Junction City, OR) were stored dessicated in the dark at -20°C. NBD-phosphatidylethanolamine (Avanti Polar Lipids, Birmingham, AL) in chloroform/methanol 2:1 was dried with nitrogen gas, resuspended at 1 mg/ml in absolute ethanol and stored in 100 microliter aliquots under nitrogen gas in sealed glass ampules. Laurylmaltoside was prepared according to Rosevear et al., (1980). Ubiquinone-$_{10}$ was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of the highest grade available.

Preparation of Giant Mitochondria

Unweaned 17-19 day old mice (Swiss Albino ICR) were fed a diet containing 3 g of cuprizone in 500 g of ground rodent chow and were supplied exclusively with deionized water. Six to twelve days after initiating the diet, giant mitochondria were prepared from the livers of these mice using a modification of the method of Tedeschi and Bowman (1983). Three to five livers per 5 mls were minced, washed four times and homogenized in isolation buffer (220 mM mannitol, 70 mM sucrose, 2 mM Hepes (Tris) pH 7.4 containing 0.5 mg/ml BSA) with a loose fitting glass homogenizer. The homogenate was centrifuged at 120 x g for 1 min and the supernatant was saved. The pellet was homogenized in isolation buffer, filtered through two layers of cheesecloth and centrifuged as before. The supernatants were pooled, layered on top

of 5 mls of 0.5 M sucrose and centrifuged for 10 min at 410 x g. The top layer which contained smaller mitochondria and other membranes was discarded and the pellet was gently resuspended into the 0.5 M sucrose (lower) layer. The sample was diluted to 0.3 M sucrose with glass distilled water and pelleted at 750 x g for 5 minutes. The pellet was resuspended in 5 mls of isolation buffer, layered on 0.5 M sucrose and centrifuged at 240 x g for 3 min. The top layer was collected and the giant mitochondria were pelleted at 750 x g for 5 minutes.

Unless indicated otherwise, whole mitochondria were depleted of outer membrane using the procedure of Sottocasa et al., (1967) as modified by Felgner et al., (1979), and the mitoplasts were pelleted at 3,000 x g for ten min. Digitonin-treated mitoplasts were prepared according to Schnaitman and Greenawalt (1968) and were washed three times. Mitoplasts prepared by either technique were resuspended in 250 mM mannitol, 50 mM Hepes pH 7.2 and swollen by the gradual addition of five volumes of double distilled water over 10 minutes. In cases where cytochrome c diffusion was measured, whole mitochondria were depleted of cytochrome c by swelling in 20 mls of 10 mM Tris phosphate pH 7.5, followed by addition of 3 mls of 1 M KCl and pelleting prior to the removal of the outer membrane.

Mitochondrial protein concentrations were determined on deoxycholate-solubilized membranes using the biuret procedure. The concentrations of the cytochromes were determined from difference spectra (dithionite reduced minus ferricyanide oxidized) of deoxycholate solubilized membranes using $\Delta\varepsilon_{552-542}$ = 18.7 $mM^{-1}cm^{-1}$ for cytochrome c + c_1; $\Delta\varepsilon_{560-575}$ = 23.4 $mM^{-1}cm^{-1}$ for cytochrome b and $\Delta\varepsilon_{605-630}$ = 24 $mM^{-1}cm^{-1}$ for cytochrome aa_3.

The total phospholipid content of broken (freeze-thawed) mitoplasts was determined using the procedure of Ames (1966). The types of lipids were

determined by extracting them from the membranes according to the procedure of Awasthi et al., (1971) and chromatographing in two dimensions using the solvent system of Parsons and Patton (1967) on Supelco Redi coat 2D precoated TLC plates which had been preextracted with methanol/methylene chloride, 1:1. The lipid spots were visualized according to Thompson and Ferguson-Miller (1983).

NBD - Phosphatidylethanolamine Labeling:

Ten microliters of NBD-phosphatidylethanolamine (1 mg/ml in 100% ethanol) was added to a one ml suspension of swollen giant mitoplasts (0.5 mg mitochondrial protein) in 42 mM mannitol, 8 mM Hepes pH 7.2. The mitochondria were incubated with the fluorescent probe for five minutes, washed three times and resuspended in the mannitol-Hepes buffer. Under these conditions approximately half of the probe was incorporated into the membranes.

Preparation of Fluorescent Ubiquinone:

All of the reaction steps during the preparation of fluorescent ubiquinone were performed under nitrogen gas to prevent autooxidation of the ubiquinol. Ubiquinone-10 (100 mg in 1 ml chloroform/methanol (2:1) was reduced with a few grains of sodium borohydride until the solution was essentially colorless (no yellow). After all the hydrogen gas had bubbled off, the sample was dried under N_2 and 0.3 g of lissamine rhodamine B sulfonyl chloride in 5 mls of pyridine was added. The reaction was allowed to proceed for 6 hours, dried as before, and resuspended in chloroform/methanol 2:1.

The reaction mixture was extracted 2 times against water and the organic phase was dried. The mixture was resolubilized in 100% ethanol and the fluorescent ubiquinone was repeatedly precipitated out of 50% ethanol at 4°C and centrifuged until no dye was observed in the supernatant. The final pellet was dissolved in 0.5 mls chloroform:methanol:water 65:25:4 and applied

to a 1 ml silicic acid column (Iatrobeads, Iatron Laboratories, Tokyo, Japan) equilibrated with the same solvent mixture. The column was eluted with the chloroform:methanol:water mixture and the initial peak containing unreacted ubiquinone and the fluorescent derivative was saved. The ubiquinone mixture was dried with nitrogen gas, dissolved in 0.5 mls of 5% ethanol in n-heptane, and applied to a 1 ml silicic acid column. The unreacted ubiquinone was eluted from the column with 5% ethanol in n-heptane while the fluorescent labeled ubiquinone remained tightly bound to the top of the resin. After the unreacted ubiquinone was completely washed from the column, the fluorescent analogue was eluted with chloroform/methanol 2:1, dried under nitrogen, and dissolved in 100% ethanol at 0.03 mg/ml.

Mitoplasts in 42 mM mannitol, 5 mM Hepes pH 7.2 were labeled with 20 microliters of the ethanolic quinone solution per ml two times and were washed twice. Under these conditions the fluorescent quinone constituted less than 2% of the total lipid.

Preparation and Characterization of Fluorescent Cytochrome c:

Cytochrome c (40-80 μmoles) was bound to a column of CM-cellulose (1.5 x 7.5 cm) equilibrated with 25 mM potassium phosphate pH 6.7. The column was re-equilibrated with 10 mM sodium bicarbonate pH 9.6. The layer of CM-cellulose containing cytochrome c was removed, placed in a beaker containing tetramethyl- or morpholinorhodamine isothiocyanate (1.5 moles/mole of cytochrome c) in 10 mls of 10 mM sodium bicarbonate, and allowed to react with the dye in the dark under constant stirring for four hours. The reaction was terminated by addition of 20 mls of 1 M lysine pH 9.3, followed by gel filtration on a column of Sephadex G-10 (2.5 x 34 cm) in 25 mM potassium phosphate pH 6.5, to separate cytochrome c from non-covalently bound dye. The mixture of derivatized forms of cytochrome

c was purified by repeated chromatography on CM-cellulose columns in 65 mM potassium phosphate as described by Brautigan et al. (1979). The derivatized forms of cytochrome c eluting close to the native protein were collected separately, concentrated on CM-cellulose and eluted with 0.5 M sodium chloride. The concentrated samples were desalted on Sephadex G-10 (superfine) in 25 mM (Tris) cacodylate, pH 7.7.

Cytochrome c concentrations were determined from the spectra of the reduced protein using $\varepsilon_{550-610} = 25\ mM^{-1}cm^{-1}$. The activity of native cytochrome c and the derivatives was measured with cytochrome c depleted rat liver mitoplasts in 25 mM (Tris) cacodylate, 250 mM sucrose pH 7.9 using the method of Ferguson-Miller et al., (1976).

The positions of the lysine modifications were determined from chymotryptic maps by a modification of the procedure of (Brautigan et al. 1978b). Native and derivatized cytochrome c were desalted by dialysis against 50 mM ammonium bicarbonate using small molecular weight cut off dialysis tubing (1,000 Mr cut-off, Spectrum Medical Ind., Los Angeles, CA). The samples were lyophilized for 48 hours with several additions of double distilled water to insure complete removal of the ammonium bicarbonate, and the cytochrome c was resolubilized at a concentration of 10 mg/ml in 100 mM ammonium bicarbonate pH 8.5. α-Chymotrypsin (10-50 µg/mg of cytochrome) was added and the samples were digested for 20 hours at room temperature. The samples were lyophilized for 2-3 days as before. Thin layer chromatography plates (20 x 20 cm precoated with Avicel to a 250 µm thickness; Analtech, Inc., Newark, DE) were sprayed with electrophoresis buffer (pyridine/acetic acid/water, 100:4:900 v/v) and pre-electrophoresed for 30 minutes. The cytochrome c digests were dissolved in the electrophoresis buffer at 100 mg/ml and 0.1 - 0.5 mg of the protein were spotted on the plates.

The plates were then electrophoresed at 400 volts (20 volts/cm) until the heme-containing peptide had migrated 1 - 1½ inches from the point of application (approximately 1½ hours). The plates were dried overnight and chromatographed in butanol/pyridine/acetic acid/water (60:40:12:48 v/v) until the solvent front was 1 inch from the top of the TLC plate. The red heme peptide(s) was easily detected and the other peptides were visualized with buffered ninhydrin (Easley, 1965). Arginine-containing peptides were identified for native cytochrome c peptide maps using the Sakaguchi staining procedure (Easley, 1965), in which a solution of 0.0125% -napthol in absolute ethanol containing 36 mM urea was substituted for solution A. The pink arginine positive spots were circled and the plates were sprayed with acid ninhydrin.

To perform the FRAP experiments swollen cytochrome c-depleted mitoplasts were labeled with 20 µM fluorescent cytochrome c in 42 mM mannitol, 8 mM Hepes pH 7.2 and were diluted ten fold or pelleted and resuspended in the same buffer or in 25 mM (tris) cacodylate pH 7.9. The initial FRAP measurements were performed with swollen cytochrome c-depleted mitochondria (with some outer membrane associated) in 42 mM sucrose, 8 mM Hepes pH 7.2.

Preparation of Fluorescent Antibodies to Cytochrome aa_3:

Cytochrome oxidase was prepared according to the method of Kuboyama et al., (1972) as modified by Suarez et al., (1984). The laurylmaltoside solubilized enzyme was dialyzed against phosphate buffered saline (136 mM sodium chloride 2.7 mM potassium chloride, 8.1 mM dibasic sodium phosphate, 1.5 mM monobasic potassium phosphate, pH 7.2) and was mixed with an equal volume of adjuvant. Rabbits were injected subcutaneously at multiple sites on the back. The initial injection was with 4 mg of enzyme in Freunds complete adjuvant and was followed by repeated injections of 1 mg of enzyme in Freunds

incomplete adjuvant at 2 week intervals. Blood samples were taken 7-10 days after each injection. Once a significant antibody response was observed the frequency of injections was reduced to 2 month intervals.

The antibodies were purified on a Protein A Sepharose (Sigma) column according to Tucker et al., (1978) and dialyzed into 0.1 M sodium bicarbonate at pH 9.5. The dialysate was centrifuged at 10,000 x g for ten minutes in order to remove any precipitated protein. The antibody-containing supernatant was reacted with 0.15 mg of morpholinorhodamine isothiocyanate per mg of antibody and the reaction was allowed to proceed for 3 hours in the dark. Free dye was separated from the labeled antibodies by gel filtration on Sephadex G-25 followed by overnight dialysis against phosphate buffered saline. The labeled antibody solution was applied to a 5 ml cytochrome oxidase affinity resin prepared from guanidine-HCl-treated cytochrome oxidase according to Hammon and Hackenbrock (1976) using cyanogen bromide activated Sepharose 4B prepared by the methods of Cuatrecasas et al., (1968) or Kohn and Wilchek (1982). The column was washed with phosphate-buffered saline until no dye could be detected in the eluent and the antibodies were eluted with 6 M guanidine-HCl pH 5.6. The more tightly binding antibodies were pooled and dialysed for three days against phosphate buffered saline with repeated buffer changes. The fluorescent antibody was stored in phosphate buffered saline at -20°C.

Prior to labeling the mitochondria, samples of the antibody preparations were dialysed against 42 mM mannitol, 8 mM Hepes pH 7.2 overnight and centrifuged at 10,000 x g for ten minutes to remove any protein which may have denatured during storage. Swollen mitoplasts were incubated in the antibody solution for 5 minutes, pelleted and washed repeatedly.

Fluorescence Redistribution After Photobleaching:

Glass slides and coverslips were washed, rinsed thoroughly with water and 95% ethanol, and allowed to dry. Ten microliters of labeled mitoplast suspensions were applied per slide and the coverslips were sealed around the edges with paraffin to prevent evaporation.

Diffusion measurements were performed on the labeled mitoplasts by the technique of fluorescence redistribution after photobleaching using the instrumentation and method developed by Koppel (1974, Koppel et al., 1980). Individual fluorescently labeled mitochondria were scanned with a low intensity laser beam. A sample profile of the fluorescence emission distribution for a mitochondrion labeled with fluorescent cytochrome c is shown in Figure 12, ($t < 0$). The peaks indicate that the fluorescent probe is associated with the membrane, giving more intense fluorescence at the edges of the organelle. A higher intensity photobleaching pulse (0.1 - 0.2 sec) is applied to one edge of the mitochondrion resulting in the profile seen in Figure 12 ($t = 120$ sec.). The redistribution of the bleached and unbleached fluorescent probe (Figure 12, $t = 600$ sec) is followed with sequential scans and the data is analyzed using a normal-mode analysis for diffusion on spherical surfaces as described by Koppel et al. (1980). Rhodamine fluorescence was monitored with an incident wavelength of 531 nm, and combination of Leitz dichroic mirror TK 580 and barrier filter K520 or a sharp cut filter made from Corning Glass 3482 (< 1% transmittance below 544 nm; > 90% transmittance above 567 nm). NBD fluorescence was monitored with an incident wavelength of 477 nm, and a combination of Leitz dichroic mirror TK 510 and barrier filter K 530.

Figure 12. A representative FRAP experiment. Scans of fluorescence distribution of TMR labeled cytochrome c on an intact inner mitochondrial membrane before (t < 0), soon after (t = 120s) and a longer time after (t = 600s), irradiation of the leading edge of the organelle with a bleaching pulse of 0.2 seconds duration. A series of 21 scans at 2 minute intervals were taken per mitochondrion. The mitochondria were repleted with TMR cytochrome c and were monitored on a glass slide with a coverslip sealed with paraffin to prevent evaporation. The arrow indicates the location of the center of the focussed bleaching beam (1 μ diameter).

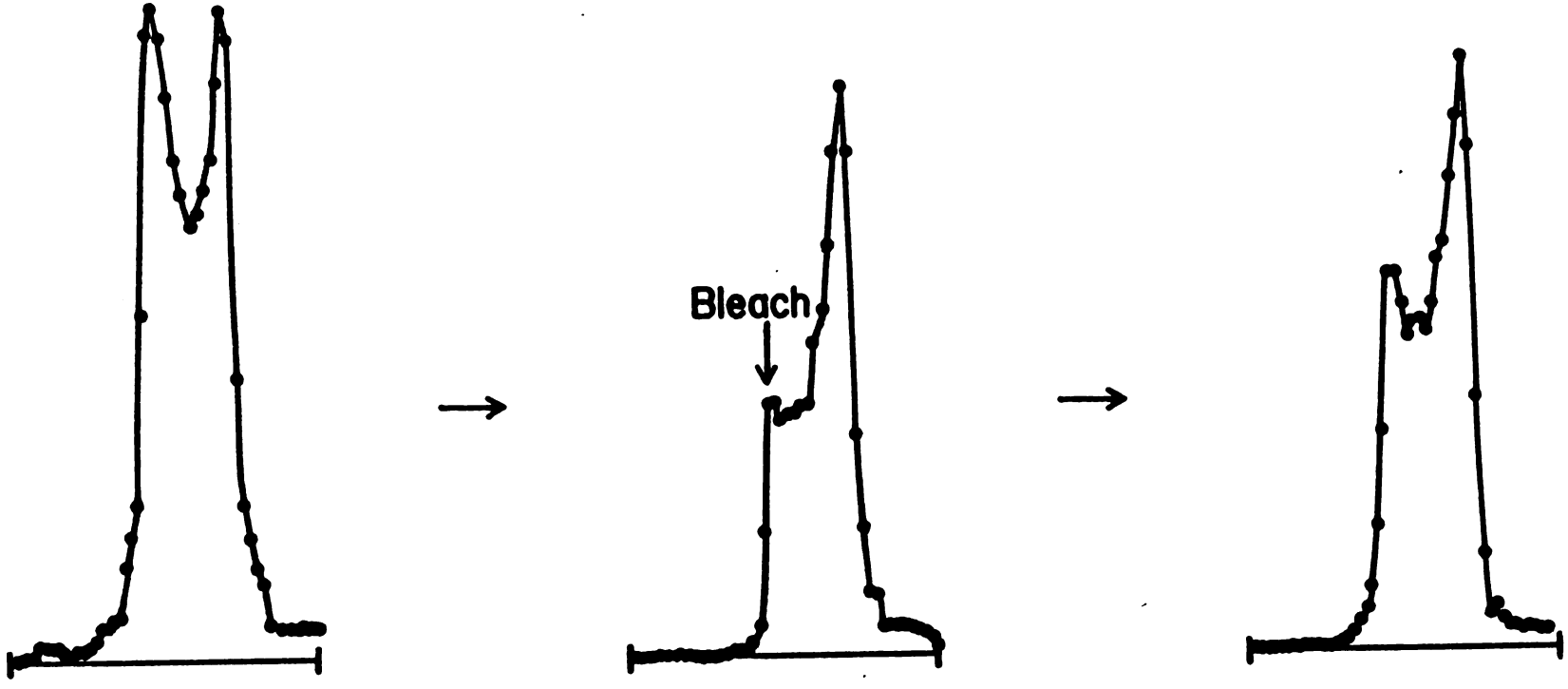


Figure 12

Results

Isolation and Characterization of Giant Mitochondria:

In order to obtain mitochondrial membranes large enough to be used in FRAP studies, giant mitochondria were prepared from the livers of mice that have been fed a diet containing the copper chelator, cuprizone (Bowman and Tedeschi, 1983). Mitochondria isolated 6-12 days after the initiation of the diet were routinely as large as 5-10 microns in diameter when observed by phase contrast microscopy (Figure 13a). Although some smaller mitochondria were also present, the relative amount of these mitochondria appeared to be dependent on the extent and harshness of the tissue homogenization, suggesting that they may represent fragmented giant mitochondria. Upon swelling in 42 mM mannitol (or sucrose), 8 mM Hepes, the mitochondria increased in size and became more transparent (Figure 13b). Electron micrographs revealed that the unswollen giant mitochondria (Figure 14a), showed characteristic cristae structures (also see Tandler and Hoppel, 1973) while the osmotically swollen mitochondria were spherical with little outer membrane and few cristae (Figure 14b). In the swollen mitochondria regions where the outer and inner mitochondrial membranes are still associated are seen to have cristae invaginations by electron microscopy and dark patched areas in phase contrast. This is particularly evident in Figures 13b and 14b where the mitochondria had not been depleted of outer membrane. For the most part, mitochondria depleted of outer membrane did not exhibit these regions. In cases where some outer membrane was still associated with the mitochondria the areas where these associations occurred were avoided in the FRAP experiments in order to eliminate the possibility that diffusion on the outer membrane could contribute to the measurements.

To determine whether photobleaching results obtained with these unusually

Figure 13. Phase contrast micrographs of unswollen (a) and swollen (b) giant mitochondria. The bars correspond to 10 μm.

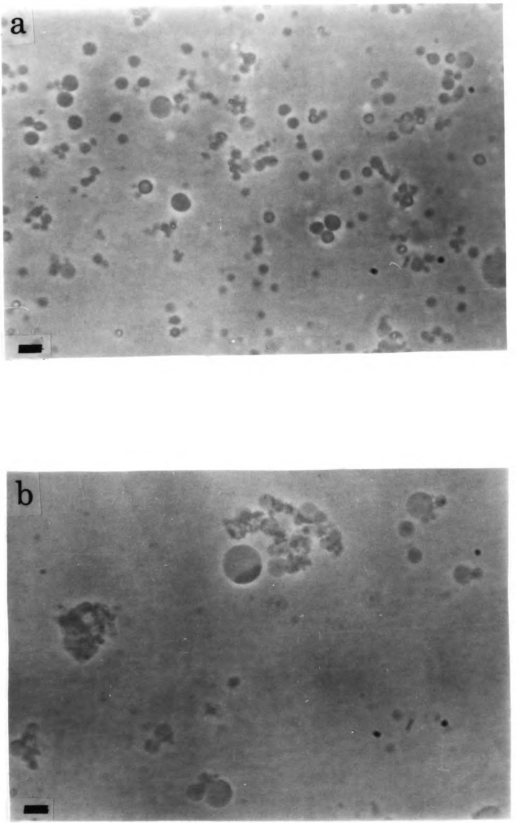


Figure 13

Figure 14. Electron micrographs of unswollen (a) and swollen (b) giant mitochondria. Mitochondria were swollen in 42 mM sucrose, 8 mM Hepes, pH 7.2, repleted with 20 μM cytochrome c, and pelleted at 1200 x g for 30 sec. The pellet was fixed for 10 hours in 0.4% glutaraldehyde, 10 mM Hepes pH 7.2, washed in 50 mM Hepes and post fixed for three hours in 1% osmium tetroxide, 10 mM Hepes. Unswollen mitochondria were fixed in an analogous manner in the presence of 250 mM sucrose. The fixed samples were dehydrated in graded ethanol and embedded in Epon 812:Araldite 502:dodecylsuccinic anhydride (25:20:60) and stained with saturated uranylacetate and 4% lead citrate.

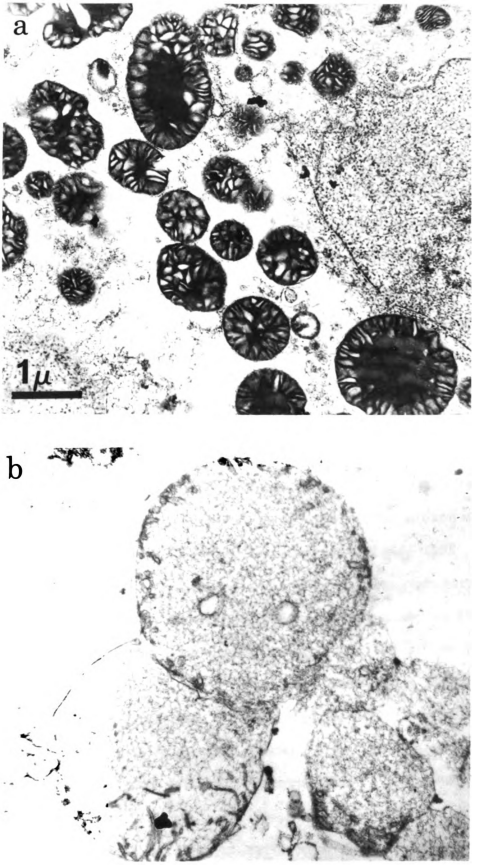


Figure 14

large mitochondria could directly apply to normal sized mitochondria (0.5 - 1 microns in diameter) it was necessary to characterize the membranes with respect to their composition and activity. The total phosphorus content of broken giant mitoplasts was 11-13 μg phosphorus per mg of protein suggesting that the membranes are composed of 21 - 25% phospholipid by weight. Thin layer chromatography of lipid extracts from these membranes revealed that the lipid composition was analogous to rat liver mitochondria, consisting primarily of phosphatidylcholine, phosphatidylethanolamine and cardiolipin.

These mitochondria also had normal levels of cytochromes as determined from their visible spectra (Figure 15a) and their calculated content of heme a, as well as normal cytochrome oxidase and succinate oxidase activities. In agreement with the values obtained with rat liver mitochondria, intact giant mitochondria had 0.24 n moles of heme a per mg of mitochondrial protein. In 42 mM mannitol (or sucrose) 8 mM Hepes, pH 7.2 (the conditions used for the FRAP experiments) electron transfer through the respiratory chain from succinate to oxygen (succinate oxidase activity) occured at a rate of 18 - 30 electrons per second per cytochrome oxidase for both the swollen undepleted and giant mitochondria repleted with cytochrome c (Figure 15b). When the giant mitochondria were resuspended in 50 mM potassium phosphate, pH 6.5, the turnover number of cytochrome oxidase (electrons per sec per cytochrome aa_3) in the presence of ascorbate and TMPD was 480 s^{-1} in the intact membranes, and 1200 s^{-1} in the membranes dissolved in laurylmaltoside. These values are comparable to those obtained with normal rat liver mitochondria (Thompson et al., 1982, Nicholls, 1974). In agreement with the results of Maloff (1978) these mitochondria also exhibited respiratory control.

Based on the results presented above it is reasonable to conclude that

Figure 15. Spectra and succinate oxidase activity of giant mitochondria. a) Spectra of giant mitochondria prior to (—) and after (—) depletion of cytochrome c with 50 mM potassium phosphate, pH 6.5. b) Succinate oxidase activity of cytochrome c depleted (A and B) and undepleted giant mitochondria (C) in the same state and under the same conditions as those used for the FRAP measurements. Oxygen consumption was monitored in 8 mM Hepes (Tris), 42 mM sucrose, pH 7.2 with 6 mM succinate (succ). Succinate oxidase activity was restored in the depleted mitochondria with addition of 4 μM fluorescent cytochrome c (TMRc) or the same amount of native cytochrome c (nat c).

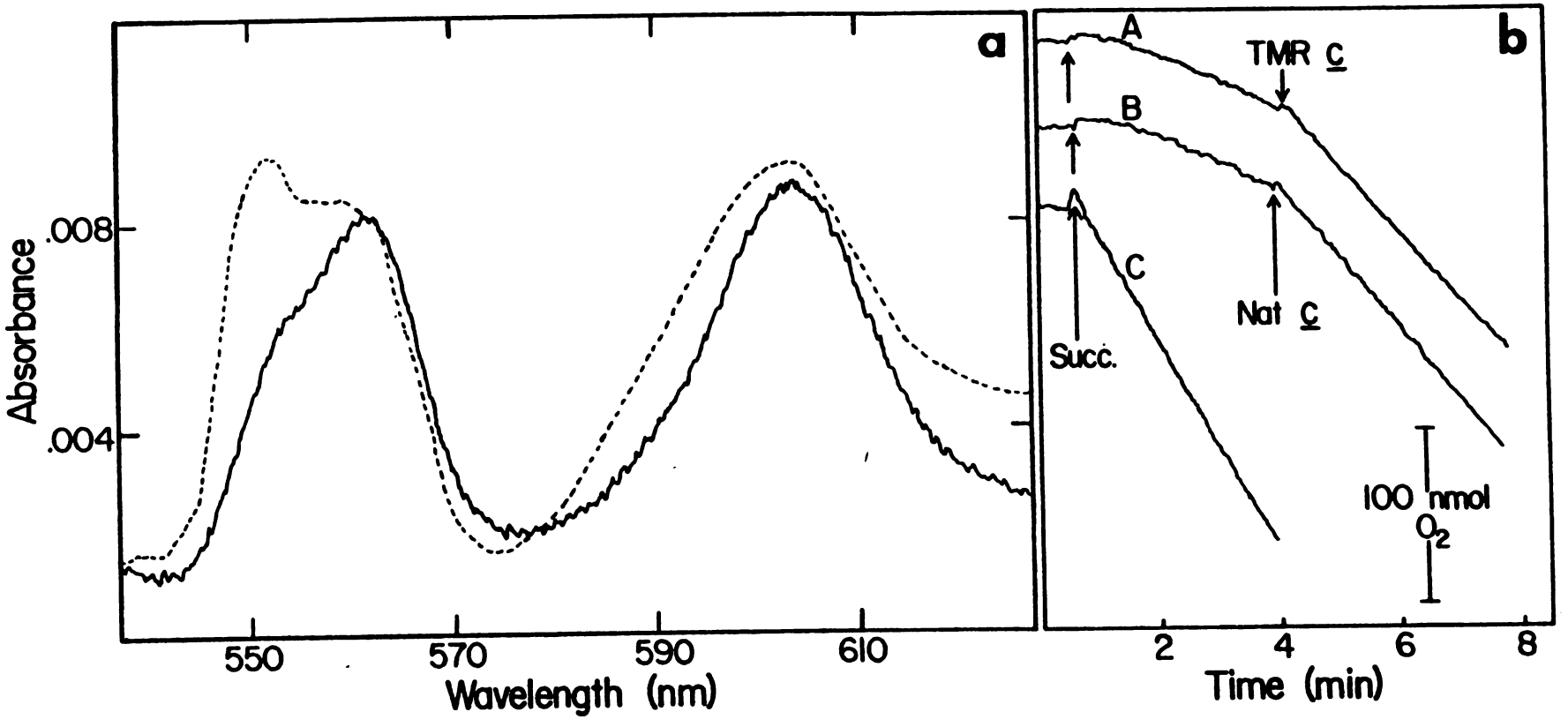

a
b
Absorbance
.008
.004
550
570
590
610
Wavelength (nm)
A
B
C
TMR C
Nat C
Succ.
100 nmol
O2
2
4
6
8
Time (min)

Figure 15

with the exception of their unusually large size these mitochondria are structurally and functionally normal. However, it should be noted that mitochondria isolated from mice which had been on the cuprizone diet for 14 days or longer had decreased levels of cytochrome oxidase and reduced electron transfer activities. Consequently, only mice which had been on the cuprizone diet for less than twelve days were used for the FRAP experiments.

NBD-Phosphatidylethanolamine Diffusion:

To obtain information about the behavior of lipids in the inner mitochondrial membrane, the fluorescent probe NBD-phosphatidylethanolamine was incorporated into the membranes of giant mitoplasts. Under conditions where the fluorescent probe represented less than 5% of the total membrane lipid the diffusion properties of the lipid were found to be dependent on the procedures used to remove the outer mitochondrial membrane. When mitochondria are depleted of outer membrane by a sonication technique (Sottocasa et al., 1967) the lipid exhibits complete (> 90%) monophasic recovery with a diffusion coefficient of 6 (± 2.1) x 10^{-9} cm^2/sec (Figure 16). In contrast to these results, mitoplasts prepared by treatment of whole mitochondria with digitonin (Schnaitman and Greenawalt, 1968) are found to have biphasic lipid recoveries (Figure 16) in which the diffusion coefficient of the first phase (D_1 = 5 (± 1.5) x 10^{-9} cm^2/sec) is approximately the same as that for untreated mitochondria, and that for the second phase (D_2 = 1 (± 0.6) x 10^{-9}) is significantly slower. Although the proportion of lipid probe in the two phases varies from one mitochondrion to another, FRAP measurements on mitochondria treated with 0.7, 1.2 and 1.5 mg of digitonin per 10 mg of mitochondrial protein revealed that there is a quantitative correlation between the amount of digitonin used and the amount of second phase observed.

Figure 16. Comparsion of the fluorescence recovery of NBD-phosphatidylethanolamine in mitoplasts prepared by digitonin treatment (●) and by mild sonication (Δ). Digitonin treated mitoplasts were prepared by incubation of whole giant mitochondria at 50 mg protein/ml with 0.12 mg of digitonin per mg of mitochondrial protein for 15 minutes. Scans were taken at 1 sec intervals. $\mu_1(t)$ is the normalized first moment of the unbleached fluorophore concentration distribution.

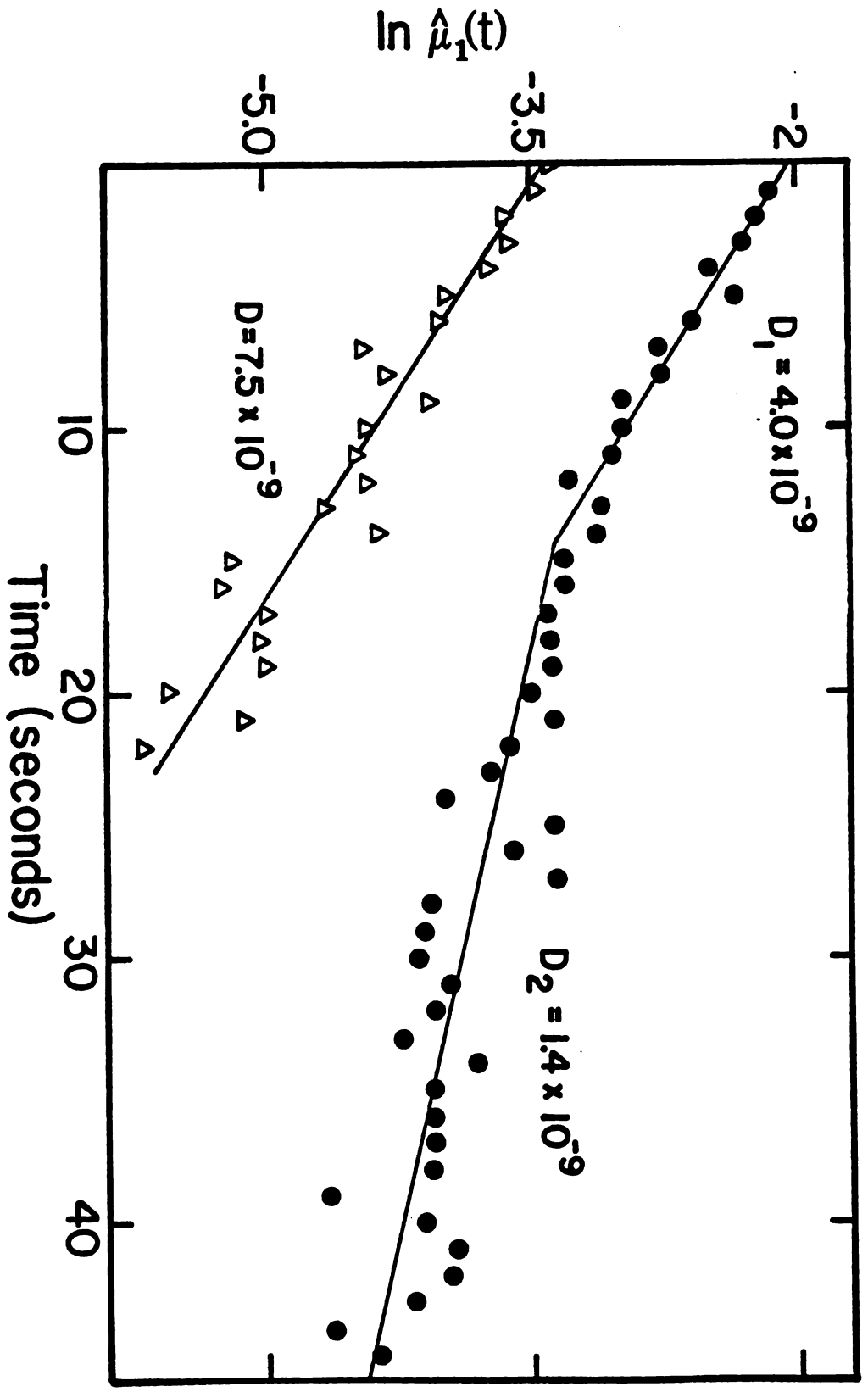

ln $\hat{\mu}_1(t)$
-5.0
-3.5
-2
10
20
30
40
Time (seconds)
D=7.5 x 10^{-9}
D_1 = 4.0x10^{-9}
D_2 = 1.4 x 10^{-9}

Figure 16

While digitonin appears to inhibit lipid mobility, at the same time accelerated rates of electron transfer are observed in the succinate oxidase sequence of reactions (Figure 17). The activity of cytochrome oxidase itself, as measured with the artificial electron donors ascorbate and TMPD, is essentially unaffected by the digitonin treatment indicating that accelerated electron transfer through the succinate oxidase chain was not due to a direct effect of digitonin on cytochrome oxidase.

Schneider et al., (1980, 1982a) previously reported a similar increase in mitochondrial electron transfer activity when cholesterol was incorporated into inner membranes of rat liver mitochondria. Since digitionin is a steroid molecule solubilized by a hydrophilic polysaccharide chain, it is possible that it incorporates into the inner membranes of mitochondria and acts in a manner analogous to cholesterol, producing patching of the lipids and resultant aggregation of the proteins. Indeed, lipid extracts from digitonin-prepared mitoplasts reveal a spot corresponding to digitonin on TLC plates even after repeated washing of the membrane (Figure 18).

Ubiquinone Diffusion

Procedures for preparing fluorescently labeled ubiquinone analogues have been reported in which fluorescent probes are attatched to a ten carbon alkyl tail bound to a substituted benzoquinone head group (Gu et al., 1983). While these analogues may prove useful for studying the interactions of ubiquinone with its redox partners, their behavior in FRAP studies may not be representative of the native molecule, since they do not have the long rigid isoprene tail of the native molecule, which could be important in determining their diffusion characteristics. Consequently it was of interest to prepare a fluorescent derivative of ubiquinone that more closely resembles the native molecule.

Figure 17. Effects of digitonin on succinate oxidase (●) and cytochrome oxidase (▲) activity. Whole giant mitochondria (50 mg/ml) were incubated with 0.03, 0.07, 0.12 or 0.15 mg of digitonin per mg of mitochondrial protein as detailed previously. In the case of zero digitonin, the outer membrane was removed by the sonication procedure described in Experimental. Turnover numbers are expressed as electron/sec cytochrome oxidase and were obtained with mitoplasts at concentrations of 0.019 and 0.098, 0.099, 0.043, 0.085, 0.033 nmoles cyt $\underline{aa_3}$/ml for mitochondria treated with 0, 0.3, 0.7, 1.2 and 1.5 mg of digitonin/mg of protein. Succinate oxidase activity was measured in 42 mM mannitol-8 mM Hepes pH 7.2 with 5.7 mM succinate, 2.3 μM cytochrome $\underline{c}$, 10 μM CCCP. Cytochrome oxidase activity was monitored by the subsequent addition of ascorbate and TMPD to final concentrations of 2.8 mM and 0.56 mM respectively.

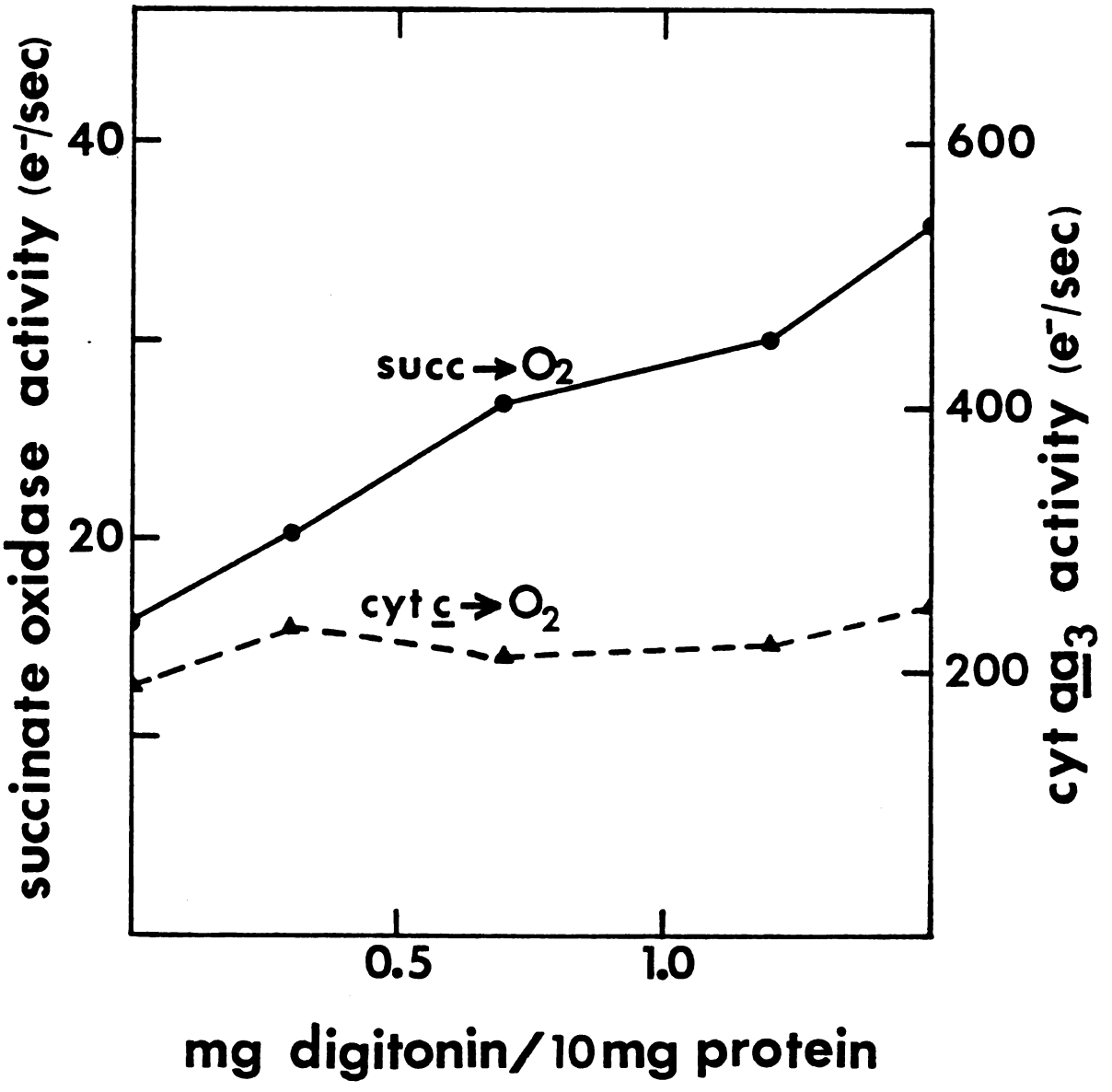

succinate oxidase activity (e⁻/sec)
40
20
succ → O2
cyt c → O2
600
400
200
cyt aa3 activity (e⁻/sec)
0.5
1.0
mg digitonin/10mg protein

Figure 17

Figure 18. Two dimensional thin layer chromatography of lipids extracted from mitoplasts treated with 1.2 mg of digitonin/10 mg of protein. Lipid extracts were applied to the activated silica gel plates and chromatographed in the vertical direction in chloroform : methanol : water : aqueous ammonia, 130:70:8:0.5, air dried, then developed in the horizontal direction with chloroform : acetone : methanol : acetic acid : water, 100:40:20:20:10. The components were visualized as in Experimental. O, origin; DPG, diphosphatidylglycerol (cardiolipin); PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DIG, digitonin.

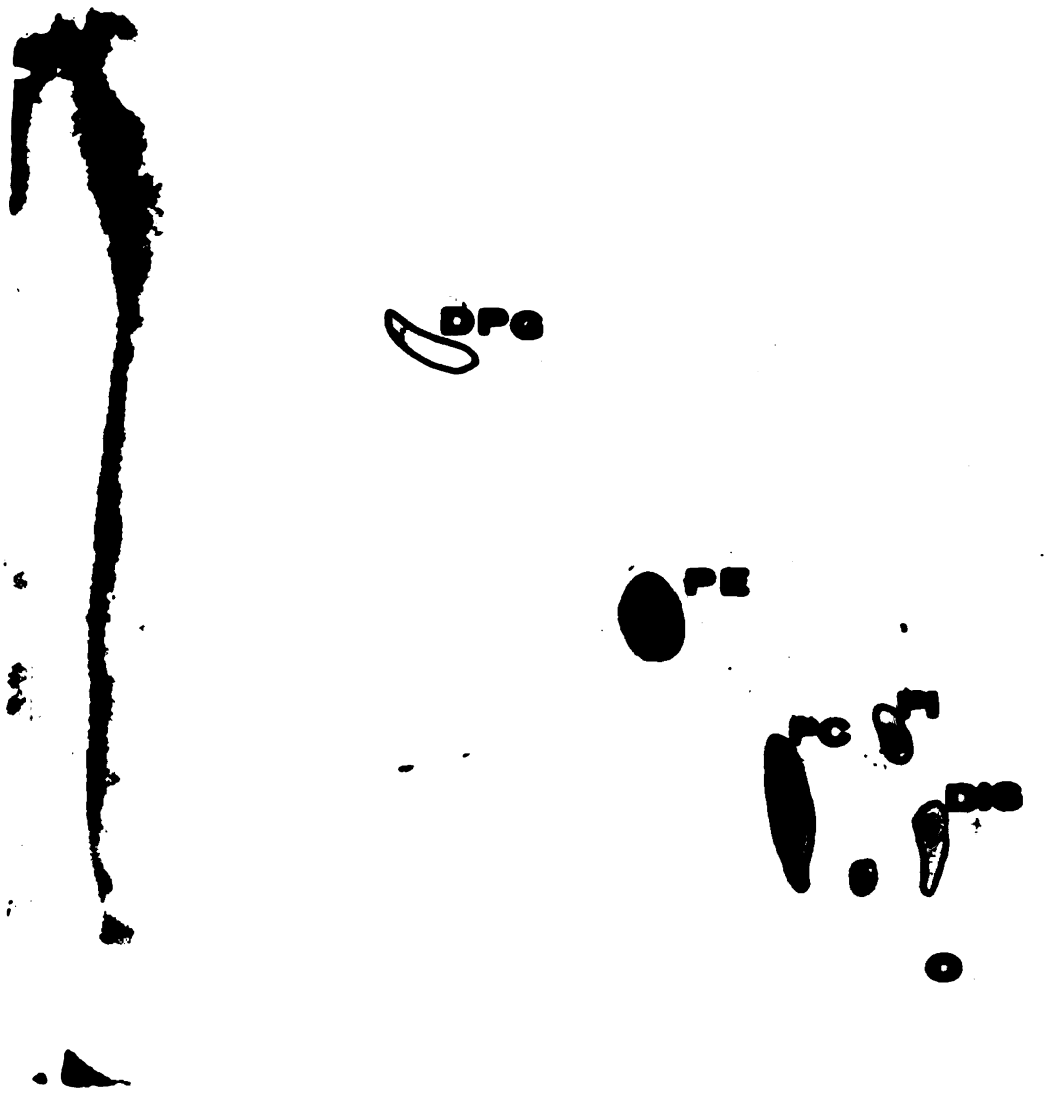


Figure 18

The reaction scheme used for preparing fluorescent ubiquinone is illustrated in Figure 19. The reactions were run under a nitrogen atmosphere to prevent auto-oxidation of the quinol. Attempts to reduce the ubiquinone directly in the pyridine resulted in the production of a blue breakdown product. Therefore, the reduction step was performed in chloroform/methanol 2:1 and dried down with nitrogen gas.

Lissamine rhodamine B sulfonyl chloride was added to the reduced ubiquinol in pyridine and the dye was allowed to react with the free hydroxyl groups. Although the dye can potentially react with both hydroxyls on the quinol head group, the accessibility of the hydroxyl group on carbon-2 should be limited due to steric hinderance from the neighboring isoprene chain and the hydroxymethyl group.

The conditions used for purifying the fluorescent ubiquinone were initially determined by thin layer chromatography on silicic acid plates. Chromatography of the crude reaction mixture in chloroform/methanol/water 65:24:4 revealed the yellow unreacted ubiquinone migrating at the solvent front immediately followed by a pink spot (LRQ10) that was absent in dye mixture, unreacted lissamine rhodamine, and a number of dye contaminants. After repeated precipitations out of 50% ethanol only three components remained: the unreacted ubiquinone, r_f = 0.95, LRQ10, r_f = 0.92 and unreacted dye r_f = 0.19. The native ubiquinone and the LRQ10 were extracted from the plate and chromatographed with 5% ethanol in n-heptane. In this solvent system the LRQ10 did not move while the underivatized ubiquinone migrated at the solvent front.

Subsequent preparations were purified on silicic acid columns. The elution profile of the ethanol-precipitated mixture chromatographed in chloroform/methanol/water 65:24:4 is shown in Figure 20. The initial peak

Figure 19. Reaction scheme for preparation of lissamine rhodamine labeled ubiquinone.

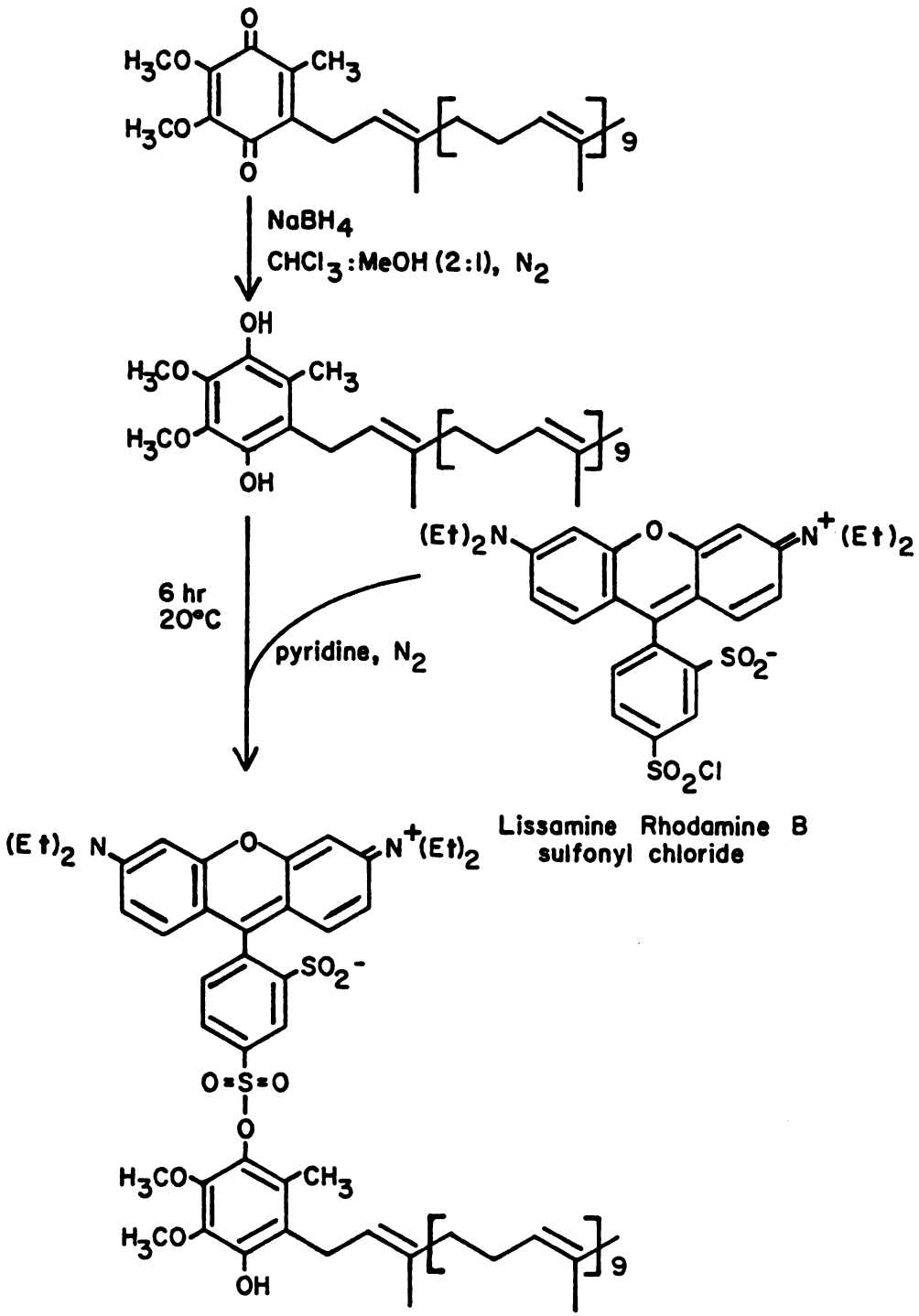

NaBH4
CHCl3:MeOH (2:1), N2
6 hr
20°C
pyridine, N2
Lissamine Rhodamine B
sulfonyl chloride

Figure 19

Figure 20. Purification of LRQ10 on a silicic acid column in chloroform/methanol/water, 65:25:4. The reaction mixture was precipitated with 50% ethanol, dried and applied to a 1 ml silicic acid column in chloroform/methanol/water. 0.2 ml fractions were collected and the initial peak containing ubiquinone and LRQ10 was saved. Fractions were monitored for absorbance at 270 nm (●) and 560 nm (△).

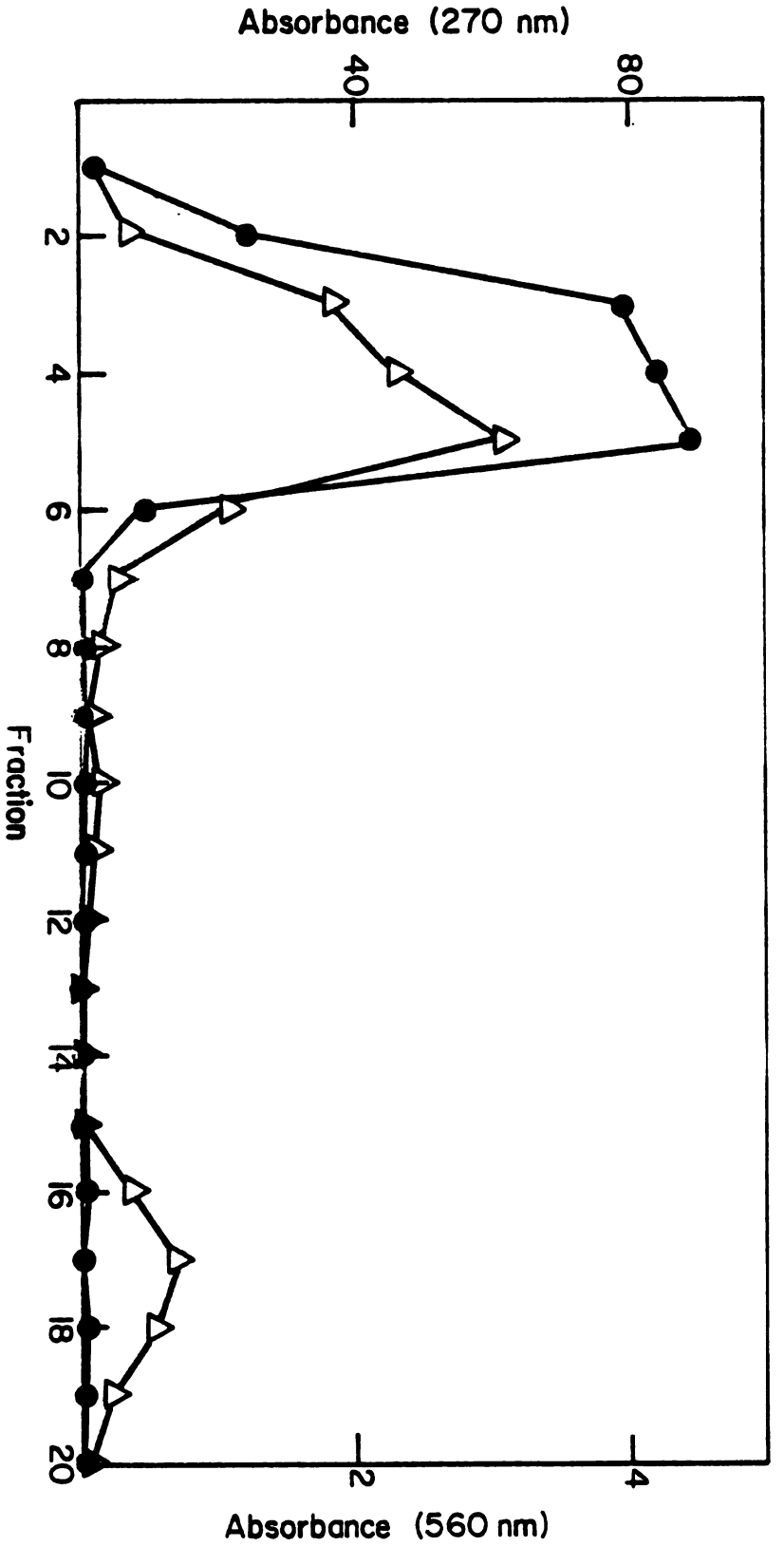

Absorbance (270 nm)
40
80
2
4
6
8
10
12
14
16
18
20
Fraction
2
4
Absorbance (560 nm)

Figure 20

contained LRQ10 and native ubiquinone while the unreacted dye eluted much later. The initial peak was rechromatographed on silicic acid with 5% ethanol in n-heptane (Figure 21). The unreacted ubiquinone did not stick to the column while LRQ10 was bound tightly at the top of the resin. The column was washed with 5% ethanol in n-heptane until all the unreacted ubiquinone had eluted as determined by its intense yellow color and absorbance at 270 nm. The LRQ10 was then eluted with chloroform/methanol 2:1.

The absorption spectra of the purified LRQ10 (solid line) and the crude dye (dashed line) is shown in Figure 22. If we assume that LRQ10 is the product predicted in Figure 19, then based on the absorbance at 560 nm the yield from the reaction is approximately 1%. Although this yield is low the quantity of product obtained was sufficient for the photobleaching measurements. Moreover, chormatography of the 50% ethanol extracts revealed that the majority of native ubiquinone was not precipitated suggesting that a considerable amount of LRQ10 may have been lost during this step. However, since the purified LRQ10 has an absorption spectra in the U.V. region that is very distinct from that of the free dye it may be possible to eliminate the ethanol precipitation step and use the relative absorbances at 270 and 560 nm to follow LRQ10 in the chromatographic steps.

Infrared, proton NMR and mass spectra of the LRQ10 showed analogies with those of unreacted ubiquinone and lissamine rhodamine. During the analysis of LRQ10 by mass spectrometry, the compound thermally degraded to two components which vaporized at different temperatures. The fragmentation pattern obtained for the first component (Figure 23a) was similar to that for lissamine rhodamine with ions at m/z 249, 173, 149, and 100. The fragmentation pattern obtained for the second component (Figure 23b) showed characteristics of the pattern observed for ubiquinone, with a cluster of ions

Figure 21. Separation of LRQ10 from unreacted ubiquinone on a silicic acid column. Unreacted ubiquinone was eluted from the column with 5% ethanol in n-heptane followed by elution of the LRQ10 with chloroform/methanol, 2:1 (indicated by arrow). 0.2 ml fractions were collected and absorbance was monitored at 270 nm (●) and 560 nm (△).

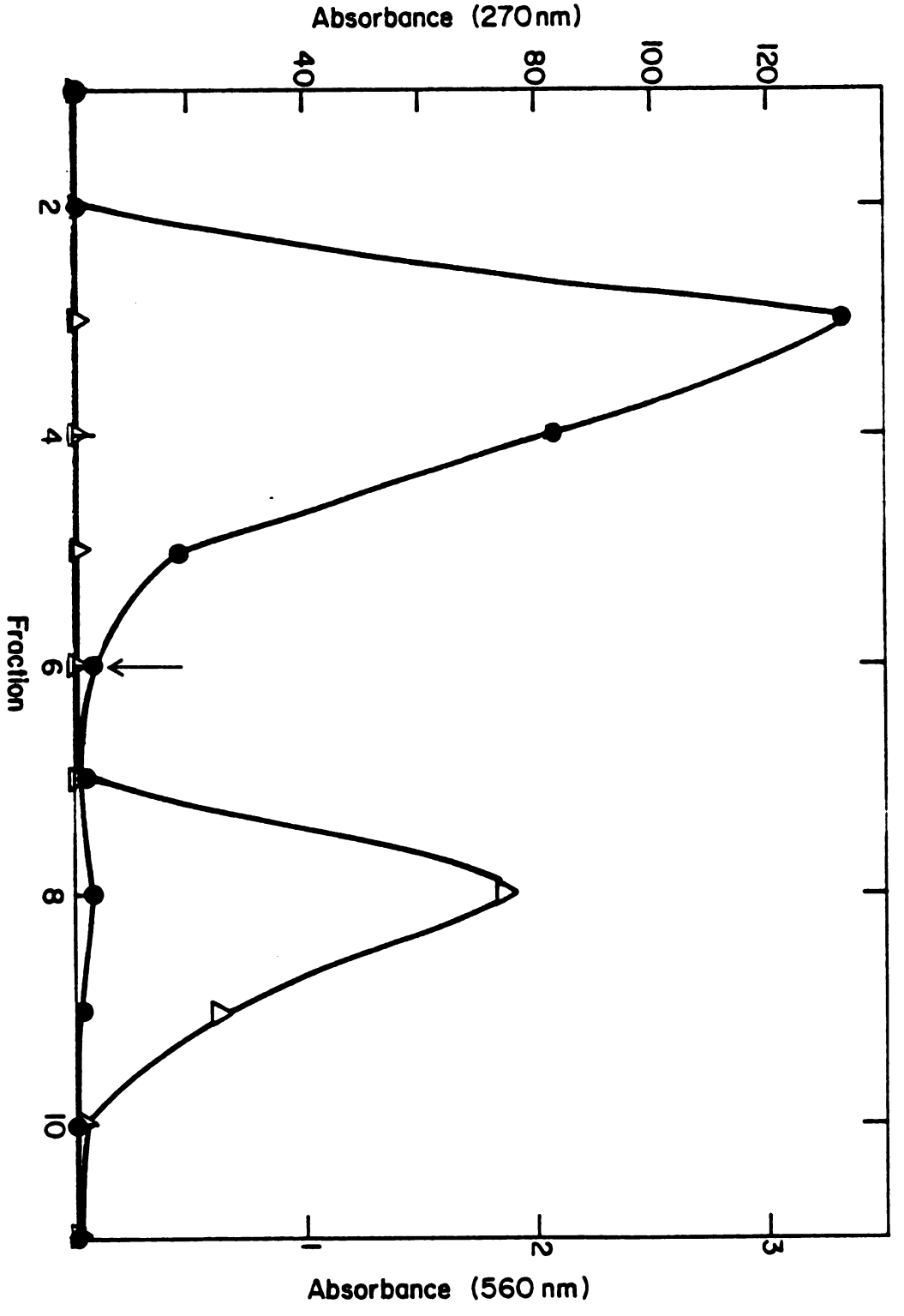

Absorbance (270nm)
40
80
100
120
2
4
6
8
10
Fraction
1
2
3
Absorbance (560 nm)

Figure 21

Figure 22. Comparison of the absorption spectra in the ultraviolet and visible regions for LRQ10 (solid line) and lissamine rhodamine B sulfonylchloride (dashed line). The full scale absorbance is 0.1 units for the LRQ10 and 1.0 units for the lissamine rhodamine spectra.

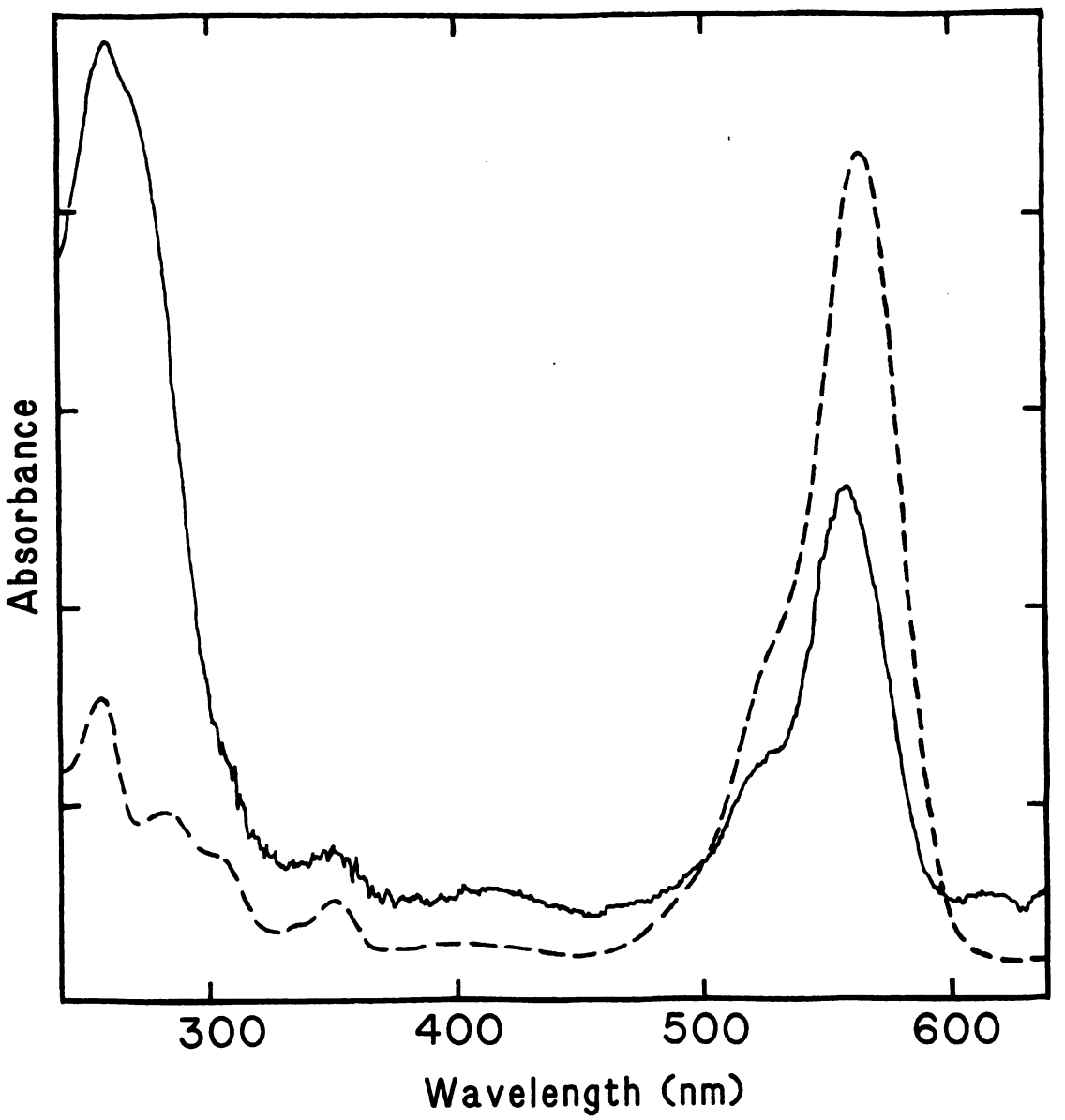
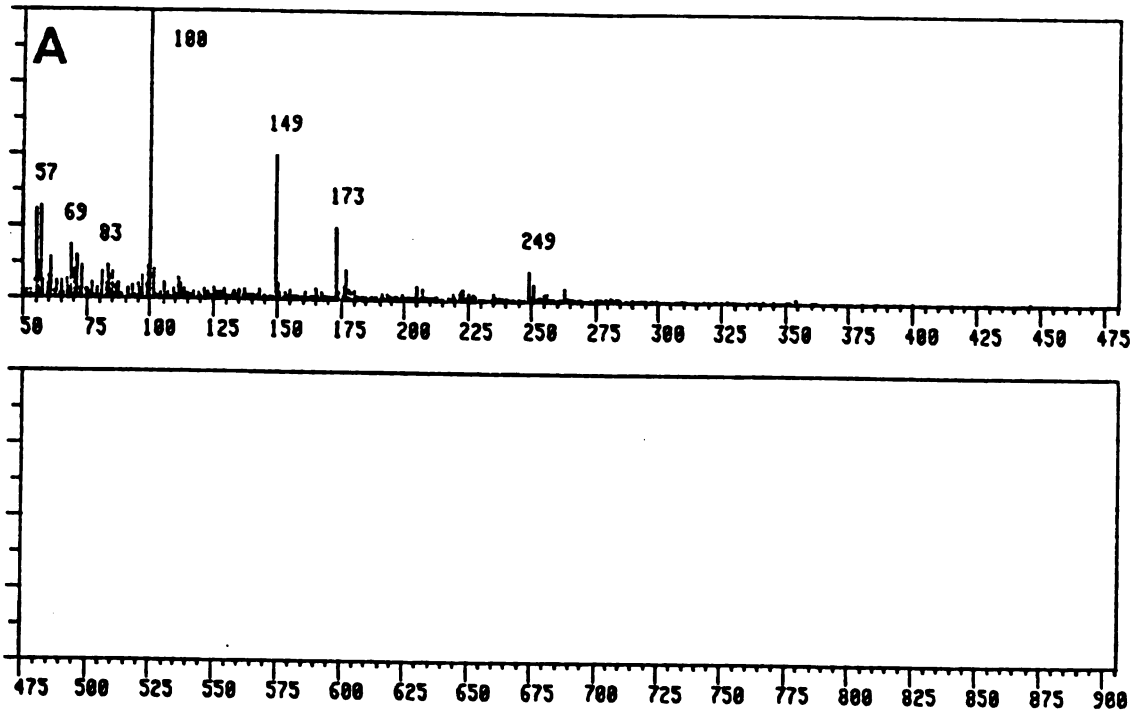


Figure 22

Figure 23. Mass spectra of the thermal degradation products from LRQ10. Mass spectra were performed using electron impact ionization. The sample was vaporized using direct insertion probe which was heated from 40°C to 200°C. Fragmentation patterns for the first (A) and the second (B) component to vaporize were obtained with a Hewlett Packard 5985 A mass spectrometer.


A
100
149
57
173
69
83
249
50 75 100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475
475 500 525 550 575 600 625 650 675 700 725 750 775 800 825 850 875 900

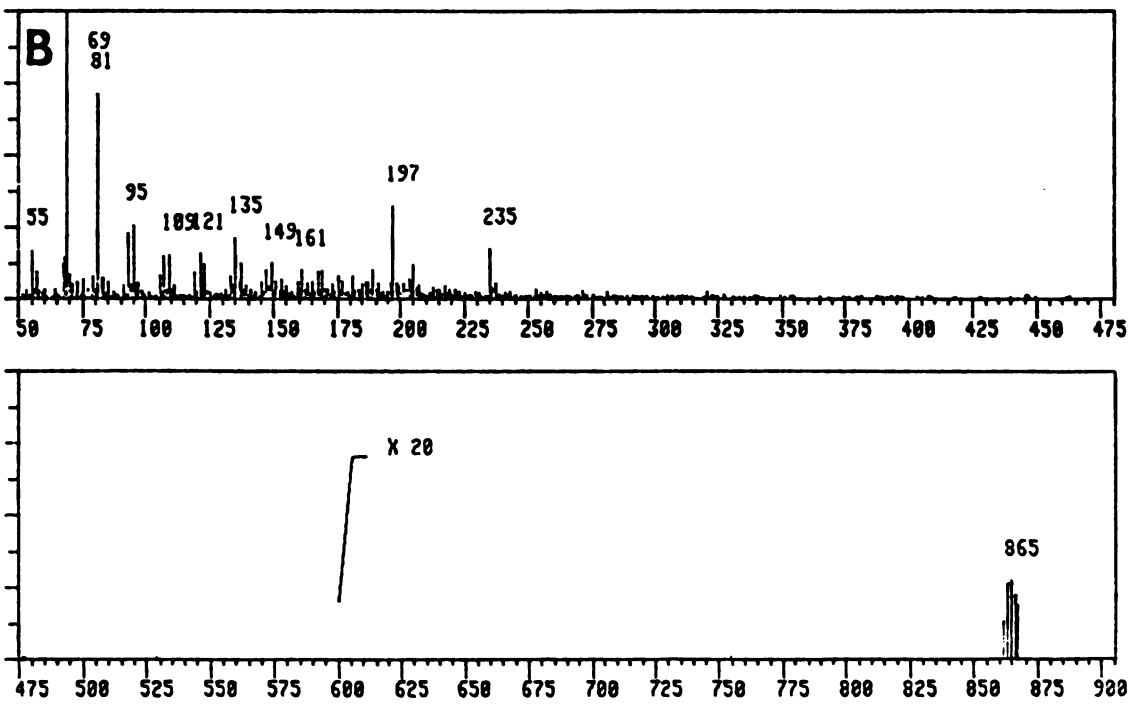

B
69
81
95
55
105121
135
149
161
197
235
50 75 100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475
X 20
865
475 500 525 550 575 600 625 650 675 700 725 750 775 800 825 850 875 900

Figure 23

at m/z = 861-867 and an ion at m/z = 235. No mass forms higher than the ubiquinone parent ion could be detected due to the labile linkage between the dye and the ubiquinone. The infrared spectra of lissamine rhodamine sulfonyl chloride, ubiquinone and LRQ10 are shown in Figures 24a,b, and c, respectively. The LRQ10 had absorbances centered at 1420 cm^{-1} and 620 cm^{-1} from ubiquinone, 1340 cm^{-1} from lissamine rhodamine, and bands at 1580 cm^{-1} and 660 cm^{-1} which had contributions from both compounds. In this spectrum the resolution of a shoulder at 1670 cm^{-1} which is attributable to the carbon-carbon double bonds in the isoprene chain is particularly interesting since this shoulder is not discernable with native ubiquinone due to the absorbance of the carbonyls of the oxidized molecule at 1650 cm^{-1} (Pennock, 1965), suggesting that these groups are diminished in LRQ10. The infrared spectrum of the unreacted dye had a sharp peak at 1180 cm^{-1} corresponding to sulfonyl chloride which was absent in the LRQ10 spectra, indicating that the majority of dye present in the derivative had reacted.

The ^{1}H-NMR spectra of oxidized ubiquinone is shown in Figure 25b. The peaks have been assigned previously (Tsukida, 1972; Kingsley and Feigenson, 1981) as follows: -1.598 ppm, isoprenoid CH_3; -1.682 ppm, terminal trans CH_3; -1.741 ppm, CH_3 on first isoprene unit; -1.974 ppm $C\underline{H_2}$-$C(CH_3)$ = CH; -2.014 ppm, -CH_3 on quinol ring; -2.059 ppm, $C\underline{H_2}$-CH=C; -3.186 ppm quinol -$C\underline{H_2}$-$C=C(CH_3)$; -3.981 ppm and -3995 ppm, OCH_3; -5.119 ppm, vinyl CH. The ^{1}H NMR spectrum for LRQ10 (Figure 24c) appears to have little contribution from the dye itself (Figure 24a). A comparison of the LRQ10 spectrum with that of the native ubiquinone (Figure 25b) indicate that the only pronounced changes occur at the -3.186; -3.981 and -3.995 ppm signals. Since the groups assigned to these signals are directly associated with the quinol ring it is reasonable that the signal perturbations are due to the

Figure 24. Infrared spectra of lissamine rhodamine B sulfonyl chloride (a), ubiquinone-10 (b) and LRQ10 (c). The spectra were performed on the samples as potassium bromide pellets.

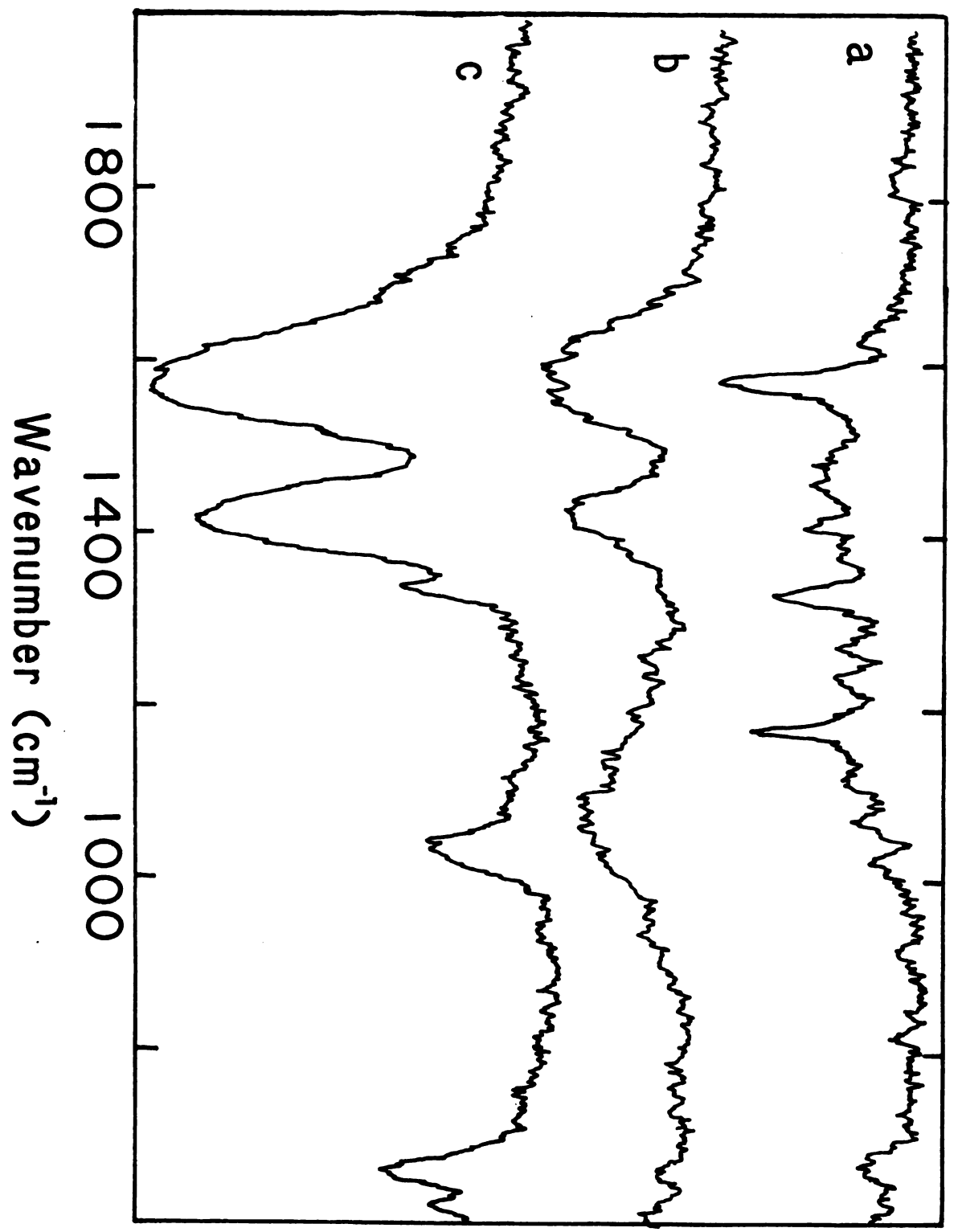


Figure 24

Figure 25. ^{1}H-NMR spectrum of lissamine rhodamine B sulfonyl chloride (top), ubiquinone-10 (center), and LRQ10 (bottom) in $CDCl_3$ Spectra were obtained with a 250 MHz NMR at 27°C.

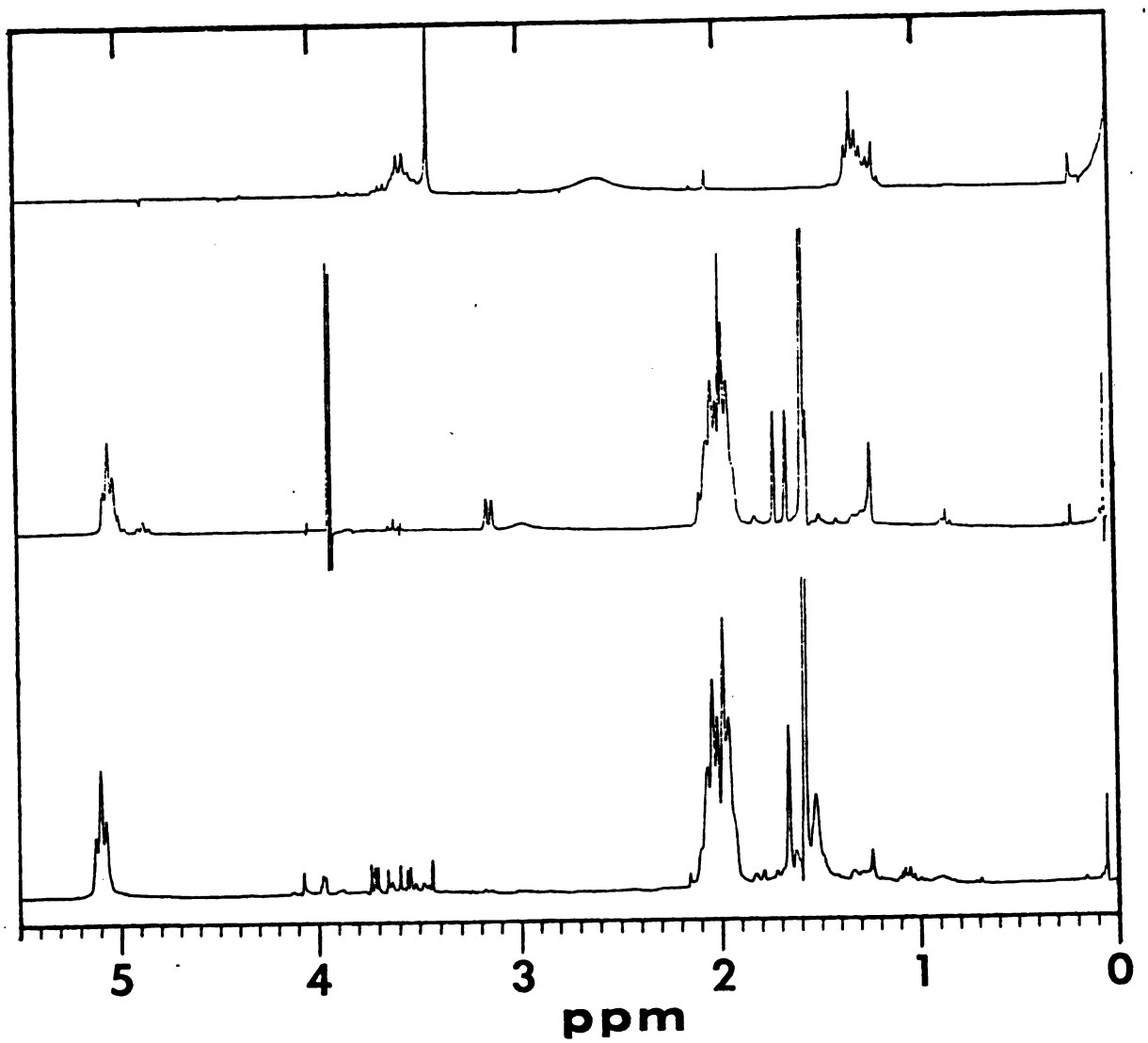


Figure 25

attachment of the fluorescent probe. It should be emphasized that the peaks attributed to groups on the isoprenoid tail are essentially the same, indicating that the isoprene chain was not altered during the reaction.

Based on the results presented above we conclude that the compound isolated is a fluorescently labeled ubiquinone derivative with an unaltered isoprenoid tail. FRAP measurements with this derivative should be representative of the diffusional properties of native ubiquinone.

Swollen mitoplasts prepared by the sonication technique (Sottocasa, 1967) were labeled with the fluorescent ubiquinone such that the probe constituted less than 2% of the total lipid and was present in a large excess (approximately 20-40 fold) over its redox partners. The diffusion coefficients obtained should therefore represent the free diffusion of ubiquinone in the membrane and should not be influenced by binding to specific proteins. FRAP experiments on these mitochondria showed monophasic recovery for the ubiquinone probe with a diffusion coefficent of 8.5 (± 0.6) x 10^{-9} cm^2/sec (84% recovery). In membranes labeled with both LRQ10 and fluorescent phospholipid and in those where the ubiquinone probe was absent, the diffusion of NBD-phosphatidylethanolamine was monophasic with diffusion coefficients of 5.5 (± 2.7) x 10^{-9} and 5.6 (± 2.4) x 10^{-9} respectively. Thus under the conditions used in these experiments the LRQ10 labeling did not appear to significantly alter the membrane. Moreover, despite the unique structural features of ubiquinone, its diffusion under these conditions is analogous to lipid.

Since digitonin treatment of mitochondria results in slower biphasic diffusion of lipids coincident with enhanced rates of electron transfer, it was of interest to determine if ubiquinone diffusion is also inhibited. When mitoplasts were prepared by treatment of whole mitochondria with digitonin

(1.2 mg/10 mg of mitochondrial protein) the diffusion of ubiquinone was significantly slower than in the untreated membranes, exhibiting monophasic diffusion with 88% recovery and a diffusion coefficient of 1.7 (± 0.7) x 10^{-9} cm^2/sec.

Cytochrome c Diffusion

It is well established that a number of lysine residues surrounding the heme crevice are important in the binding of cytochrome c to cytochrome oxidase and cytochrome bc_1. Since the isothiocyanate-conjugated dyes that were used to prepare the fluorescent cytochrome c modify amino groups, careful purification and characterization of the cytochrome c derivatives was essential in order to insure that the mobilities obtained are representative of the native protein. Singly-modified derivatives of cytochrome c with native activity were prepared as described in Experimental. The reaction of cytochrome c with tetramethylrhodamine isothiocyanate yielded a mixture of derivatives that were separated from each other and from unreacted dye by a series of gel filtration and ion-exchange chromatography steps. The elution profile from CM-cellulose for derivatized species whose chromatographic properties were similar to unmodified cytochrome c is shown in Figure 26a. More highly modified cytochrome c derivatives were not retained on the CM-cellulose column. Cytochrome c from peak B was found to be fluorescent and to have kinetic parameters similar to native cytochrome c (Km_1 = 3.5 x 10^{-8} M; Km_2 = 0.7 x 10^{-6} M) when assayed with rat liver mitochondrial inner membranes (Figure 26c). This derivative was also active in communicating electrons between cytochromes bc_1 and aa_3 as indicated by its ability to completely restore (94%) succinate oxidase activity to cytochrome c depleted mitochondria (see Figure 15).

Since the tetramethylrhodamine-labeled cytochrome c in peak B had native-like activity, it's diffusion should be representative of that for the

Figure 26. Purification and kinetic characterization of TMR labeled cytochrome c. a) Elution profile of TMR cytochrome c mixture chromatographed on a CM cellulose column as described in Methods. Absorbance was monitored at 410 nm. Peak B, indicated by the bar, was used for FRAP analysis b) Elution profile of peak B re-chromatographed on a column of CM cellulose (17 x 0.5 cm) as described in a) except the buffer concentration was raised to 100 mM, as indicated by the arrow. Absorbance was monitored at 410 nm (●) and 280 nm (△). c) Kinetics of reaction of peak B with rat liver mitoplasts (0.025 nmole heme aa_3) in 25 mM (Tris) cacodylate pH 7.9. Cytochrome c concentrations were: Peak B(△), 0.019 - 4.1 μM; native (○), 0.089 - 4.4 μM. d) Kinetics of reaction of the purified components of peak B with rat liver mitoplasts (0.1 nmol heme aa_3). Cytochrome c concentrations were: component I (■), 0.019 - 0.46 μM; component II (▲), 0.009-0.093 μM; native (○), 0.009 - 0.093 μM.

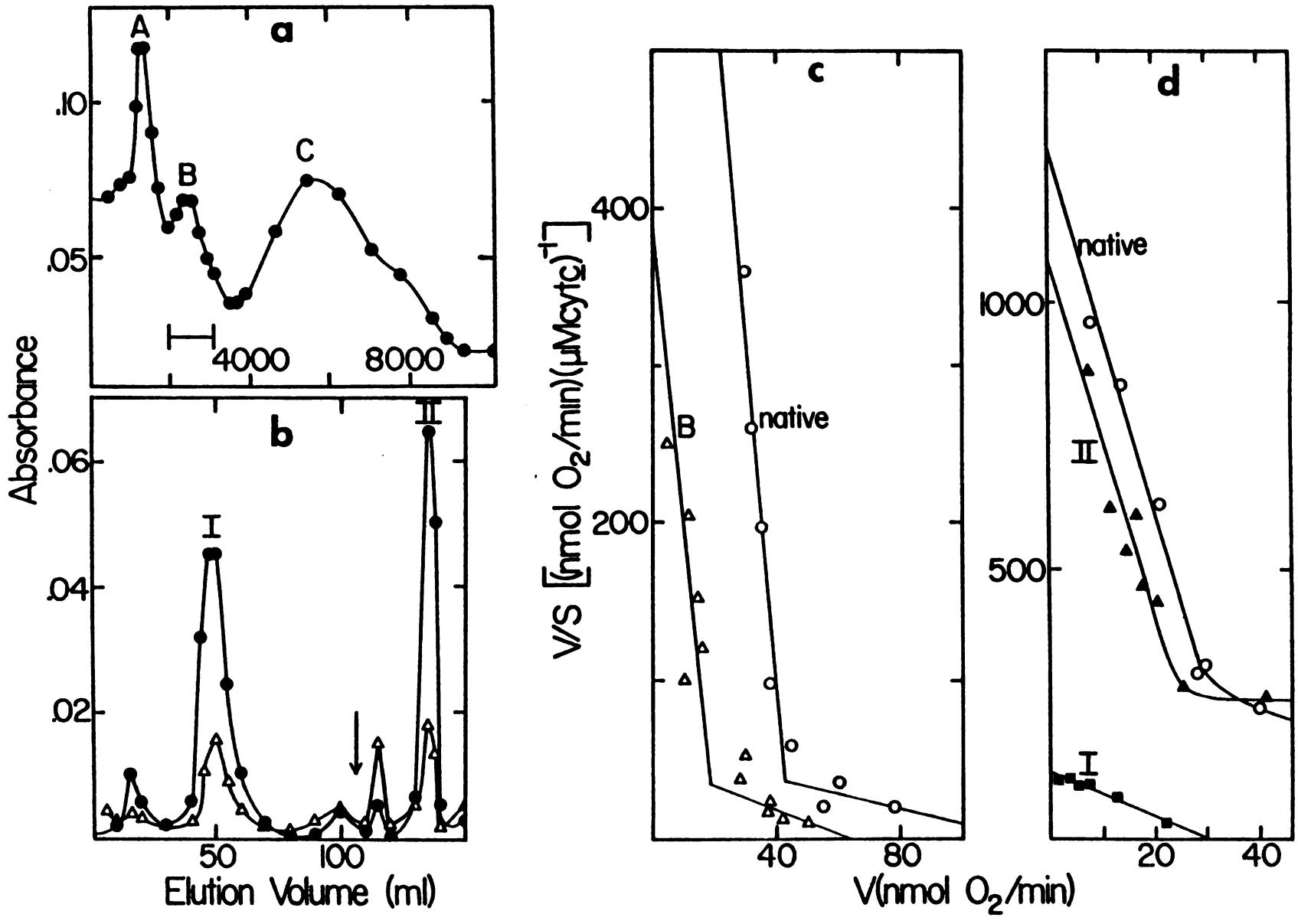

a
A
B
C
.10
.05
4000
8000
b
I
II
.06
.04
.02
50
100
Absorbance
Elution Volume (ml)
c
d
native
B
native
400
200
1000
500
II
I
40
80
20
40
V/S [nmol O2/min)(μMcyt c)-1]
V(nmol O2/min)

Figure 26

native protein. This derivative was used for the initial FRAP measurements.

Swollen, cytochrome c-depleted giant mitochondria (with some outer membrane associated) were repleted with the cytochrome c from peak B in the sucrose-Hepes buffer as described in Experimental. Under these conditions the derivatized cytochrome c was associated with the membrane in a 10-20 fold excess to cytochrome oxidase, indicating that the majority of the diffusing species should be bound to the membrane rather than to specific protein complexes. Thus the diffusion measurements obtained are for cytochrome c diffusing between redox partners as would be required for a random diffusion mechanism. Under these conditions a diffusion constant for cytochrome c of 1.6 (± 1.0) x 10^{-10} cm^2s^{-1} was obtained. The recovery was monophasic, greater than 90% complete, with correlation coefficients as high as 0.95.

Further characterization of the cytochrome c used for the FRAP experiments revealed that peak B consisted of two fluorescent cytochrome c derivatives which could be separated by chromatography on CM-cellulose (Figure 26b). The first cytochrome c derivative to elute from the column (Peak I) exhibited only low affinity activity (Km_1 = 2 x 10^{-7} M) while that from the second peak (II) demonstrated native behavior (Km_1 = 3 x 10^{-8} M) (Figure 26d). It is apparent from a comparison of Figures 26c and d, and from the succinate oxidase activity of the mixture, that the lower binding form, peak I, does not inhibit the binding or activity of peak II when the two are combined in fraction B. Therefore, it is concluded that the majority of the fluorescent cytochrome c bound to the repleted mitochondrial membranes was the more native, tight-binding fraction, peak II. Furthermore, the relative fluorescence intensity of peak II was 4x that of peak I, indicating that even if the two components bound to the mitochondria in the ratios that

they were present in the mixture, at least 80% of the observed fluorescence was due to the more native like component (peak II).

Although it is reasonable that the diffusion coefficient obtained for cytochrome c using the derivative in peak B is accurate, the amount of cytochrome c in peaks I and II were too small to sufficiently characterize the derivatives. To verify these results, additional cytochrome c derivatives were prepared, modified with tetramethylrhodamine isothiocyante or with the more hydrophilic dye morpholinorhodamine isothiocyanate. Following the removal of free dye from cytochrome c by gel filtration, singly-modified derivatives were separated from native and multilabeled cytochromes c by chromatography on CM-cellulose. Rechromatography of the singly modified derivatives obtained from reaction with either dye gave similar elution profiles, as shown for the morpholinorhodamine derivatives in Figure 27. Cytochrome c from peak D was found to be highly fluorescent (excitation maximum = 510 nm; emission maximum = 550 nm) and exhibited native-like activity with cytochrome oxidase in rat liver mitochondria (Km_1 = 4.3 x 10^{-9} M, Km_2 = 2 x 10^{-7} M) (Figure 27, inset). Two dimensional chymotryptic peptide maps revealed that peak D contained a mixture of three derivatives modified at lysine 22, 39 and 99. Since these modifications are located toward the back of the cytochrome c molecule, and do not significantly alter its activity with cytochrome oxidase (Brautigan et al., 1978a,b; Ferguson-Miller et al., 1978; Osheroff et al., 1980) or with cytochrome bc_1 (Speck et al., 1981; Konig et al., 1980), the diffusion coefficient of the derivative should represent that of the native protein.

A peak containing a similar mixture of derivatives was obtained by reaction of cytochrome c with tetramethylrhodamine isothiocyanate. When either of these derivative mixtures were stored for long periods of time (9 months) at -20°C some loss of activity and fluorescence was observed. The elution

Figure 27. Purification and kinetic characterization of MR cytochrome c. Elution profile of MR cytochrome c mixture chromatographed on CM-cellulose as described in Experimental. Absorbance was monitored at 410 nm (●) and 280 nm (△). (inset) Kinetics of native cytochrome c (0.0127 to 6.45 μM) and derivative in peak D (0.0055 to 5.03 μM) with rat liver mitoplasts (3.13×10^{-2} nmoles aa_3) in 250 mM sucrose, 25 mM (Tris) cacodylate pH 7.9.

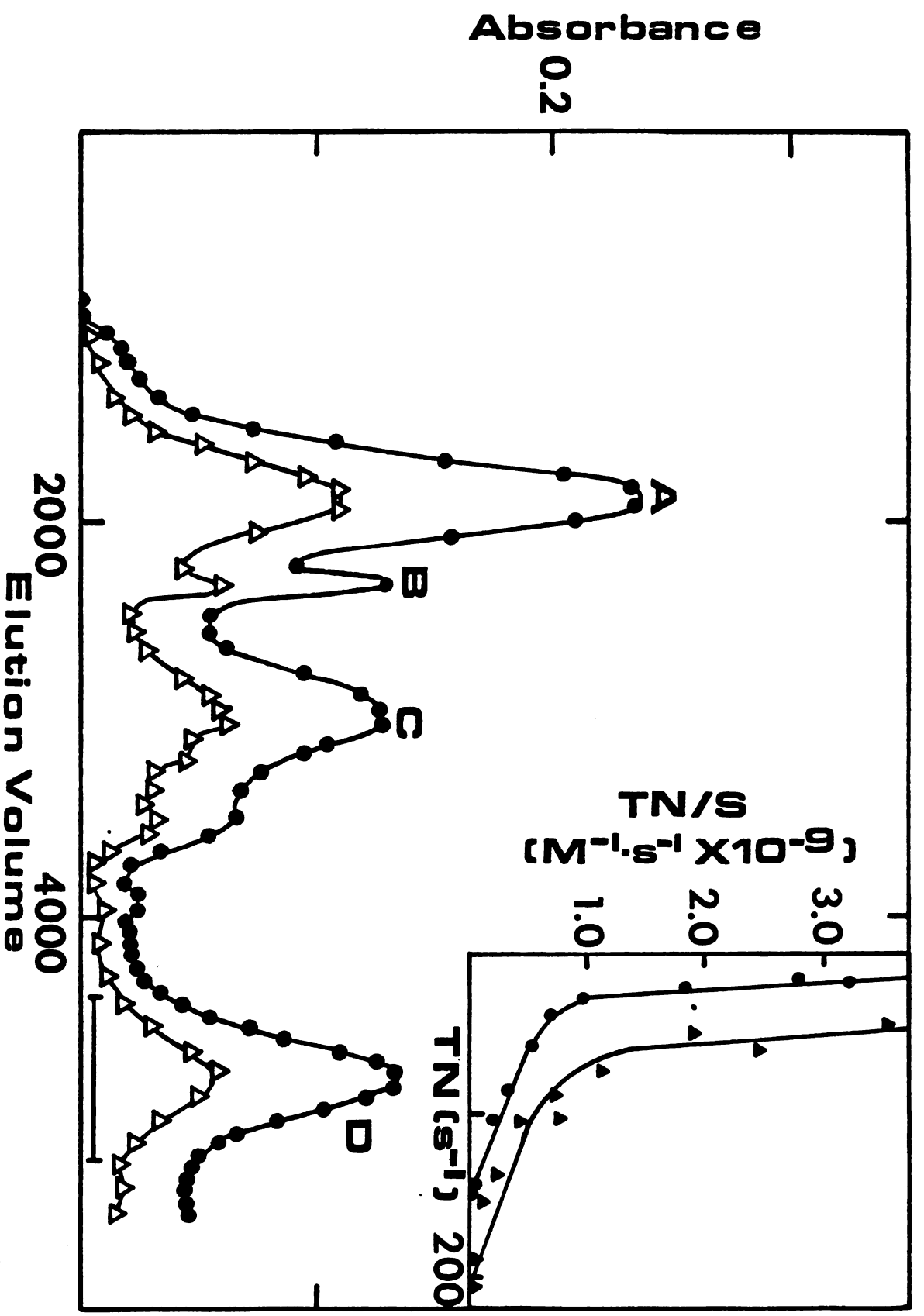

Absorbance
0.2
2000
4000
Elution Volume
A
B
C
D
TN/S
(M⁻¹·s⁻¹ X10⁻⁹)
1.0
2.0
3.0
TN (s⁻¹) 200

Figure 27

profile from rechromatography of tetramethylrhodamine cytochrome c peak D (after storage) is shown in Figure 28. Peaks A and B showed diminished electron transfer activity, but peak C had good fluorescence and native activity with cytochrome oxidase in cytochrome c-depleted mitochondria (Km_1 = 1 x 10^{-8} M, Km_2 = 2 x 10^{-7} M) (Figure 28 inset). The repurified derivative was also effective in restoring succinate oxidase activity to cytochrome c depleted mitochondria. Chymotryptic peptide maps of peak C (Figure 29) revealed that the primary site of modification is at lysine 39, as indicated by the absence of peptide 5, and the appearance of a modified peptide in a position consistent with loss of a positive charge. The inability to detect peptide 5 by ninhydrin staining of the thin-layer plate is indicative of greater than 95% purity (Brautigan et al., 1978b).

Giant mitoplasts depleted of outer membrane by the sonication technique were labeled with fluorescent cytochrome c (either the 22-99-39 mixture of morpholinorhodamine derivatives, or the lysine-39 tetramethylrhodamine derivative) under low ionic strength where cytochrome c was bound to the membranes in about a 10 fold excess. FRAP measurements on these mitochondria showed complete monophasic recovery (90%) for both the cytochrome c derivatives with a diffusion coefficient of 3.5 (± 1.5) x 10^{-10} cm^2/sec for the mixture, and 3.7 (± 1.6) x 10^{-10} cm^2/sec for the derivative of cytochrome c at position 39 (Figure 30). These values confirm the previous results with the less highly purified derivative, and are consistent with values predicted for cytochrome c diffusing on phospholipid vesicles, from kinetic measurements under similar conditions (Overfield and Wraight, 1980). At higher ionic strength (25 mM Tris cacodylate pH 7.9) the mitochondria were not labeled as intensely and more scatter was observed in the data. The diffusion was observed to be monophasic with 84% recovery and a diffusion coefficient of 7.4 (± 4.1)

Figure 28. Purification and kinetic characterization of Lysine 39-TMR cytochrome c. Rechromatography of peak D from a TMR cytochrome c preparation on CM-cellulose after prolonged storage. (inset) Kinetics of native cytochrome c (0.008 - 2.63 μM) (△) and derivative in peak C (0.0044 - 0.482 μM) (●) with rat liver mitoplasts (2.5×10^{-2} nmoles aa_3).

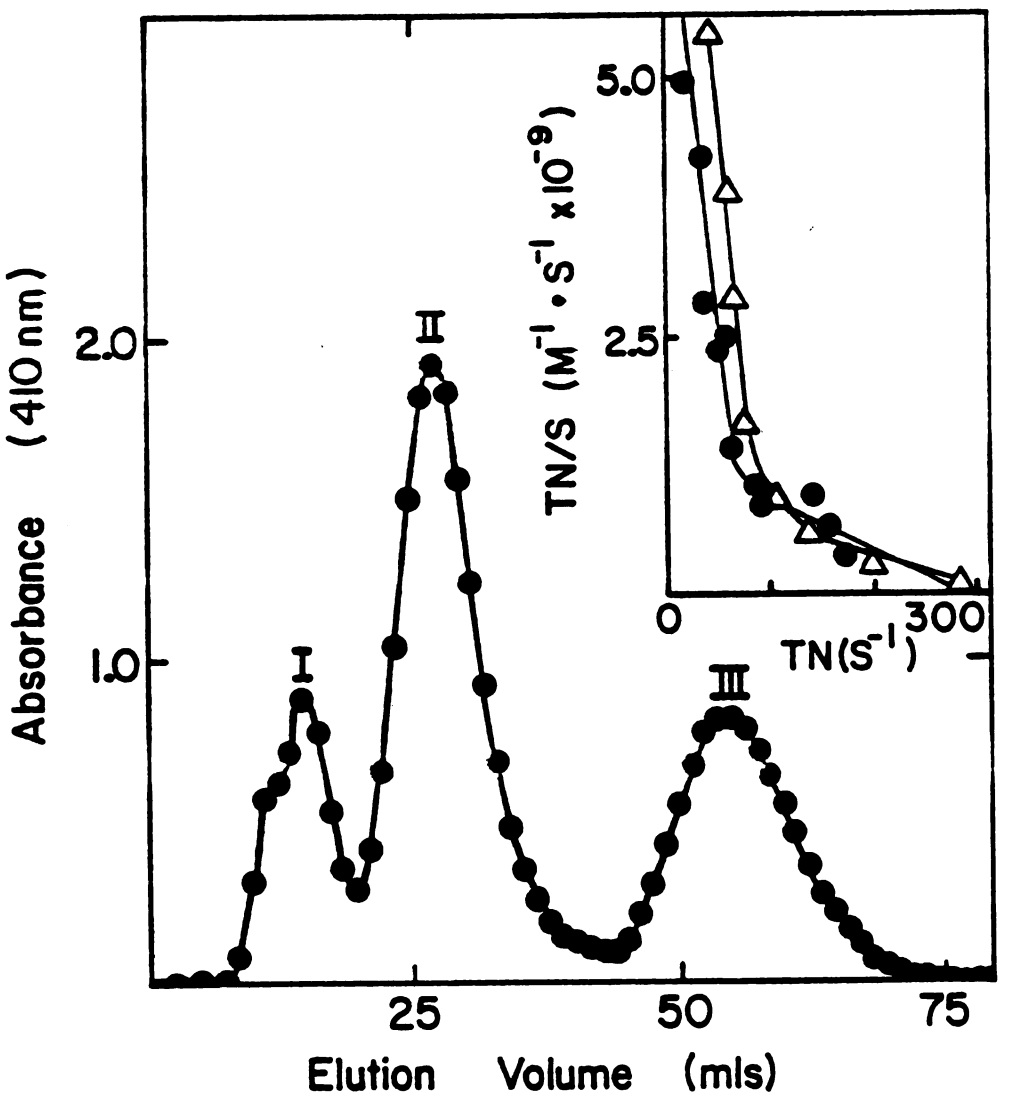

Absorbance (410 nm)
2.0
1.0
I
II
III
25
50
75
Elution Volume (mls)
TN/S ($M^{-1} \cdot S^{-1} \times 10^{-9}$)
5.0
2.5
0
300
TN(S^{-1})

Figure 28

Figure 29. Chymotryptic peptide map of native (A) and lysine-39-TMR cytochrome c. (B) The peptide maps were performed and the peptides were visualized as in Experimental. The peptides are assigned as follows:

Acetyl-Gly-Asp-Val-Glu-[1]Lys-Gly-Lys-Lys-Ile-Phe|Val-Gln-Lys-

Cys-Ala-Gln-[2]Cys-His-Thr-Val-Glu-Lys-Gly-Gly-Lys-His|Lys-[3]Thr-

(Cys ... Cys = HEME)

Gly-Pro-Asn-Leu-His|Gly-[4]Leu-Phe|Gly-Arg-Lys-Thr-[5]Gly-Gln-Ala-

-Pro-Gly-Phe|[6]Thr-Tyr|Thr-Asp-Ala-[7]Asn-Lys-Asn|Lys-[8]Gly-Ile-

Thr-Trp|Lys-Glu-Glu-[9]Thr-Leu-Met|[10]Glu-Tyr|Leu-Glu-[11]Asn-Pro-Lys-

-Lys-Tyr|Ile-Pro-Gly-[12]Thr-Lys-Met|[13]Ile-Phe|Ala-Gly-Ile-[14]Lys-Lys-

-Lys|Thr-Glu-Arg-[15]Glu-Asp-Leu|Ile-[16]Ala-Tyr|Leu-Lys-[17]Lys-Ala-Thr-

-Asn-GluCOOH

Peptide 7a is the deamidated form of peptide 7. Peptides 14, 14a and 14b are terminated at lysine 88, 87 and 86 respectively.

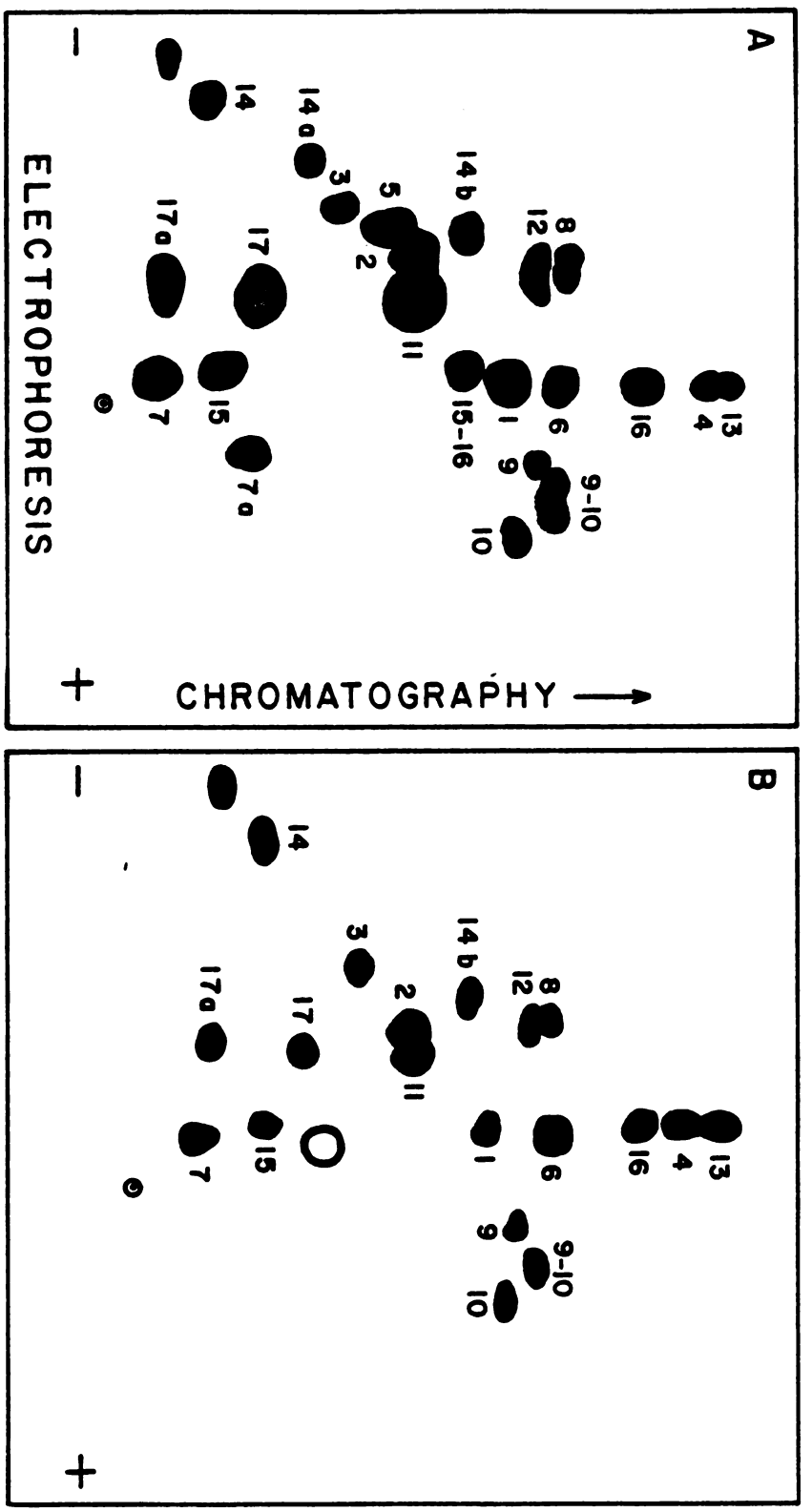

A
B
ELECTROPHORESIS
CHROMATOGRAPHY
14
14a
3
5
2
11
14b
8
12
17a
17
7
15
7a
15-16
1
6
16
13
4
9
9-10
10

Figure 29

Figure 30. Recovery curve for lysine-39-TMR cytochrome c on a swollen giant mitoplast in 42 mM mannitol, 8 mM Hepes (Tris) pH 7.2. Scans were taken every 5 seconds. $D = 3.8 \times 10^{-10}$ cm^2/sec.

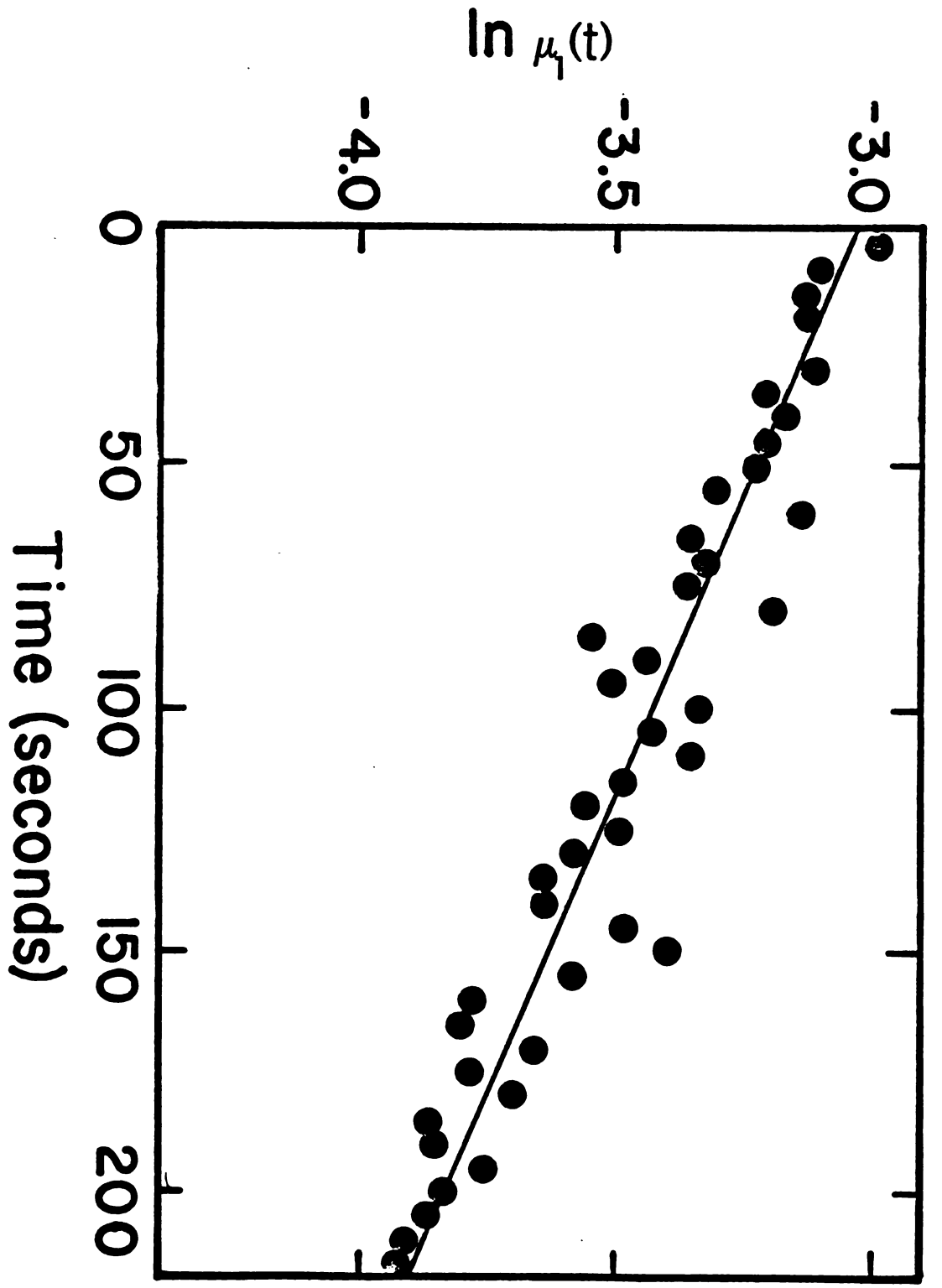


Figure 30

x 10^{-10} cm^2/sec. was obtained.

Cytochrome Oxidase Diffusion

The mobility of cytochrome oxidase was investigated using morpholinorhodamine-labeled antibodies which had been purified by gel filtration followed by chromatography on a cytochrome oxidase affinity column. The antibodies eluted as a single peak with 6 M guanidine HCl. The labeled antibodies eluting toward the back of the peak were more effective in binding to the mitochondria and were used for the FRAP measurements. Antibodies prepared in this manner did not label red blood cells, nor bind to mitochondria that had been pre-incubated with non-fluorescent antibodies. These results indicate that the antibodies are specific for cytochrome oxidase and are not associating with membranes or unrelated (red blood cell) proteins.

To avoid crosslinking of the oxidase by means of the bivalent antibodies, mitoplasts were incubated with a large excess of antibodies and washed to remove any non-specifically bound forms. Diffusion measurements on these mitochondria showed complete monophasic recovery (87%) indicating that there was no significant crosslinking induced by the antibodies. From these data a diffusion coefficient of 1.5 (± 1.0) x 10^{-10} cm^2/sec was obtained. Similar results were obtained with digitonin-treated mitoplasts (D = 1.7 (± 0.8) x 10^{-10} cm^2/sec with 85% recovery).

By following the redistribution of proteins in rat liver mitochondrial membranes after electrophoretic displacement, Sowers and Hackenbrock (1981) calculated a diffusion coefficient of 8 x 10^{-10} cm^2/sec for the large integral membrane complexes visualized by freeze-fracture electron microscopy. This value is significantly higher than the value obtained from the FRAP measurements. Considering the possible artifacts induced by preparation for electron microscopy, and by electrophoretic displacement

of the proteins the value obtained from the photobleaching measurements seem more tenable.

Determination of the Concentration of Cytochrome Oxidase in the Inner Mitochondrial Membrane:

To assess whether the diffusion coefficients for the redox components are compatible with a random diffusion mechanism for electron transfer, it is necessary to determine the concentrations of the redox components in the membrane. To determine the concentration of cytochrome oxidase in the inner membranes of rat liver mitochondria, mitoplasts were osmotically shocked in 10 mM Tris phosphate at protein concentrations less than 5 mg/ml, sonicated extensively, and the broken membranes were pelleted at 40,000 x g for 1 hour. This procedure depletes the mitochondria of matrix proteins and yields membranes which have 0.4 to 0.45 nmoles heme a per mg of protein. A similar value is obtained by freeze-thawing mitoplasts, washing, and pelleting the membranes (Thompson, 1984). Comparing this value with the heme a to protein ratio for the purified enzyme (10 nmoles heme a/mg protein) we calculate that cytochrome oxidase comprises 4.5% of the protein in rat liver inner membranes. Since the total protein content of the inner mitochondrial membrane can be packed into half of the total area (Sowers and Hackenbrock, 1981) then as 4.5% of the protein, oxidase will occupy 2.25% of the membrane area. Thus given that the area of a cytochrome oxidase monomer is 5,900 A^2 (Deathridge et al., 1982), there are 3.8×10^{10} monomers per 1 cm^2. The concentrations of the other electron transfer components can be extrapolated from their stoichiometries relative to cytochrome oxidase (2 cytochrome oxidase : 2cytochrome c : 1 cytochrome bc_1 : 10 ubiquinone : 1.5 dehydrogenase complex) (Vinogradov and King, 1979). These values are crucial to determining the validity of a random diffusion model of electron transfer (see Discussion).

Discussion

A number of models have been suggested to explain the way in which components of the electron transfer chain interact to accomplish efficient, energy-conserving electron transfer. Considering only the structural aspects of the membrane, two limiting cases can be proposed: a "solid state" model in which stable associations occur between the components in the chain forming electron transfer "units" (Lehninger, 1959; Blair et al., 1963; Klingenberg, 1968), or a random diffusion model in which electron transfer occurs by random encounters between freely diffusing components involving only transitory associations. Although several studies have addressed this problem, the precise role of diffusion in the respiratory chain has not been determined. To further elucidate the degree that structural associations may be involved in electron transfer the mobilities of phospholipids, ubiquinone, cytochrome c, and cytochrome oxidase have been measured in inner mitochondrial membranes using the technique of fluorescence redistribution after photobleaching.

Giant mitochondria from cuprizone-fed mice offer membranes of sufficient size to permit application of fluorescence photobleaching technology. These mitochondria have been observed to be coupled and are normal with respect to their electron transfer properties, levels of cytochromes, lipid composition, and protein to lipid ratios. Thus, giant mitochondria appear to be structurally and functionally analogous to the smaller mitochondria more commonly observed and the mobilities measured can be directly related to those in normal mitochondria.

The diffusion properties of lipid and ubiquinone were found to be dependent on the technique used to remove the outer mitochondrial membrane. In mitochondria depleted of outer membrane by sonication, rapid monophasic recoveries were observed for the two components. In contrast, removal of

the outer membrane by treatment of whole mitochondria with digitonin resulted in the appearance of a second, slower-diffusing lipid component, and an inhibited rate of diffusion of ubiquinone. Coincident with the decreased mobilities, digitonin was found to incorporate into the inner membranes and enhanced the rates of electron transfer in a manner reminiscent of the effects of cholesterol in the experiments of Schneider et al. (1980, 1982a). It seems likely that digitonin is acting in a manner analogous to cholesterol, producing patching of lipids with resultant aggregation of the proteins. In the cholesterol incorporation experiments, Schneider et al. (1980, 1982a) accounted for the increased electron transfer activities on the basis of decreased distances between the redox centers, shortening the path that cytochrome c and ubiquinone would have to travel to accomplish diffusion mediated electron transfer. However, the findings with digitonin suggest that a dual effect of cholesterol may also occur, such that the decreased distances between the complexes may be counterbalanced by reduced ubiquinone mobility, raising some question as to the validity of this argument.

The diffusion constants for the mitochondrial membrane components measured in mitoplasts prepared in the absence of digitonin are shown in Table I.

Using these values and the calculated concentration of cytochrome oxidase in the membrane (C_{aa_3} = 3.8 x 10^{10} monomers/cm^2), it is possible to calculate the minimal diffusion coefficient for cytochrome c that would be required to account for the observed electron transfer activity by a random diffusion mechanism. Since only 10-15% of the cytochrome c is reduced under normal steady state conditions in mitochondria (Nicholls, 1974; 1976), the concentration of cytochrome c involved in transferring electrons to oxidase (C_c^{2+}) is 3.8 - 5.8 x 10^9 molecules/cm^2. If we assume that the target area for cytochrome

Table 1. Diffusion Coefficients for Mitochondrial Membrane Components

	Probe	Buffer	Diffusion Coefficient[a] (cm^2/sec)	Recovery
Lipid	NBD-phosphatidylethanolamine	42 mM Mannitol 8 mM Hepes, pH 7.2	$6.0\ (\pm 2.1) \times 10^{-9}$	> 90%
Ubiquinone	LR-ubiquinone-10	42 mM Mannitol 8 mM Hepes, pH 7.2	$8.5\ (\pm 0.6) \times 10^{-9}$	84%
Cytochrome $\underline{aa_3}$	MR-rabbit anti-cytochrome $\underline{aa_3}$ IgG	42 mM Mannitol 8 mM Hepes, pH 7.2	$1.5\ (\pm 1.0) \times 10^{-10}$	85%
Cytochrome $\underline{c}$	TMR-cytochrome $\underline{c}$	42 mM Sucrose 8 mM Hepes, pH 7.2	$1.6\ (\pm 1.0) \times 10^{-10}$	> 90%
	MR-cytochrome $\underline{c}$ (Lysine-22,39,99 mixture)	42 mM Mannitol 8 mM Hepes, pH 7.2	$3.5\ (\pm 1.5) \times 10^{-10}$	90%
		25 mM Tris Cacodylate, pH 7.9	$7.4\ (\pm 4.1) \times 10^{-10}$	84%
	TMR cytochrome $\underline{c}$ Lysine-39	42 mM Mannitol 8 mM Hepes, pH 7.2	$3.8\ (\pm 1.6) \times 10^{-10}$	89%

[a] Values are expressed as ± standard deviation.

c reacting with cytochrome oxidase has a radius of 40 Å (a = 4 x 10^{-7} cm), then using the equation of Hardt (1979):

$$\bar{\Phi} = 2\pi C_{aa_3} C_c \left\{ \frac{D_{aa_3}}{\ln[(\pi C_c)^{-1/2}/a]} + \frac{D_c}{\ln[(\pi C_{aa_3})^{-1/2}/a]} \right\}$$

where is the frequency of collisions between cytochrome c and cytochrome oxidase, we calculate that for the steady-state electron transfer rate observed under the conditions of the diffusion measurements (TN = 20 e^-/cytochrome aa_3/sec, Φ = 7.6 x 10^{11} e^-/cm^2 sec), the minimal diffusion coefficient that would be compatible with random diffusion mechanism is:

$$D_c(\text{minimum}) = 1.0 - 1.6 \times 10^{-9} \text{ cm}^2/\text{sec}.$$

To make this calculation we assume that the electron transfer reactions occur by random collisions and do not involve the formation of any long-lived complexes (i.e. dissociation rates of cytochrome c from the complexes are very fast) and that every collision between a reduced cytochrome c and cytochrome oxidase will result in an electron transfer event (100% efficiency). These assumptions bias the calculation in the direction of a considerable underestimate of the required diffusion coefficient, giving the slowest possible mobility for cytochrome c that would be compatible with observed rates of electron transfer.

While the calculated diffusion coefficient is a minimal estimate, the experimentally determined coefficient is biased in the direction of a maximal

value, since potential steric hindrances are removed by swelling the mitochondria, and excess cytochrome c was used for the measurements. Native mitochondria have cristae invaginations in which the inner membranes are in close apposition, separated by approximately 150-200 Å (Srere, 1982). Intrinsic proteins extend into this space as much as 70 Å and other proteins appear to be densely packed on the outer and matrix sides of the membrane (Sjorstrand, 1979; Williams, 1983). Removal of these impediments to short and long range movements should give rise to upper limit estimates of mobilities. The use of excess cytochrome c to obtain accurate fluorescent measurements makes it impossible to distinguish the diffusional characteristics of the small amount of specifically bound cytochrome c. Thus, the measured diffusion coefficients do not take into account the potentially limiting rates of dissociation of cytochrome c from its redox partners. This is particularly important when considering the influence of ionic strength on diffusion rates, since binding to the lower affinity, non-specific sites on the membrane is likely to be more strongly affected by ionic strength than binding to the high affinity sites on the redox partners (Ferguson-Miller et al., 1979). Indeed, a preliminary report indicates that when cytochrome c is used in amounts stoichiometric with cytochrome oxidase and the mitochondria are intact and unswollen, cytochrome c diffusion is greatly diminished and is insensitive to ionic strength (Maniara et al., 1984). Thus, even though our calculation is weighted toward a lower limit estimate and our measurements are made under conditions favoring an upper limit value, a comparison of these diffusion coefficients indicates that the mobility of cytochrome c is not sufficient to explain electron transfer by a completely random diffusion mechanism.

While the measured diffusion for cytochrome c appears too low to account for the observed electron transfer, observations indicating independent diffusion

of cytochrome oxidase and cytochrome bc_1 (Hochli et al., 1981; Kawato et al., 1981) as well as the lack of one to one stoichiometry of the electron transfer components (Hatefi and Galante, 1978) indicate that a stable unit electron transfer chain is also not a tenable hypothesis. A model that would reconcile these apparently inconsistent results is shown in Figure 31. According to this dynamic aggregate model an equilibrium exists between freely diffusing, partially aggregated and completely aggregated electron transfer components. Electron transfer can occur by collisions between freely diffusing redox partners, but higher rates are achieved by formation of transitory complexes between redox partners, with lifetimes significantly longer than an electron transfer event.

In the case of cytochrome c, physical association between cytochromes bc_1 and aa_3 could create a limited domain in which cytochrome c movement is restricted, thereby increasing the efficiency of electron conductance. In Paracoccus denitrificans the components of the respiratory chain including cytochrome c appear to be tightly associated, suggesting that even though high rates of electron transfer occur in the system, (Erecinska et al., 1979) the diffusion of cytochrome c may be severely restricted. The observations of Cherry and co-workers (Kawato et al., 1980, 1982) indicate that the respiratory complexes of mammalian mitochondria are to a large extent immobilized on a short time scale suggesting a high degree of aggregation in the native membrane. The authors ascribe this phenomenon to non-specific aggregation, but it seems more reasonable from a physiological standpoint that functional aggregates would predominate.

No evidence of physical associations between cytochrome oxidase and cytochrome bc_1 was obtained in the experiments of Kawato et al. (1981) in which co-reconstitution of the two components into liposomes did not affect

Figure 31. The "dynamic aggregate" model of the mitochondrial respiratory chain. The major electron transfer components are represented diagrammatically in various reversible states of aggregation (aa_3 = cytochrome oxidase; bc_1 = complex III, or cytochrome $\underline{bc_1}$; c = cytochrome $\underline{c}$; Q = coenzyme Q; f1 = complex I or NADH dehydrogenase.) The model assumes that all components are diffusing in or on the membrane, and that aggregates with significant lifetimes are formed by random interaction between correct redox partners.

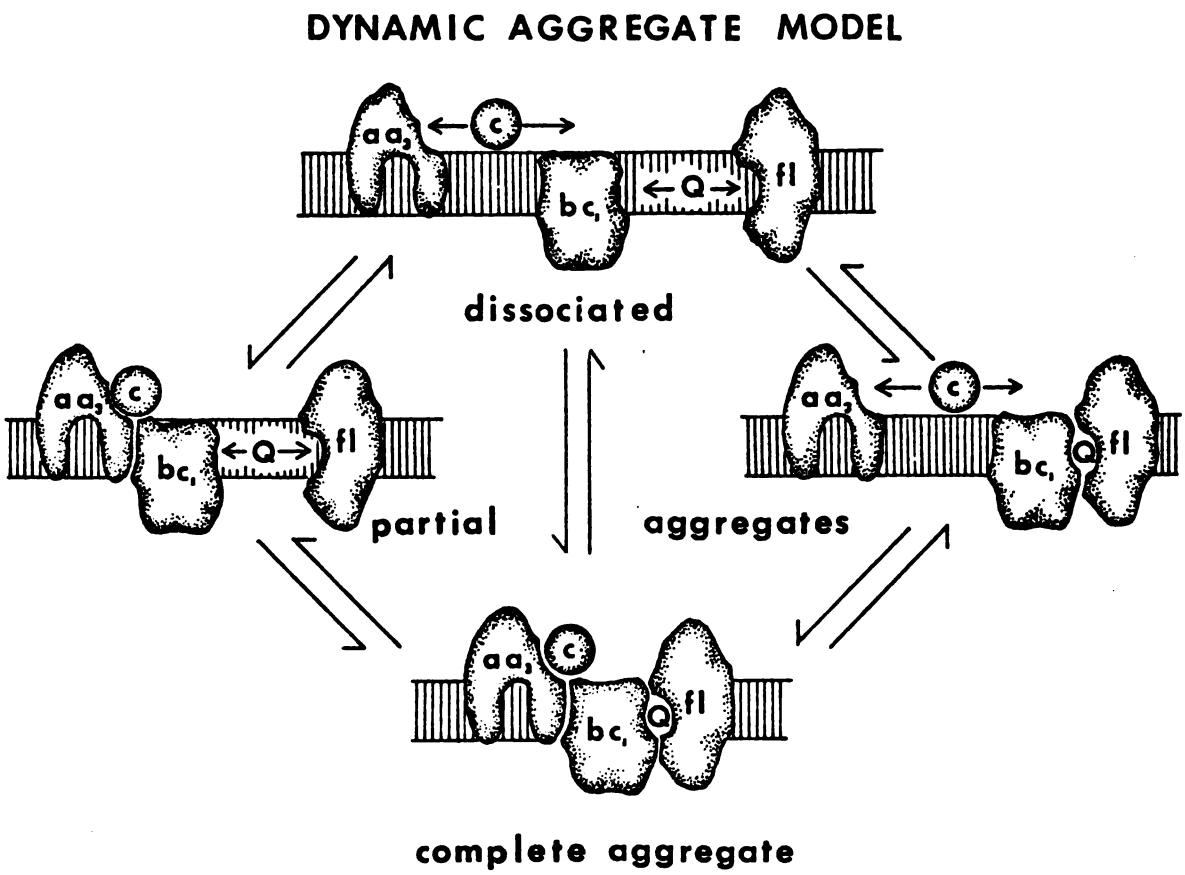

DYNAMIC AGGREGATE MODEL
aa3
c
bc1
Q
f1
dissociated
partial
aggregates
complete aggregate

Figure 31

the rotational diffusion of cytochrome oxidase. However, the lipid environment (Kaprelyants et al., 1984) and the integrity of the purified components may be crucial for complex formation to occur. Furthermore, association of NADPH-cytochrome P-450 reductase with cytochrome P-450 was found not to affect the rotational diffusion of cytochrome P-450 (Gut et al., 1983) indicating that the use of rotational diffusion as a criterion for assessing whether complexes are formed between cytochromes bc_1 and aa_3 may not be valid.

While the efficiency of electron transfer may be increased by formation of specific associations among the complexes, it is clear that the structural features of cytochrome c are also compatible with two dimensional or three dimensional diffusion, and these modes of activity may be physiologically important as well. Indeed, Froud et al., (1984) recently reported that cytochrome c restores ubiquinol oxidase activity in a manner consistent with a mobile pool mechanism in a reconstituted system containing complexes III and IV and excess cytochrome c. While this study demonstrates the feasibility of such a mechanism it does not show that this is the exclusive or even predominant mode of electron transfer by cytochrome c in native mitochondria, where stoichiometric amounts of cytochrome c are sufficient to restore the full activity of the respiratory chain (Nicholls 1974, 1976).

In contrast to cytochrome c, a ten fold excess of ubiquinone over its redox partners is found in the mitochondrial membrane making a pool function for this mediator more likely. Applying the analysis used to determine the minimal diffusion coefficient for cytochrome c, we find that for 1.9×10^{10} monomers of cytochrome bc_1 per cm^2 and 10% of the ubiquinone reduced during steady state electron transfer, (Kroger and Klingenberg 1975), the minimum diffusion coefficient for ubiquinone is 6.4×10^{-10} cm^2/sec. The diffusion coefficient obtained for ubiquinone (8.5×10^{-9} $cm^2/sec.$) is considerably higher than the

minimal value calculated, indicating that ubiquinone can potentially function as a mobile electron carrier. However, some observations indicate that direct interactions between cytochrome bc_1 and complexes I (Ragan and Heron, 1978) and II (Yu et al., 1974) can occur. Therefore consideration of the dynamic aggregate model for this part of the respiratory chain is also warranted.

The dynamic aggregate model not only reconciles the apparently conflicting data concerning the requirements for associations in the electron transfer chain, but can also explain the digitonin-induced artifacts, as well as the cholesterol and lipid incorporation results of Schneider et al. (1980, 1982a,b). In the case of cholesterol or digitonin incorporation, the exclusion of protein from some regions of the membrane results in higher protein concentration in others, thus favoring the formation of the more efficient electron transfer chain aggregates. Similarly, dilution of the membrane components with added lipid shifts the equilibrium to favor dissociation of the aggregates resulting in a decreased electron transfer rate.

A corollary to the proposed model is that factors that affect the lifetime or stability of the aggregated forms may regulate electron transfer activity. To evaluate how this applies to the physiological functioning of mitochondria, it is important to consider the effects of high protein concentrations in the matrix and intermembrane space on the mobilities of membrane bound components (Srere, 1982). It has been proposed that the matrix of mitochondria have very high protein concentration when in the condensed configuration. This dense packing of protein could hinder lateral and rotational mobility of the integral complexes, resulting in reorganization on a time scale much larger than that of an electron transfer event. Association and dissociation of the complexes in this time frame would be compatible with a regulatory function. The studies of Kaback and co-workers (1984) indicate that the state of

aggregation of a bacterial membrane protein is modulated by the membrane potential. In mitochondria it is found that the membrane exhibits a non-ohmnic response to the electrochemical gradient (Nicholls, 1974), suggesting that a change in the membrane structure may occur. In light of the dynamic aggregate model it can be postulated that a high membrane potential will favor a disaggregated state of the components, while at a low potential aggregated complexes would predominate. Thus under a high membrane potential electron transfer would proceed at reduced rates and cytochrome c would be accessible to secondary electron donors (i.e. NADH-cytochrome b_5 reductase) which bypass the major energy conserving steps in the electron transfer chain. In the aggregated states, not only could higher rates of electron transfer be realized, but comformational interactions between the complexes could also yield more efficient energy coupling with higher H^+/e^- ratios.

In summary, the results presented indicate that the rate of diffusion of cytochrome c is not sufficient to account for electron transfer solely by a random diffusion mechanism, suggesting that some degree of association between cytochrome oxidase and cytochrome bc_1 is necessary. In contrast, the diffusion coefficient for ubiquinone is compatible with a diffusion-mediated role for this electron carrier; however the results obtained with digitonin-treated membranes, as well as other lines of evidence suggest that associations between the dehydrogenase complexes and cytochrome bc_1 may also be important. These findings can easily be explained by a model employing an equilibrium between associated and dispersed states of the electron transfer complexes. Considering the potential implications of this model with regard to regulation of energy production in the cell, further research directed toward isolating and characterizing associated complexes is warranted.

LIST OF REFERENCES

Adam, G. and Delbruck, M. (1968) in Structural Chemistry and Molecular Biology (A. Rich and N. Davidson, eds.) Freeman, San Francisco. pp. 198-215.

Adegite, Al and Okpanachi, M.I. (1979) J. Amer. Chem. Soc. 102, 2832-2836.

Ahmed, A., Smith, H.T., Smith, M.B. and Millet, F.S. (1978) Biochemistry 17 2479-2483.

Ames, B. (1966) Methods Enzymol. 8, 115-118.

Anderson, S., Bankier, A.T., Barrell, B.G., De Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R. and Young, I.G. (1981) Nature, 290 457-465.

Anderson, S., De Bruijn, M.H.L., Coulson, A.R., Eperon, I.C., Sanger, F., Young, I.G. (1982) J. Mol. Biol., 156 683-717.

Ashida, T., Takana, N., Yamane, T. and Kaado, M. (1973) J. Biochem. (Tokyo) 73, 463.

Awasthi, Y.C., Chuang, T.F., Keenan, T.W. and Crane, F.L. (1971) Biochim. Biophys. Acta 226, 42-52.

Azzi, A., Bill, K., and Broger, C. (1982) Proc. Natl. Acad. Sci. USA 79, 2447—2450.

Azzone, G.F., Colona, R. and Ziche, B. (1979) Methods in Enzymol., Vol. LV (Fliescher, S. and Packer, E., eds.) Academic Press, New York, pp. 46-50.

Barlow, G.H. and Margoliash, L. (1966) J. Biol. Chem. 241, 1473-1477.

Baum, H., Silman, H.I., Reiske, J.S. and Lipton, S.H. (1967) J. Biol. Chem. 242, 4876-4887.

Beechey, R.B. Raberton, A.M., Holloway, C.T. and Knight, I.G. (1967) Biochemistry 6, 3867-3879.

Bell, R.L., Sweetland, J., Ludwig, B. and Capaldi, R.A. (1979) Proc. Natl. Acad. Sci. USA 76, 741-745.

Berg, H.C. and Purcell, E.M. (1977) Biophys. J. 20, 193-219.

Bernstein, J.D., Bucher, J.R. and Pennial, R. (1978) J. Bioenerg. Biomembr. 10, 59-74.

Bibb, M.J., Van Etten, R.A., Wright, C.T., Walberg, M.W. and Clayton, D.A. (1981) Cell, 26 167-180.

Bill, K., Casey, R., Broger, C., and Azzi, A. (1980) FEBS Lett. 120, 248-250.

Bill, K. Broger, C. and Azzi, A. (1982) Biochim. Biophys. Acta 679, 28-34.

Bisson, R., Azzi, A., Gutweniger, H., Colonna, R., Montecucco, C., and Zanotti, A. (1978) J. Biol. Chem. 253, 1874-1880.

Bisson, R., Gutweniger, H., Montecucco, C., Colona, R., Zanotti, A. and Azzi, A. (1977) FEBS Lett. 81, 147-150.

Bisson, R., Jacobs, B. and Capaldi, R. (1980) Biochemistry 19, 4173-4178.

Bisson, R., Montecucco, C. Gutweniger, H. and Azzi, A. (1979) J. Biol. Chem. 254, 9962-9965.

Blair, P.V., Oda, T., Green, D.E., Fernandez-Moran, H. (1963) Biochemistry 2, 756-764.

Bonitz, S.G., Coruzzi, G., Li, M., Macino, G., Nobrega, M.P., Thalenfeld, B. and Tzagoloff, A. (1980) J. Biol. Chem. 255, 11927-11941.

Bowman C.L. and Tedeschi, H. (1983) Biochim. Biophys. Acta 731, 261-266.

Bowyer, J.R. and Trumpower, B.L. (1981) in Chemiosmotic Proton Circuits in Biological Membranes (V.P. Skulachev and P.C. Hinkle, eds.) Addison-Wesley Publishing Co., Massachusetts, pp. 105-122.

Brautigan, D.L., Ferguson-Miller, S., Margoliash, E. (1978a) J. Biol. Chem. 253, 130-139.

Brautigan, D.L., Ferguson-Miller, S., Tarr, G. and Margoliash, E. (1978b) J. Biol. Chem. 253, 140-149.

Brautigan, D.L., Ferguson-Miller, S., and Margoliash, E. (1978c) Methods Enzymol. 53, 128-181.

Briggs, M.M. and Capaldi, R.A. (1977) Biochemistry 16, 73-77.

Briggs, M.M. and Capaldi, R.A. (1978) Biochem. Biophys. Res. Commun. 80, 553-559.

Briggs, M., Kamp, P.F., Robinson, N.C., Capaldi, R.A. (1975) Biochemistry 14, 5123-5128.

Bygrave, F.L. (1978a) Biochem. J. 170, 87-91.

Bygrave, F.L. (1978b) Biochem. J. 174, 1021-1030.

Capaldi, R.A., Bell, R.L. and Branchek, T. (1977) Biochem. Biophys. Res. Commun. 74, 425-433.

Caroni, P., Schwerzmann, K., Carafoli, E. (1978) FEBS Lett. 96, 339-342.

Carrondo, M.A.A.F. de C.T., Griffith, W.P., Hall, J.P. and Skapski, A.C. (1979) Biochim. Biophys. Acta 627, 332-334.

Casey, R., Broger, C. and Azzi, A. (1981) Biochim. Biophys. Acta 638, 86-93.

Casey, R.P., Chappell, J.B., and Azzi, A. (1979) Biochem. J. 182, 149-156.

Casey, R.P., Thelen, M. and Azzi, A. (1980) J. Biol. Chem. 255, 3994-4000.

Casey, R.P., Thelen, M. and Azzi, A., (1979) Biochem. Biophys. Res. Commun. 87, 1044-1051.

Cerfetti, N. and Schatz, G. (1979) J. Biol. Chem. 254, 7746-7751.

Chan, S.H.P. and Tracy, R.P. (1978) Eur. J. Biochem. 89, 595-605.

Chance, B. (1972) in Biochemistry and Biophysics of Mitochondrial Membranes (Azzone, G.F., Carafoli, E., Lehninger, A.L., Quagliariella, E. and Siliprandi, N., eds.) Academic Press, New York, pp. 183-206.

Chance, B. (1974) Ann. N.Y. Acad. Sci. USA 78, 6246-6250.

Colbeau, A., Nachbaur, J. and Vignais, P.M. (1971) Biochim. Biophys. Acta 249, 462-492.

Crofts, A.R. and Wraight, C.A. (1983) Biochim. Biophys. Acta 726, 149-185.

Cuatrecasas, P., Wilchek, M. and Anfinsen, C. (1968) Proc. Natl. Acad. Sci. USA 61, 636-643.

Das Gupta, U. and Rieske, J.S. (1973) Biochem. Biophys. Res. Commun. 70, 73-80.

Deathridge, J.F., Henderson, R., Capaldi, R.A. (1982) J. Mol. Biol. 158 487-499.

Dickerson, R.E., Takano, T., Eisenberg, D., Kallai, O.B., Samson, L., Cooper, A. and Margoliash, E. (1971) J. Biol. Chem. 246, 1151.

Dickerson, R.E., Takano, J., Kallai, O.B. and Samson, L. (1972) in Structure and Function of Oxidation Reduction Enzyme (A. Akenson and A. Ehrenberg, eds.) Pergamon Press, Oxford, pp. 69-83.

Dixit, B.P.S.N., Waring, A.J., Wells III, K.O., Wong, P.S., Woodrow, III, G.V. and Vanderkooi, J.M. (1982) Eur. J. Biochem. 126, 1-9.

Downer, N.W., Robinson, N.C. and Capaldi, R.A. (1976) Biochemistry 15, 2930-2936.

Easley, C.W. (1965) Biochim. Biophys. Acta 107, 386-388.

Edwards, C.A. and Bowyer, J.R. (1981) in Function of Quinones in Energy Conserving Systems (Trumpower, B.C. ed.) Academic Press, New York.

Erecinska, M. (1977) Biochem. Biophys. Res. Commun. 76, 495-501.

Erecinska, M. Davis, J.S. and Wilson, D. (1979) Arch. Biochem. Biophys. 197, 463-469.

Erecinska, M., Vanderkooi, J., and Wilson, D. (1975) Arch. Biochem. Biophys. 171, 108-116.

Errede, B. and Kamen, M.D. (1978) Biochemistry 17, 1015-1027.

Ewall, R.X., and Bennett, L.E. (1974), J. Amer. Chem. Soc. 96, 940-942.

Eytan, G.D. and Broza (1978) J. Biol. Chem. 253, 3196-3202.

Eytan, G.D., Carroll, R.C., Schatz, G. and Racker, E. (1975) J. Biol. Chem. 250, 8598-8603.

Felgner, P.L., Messer J.L., and Wilson, J.E. (1979) J. Biol. Chem. 254, 4946-4949.

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

Ferguson-Miller, S., Brautigan, D.L., and Margoliash, E (1978) J. Biol. Chem. 253, 150-159.

Ferguson-Miller, S., Brautigan, D.L., and Margoliash, E. (1979a) in The Porphyrins, Vol. VII, (Dolphin, D., eds.) pp. 149-240, Academic Press, New York.

Ferguson-Miller, S., Van Aken, T., and Rosevear, P. (1982) in Interaction Between Iron and Protein in Oxygen and Electron Transport (ed. C. Ho) Elsvier/North Holland Press, Amsterdam, The Netherlands, pp. 297-303.

Ferguson-Miller, S., Weiss, H., Speck S.H. and Brautigan, D.L., Osheroff, N. and Margoliash, E. (1979b) in Cytochrome Oxidase, (eds. King, T.E., Orii, Y., Chance, B., and Okuniski, K.) (Elsevier North Holland, New York), pp. 281-292.

Fleischer, S., Klouwen, H. Brierley, G. (1961) J. Biol. Chem. 236, 2936-2941.

Fletcher, J.M., Greenfield, B.F., Hardy, C.J., Scargile, D. and Woodhead, J.L. (1961) J. Chem. Soc., (London), 2000-2006.

Fowler, L.R., Richardson, S.H., and Hatefi, Y. (1962) Biochim. Biophys. Acta 64, 170-173.

Freidkin, M. and Lehninger, A.L. (1948) J. Biol. Chem. 174, 757.

Freidmann, J.M., Rousseau, D.L., Navon, G., Rosenfeld, S., Glynn, P. and Lyons, K.B. (1979) Arch. Biochem. Biophys. 193, 14-21.

Frey, T.G., Chan, S.H.P., and Schatz, G. (1978) J. Biol. Chem. 253, 4389-4395.

Froud, R.J. and Ragan, C.I. (1984) Biochem. J. 217, 551-560.

Frye, L.D. and Edidin, M. (1970) J. Cell. Sci., 7, 319-335.

Fuller, S.D., Capaldi, R.A., and Henderson, R. (1979) J. Mol. Biol. 134, 305-327.

Futami, A., Hurt, E., and Hauska, G. (1979) Biochim. Biophys. Acta 547, 583-596.

Gellerfors, P., Johansson, T. and Nelson, B.D. (1981) Eur. J. Biochem. 115, 275-278.

Gellerfors, P., Luden, M. and Nelson, B.D. (1976) Eur. J. Biochem. 67, 463-468.

Gellerfors, P. and Nelson, B.D. (1975) Eur. J. Biochem. 52, 433-443.

Georgevich, G. and Capaldi, R.A. (1982) Biophys. J. 37, 66-67.

Georgevich, G., Darley-Usmor, V.M., Malalesta, F. and Capaldi, R.A. (1983) Biochemistry 22, 1317-1322.

Goldkorn, T., Rimon, G. Kempner, E.S., Kaback, H.R., Proc. Natl. Acad. Sci. USA 81, in press.

Goldstein, B., Wofsy, C., Bell, G. (1981) Proc. Natl. Acad. Sci. 78, 5695-5698.

Green, D.E. (1962) Comp. Biochem. Physiol. 4, 81-122.

Grosskopf, R. and Feldmann, H. (1981) Curr. Genet. 4, 151-158.

Gu, L.Q., Yu, L., and Yu, C.A. (1983) Fed. Proc. 42, 1942 pos. 1081.

Gut, J., Richter, C., Cherry, R.J., Winterhalter, K.H. and Kawato, S. (1983) J. Biol. Chem. 258, 8588-8594.

Hackenbrock, C.R. (1981) Trends Biochem. Sci. 6, 151-154.

Hackenbrock, C.R. and Hammon, K.M. (1975) J. Biol. Chem. 250, 9185-9197.

Hackenbrock, C.R., Hochli, M., and Chau, R.M. (1976) Biochim. Biophys. Acta 455, 466-484.

Hanukoglu, I. and Jefcoate, C.R. (1980) J. Biol. Chem. 255, 3057-3061.

Hare, J.F., Ching, E., and Attardi, G. (1980) Biochemistry 19, 2023-2030.

Hardt, S.L. (1979) Biophysical Chemistry 10, 239-243.

Hatefi, Y. (1966) in Comprehensive Biochemistry (Florkin, M. and Stotz, E., eds.) Vol. 14, Elsevier, New York, pp. 199-231.

Hatefi, Y., Haavik, A.G., and Griffith, D.E. (1962) J. Biol. Chem. 237, 1681-1685.

Hauska, G. (1977a) FEBS Lett. 79, 345-347.

Hauska, G. (1977b) in Bioenergetics of Membranes (Packer, L., Papageorgiou, G.C. and Trebst, A., eds.) Elsevier/North-Holland, Amsterdam, pp. 189-198.

Haworth, P., Kyle, D.J., Horton, P. and Arntzen, C.J. (1982) Photochem. Photobiol. 36, 743-748.

Haworth, R.A., Hunter, D.R., and Berkoff, H.A. (1980), FEBS Lett. 110, 216-218.

Henderson, R., Capaldi, R.A., and Leigh, J.S. (1977) J. Mol. Biol. 134, 305-327.

Heron, C., Smith, S., and Ragan, C.I. (1979) Biochem. J. 181, 435-443.

Hinkle, P. (1973) Fed. Proc. 32, 1988-1992.

Hinkle, P.C., Kim, J.J. and Racker, E. (1972) J. Biol. Chem. 247, 1338-1342.

Hochli, M. and Hackenbrock, C.R. (1976) Proc. Natl. Acad. Sci. USA, 73, 1636-1640.

Hochli, M. and Hackenbrock, C.R. (1977) J. Cell Biol. 72, 278-291.

Hochli, M. and Hackenbrock, C.R. (1978) Proc. Natl. Acad. Sci. USA 76, 1236-1240.

Hovmoller, S., Leonard, K., and Weiss, H. (1981) FEBS Lett. 123, 118-122.

Jagendorf, A.T. and Uribe, E. (1966), Proc. Natl. Acad. Sci. USA 55, 170-177.

Johnson, D., and Lardy, H.A. (1967) Methods Enzymol. 10, 94-96.

Kadenbach, B., Jarausch, J. Hartmann, R. and Merle, P. (1983) Anal. Biochem. 129, 517-521.

Kadenbach, B., and Merle, P. (1981) FEBS Lett. 135, 1-11.

Kagawa, Y. (1967) Biochim. Biophys. Acta 131, 586-588.

Kaprelyants, A.S., Dergunov, A.D. and Ostrovky, D.N. (1983) Biokhimiya 48, 2049-2055.

Katan, M.B., Poal, L., and Groot, G.S.P. (1976) Eur. J. Biochem. 65, 95-105.

Katsikas, H., and Quinn, P.J. (1982) Eur. J. Biochem. 124, 165-169.

Kawato, S., Lehner, C., Muller, M. and Cherry, R.J. (1982) J. Biol. Chem. 257, 6470-6476.

Kawato, S., Sigel, E., Carafoli, E., and Cherry, R.J. (1980) J. Biol. Chem. 255, 5508-5510.

Kawato, S., Sigel, E., Carafoli, E., Cherry, R. (1981) J. Biol. Chem. 256, 7518-7527.

Keilin, D. (1966) The History of Cell Respiration, Cambridge University Press, Cambridge.

Keilin, D. (1925) Proc. Roy. Soc., B 98, 312-339.

Keilin, D. and Hartree, E.F. (1945) Biochem. J. 39, 289-292.

King, T.E. (1967) Methods Enzymol. 10, 202-208.

King, T.E. (1981) in Chemiosmotic Proton Circuits in Biological Membranes (V.P. Skulachev and P.C. Hinkle eds.) Addison-Wesley Publishing, Co., Massachusetts, pp. 147-159.

Kingsley, P.B. and Feigenson, G.W. (1981) Biochim. Biophys. Acta 635, 602-618.

Kohn, J. and Wilcheck, M. (1982) Biochem.. Biophys. Res. Commun. 107, 878-884.

Koppel, D.E., Scheetz, M.P. and Schindler, M. (1980) Biophys. J. 30, 187-192.

Konig, B.W., Osheroff, N., Wilms, J., Muipers, A.O., Dekker, H.L., and Margoliash, E. (1980) FEBS Lett. 111, 395-398.

Koppenol, W.H. and Margoliash, E. (1982) J. Biol. Chem. 257, 4426-4437.

Koppel, D.E. (1979) Biophys. J. 28, 281-292.

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

Krebs, J.J.R., Hauser, H. and Carafoli, E. (1979) J. Biol. Chem. 254, 5308-5316.

Kroger, A. and Klingenberg, M. (1973a) Eur. J. Biochem. 34, 358-368.

Kroger, A. and Klingenberg, M. (1973b) Eur. J. Biochem. 39, 313-323.

Kuboyama, M., Yong, F.C. and King, T.E. (1972) J. Biol. Chem. 247, 6375-6383.

Lehninger, A.L. (1955) Harvey Lect. 49, 176-215.

Lehninger, A.L. (1959) Rev. Mod. Phys. 31, 136-146.

Leigh, J.S., Jr., and Erecinska, M. (1975) Biochim. Biophys. Acta 387, 95-106.

Li, Y., Leonard, K. and Weiss, H. (1981) Eur. J. Biochem. 116, 199-205.

Leebman, P.A., Pugh, Jr., E.N. (1979) Vision Research 19, 375-380.

Lin, L.F.H. and Beattie, D.S. (1978) J. Biol. Chem. 253, 2412-2418.

Lipmann, F. (1946) in Currents in Biochemical Research. D.E. Green, editor. Wiley-Interscience, New York, 137-148.

Lipmann, F. (1941) Adv. Enzymol. Relat. Areas. Mol. Biol. 1, 99.

Ludwig, B. (1981) Biochim. Biophys. Acta 594, 177-189.

Ludwig, B., Downer, N.W., and Capaldi, R.A. (1979) Biochemistry 18, 1401-1407.

Ludwig, B., and Schatz, G. (1980) Proc. Natl. Acad. Sci. 77, 196-200.

Luft, J.H. (1971) Anat. Rec. 171, 347-368.

MacLennan, D.H., and Tzagoloff, A. (1965) Biochim. Biophys. Acta 96, 166-168.

McCarty, R.E. and Racker, E. (1967) J. Biol. Chem. 242, 3435-3439.

McCloskey, M. and Poo, M. (1984), Inter. Rev. of Cytol. 87, 19-81.

Madden, T.D., Vigo, C., Bruckdorfer, K.R. and Chapman, D. (1980) Biochem. Biophys. Acta 599, 528-537.

Maloff, B.L., Scordilis, S.P., Reynolds, C. and Tedeschi, H. (1978) J. Cell. Biol. 78, 199-213.

Maniara, G., Vanderkooi, J.M., and Erecinska, M. (1984) Biophys. J. 45, 90a.

Margoliash, E. and Bosshard, H.R. (1983) Trends Biochem. Sci. 8, 316-320.

Marres, C.A.M., and Slater, E.C. (1977) Biochim. Biophys. Acta 462, 531-548.

Mason, T.L., and Schatz, G. (1973) J. Biol. Chem. 248, 1355-1360.

Mendel-Hartvig, I., and Nelson, B.D. (1978) FEBS Lett. 92, 36-40.

Mendel-Hartvig, I., and Nelson, B.D. (1981) Biochim. Biophys. Acta 636, 91-97.

Merle, P. and Kadenbach, B. (1980) Eur. J. Biochem. 105, 499-507.

Merle, P. and Kadenbach, B. (1982) Eur. J. Biochem. 125, 239-244.

Merle, P., Jarausch, J., Trapp, M., Scherka, R., and Kadenbach, B. (1981), Biochim. Biophys. Acta 669, 222-230.

Meyer, T.J. and Taube, H. (1968) Inorg. Chem. 7, 2369-2379.

Millett, F., Darley-Usmar, V., and Capaldi, R.A. (1982) Biochemistry 21, 3857-3862.

Millett, F., deJong, C., Paulson, L., and Capaldi, R.A. (1983) Biochemistry 22, 546-552.

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

Mitchell, P. (1966) Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation, Glynn Research, Bodmin.

Mitchell, P. (1976) J. Theor. Biol. 62, 327-367.

Moore, C.L. (1971) Biochem. Biophys. Res. Commun. 42, 298-305.

Moroney, P.M., Scholes, T.A. and Hinkle, P.C. (1984) Biochemistry, in press.

Morton, R.A. (1965) in Biochemistry of Quinones (Morton, R.A., ed.), Academic Press, New York, pp. 1-21.

Moyle, J., Mitchell, P. (1978) FEBS Lett. 88, 268-272.

Nagoaka, S., Yu, L., and King, T.E. (1981) Arch. Biochem. Biophys. 208, 334-343.

Nalecz, K., Balli, R., and Azzi, A. (1983) Biochem. Biophys. Res. Commun. 114, 822-828.

Nalecz, M.J. and Azzi, A. (1984) Biophys. J. 45, 297a.

Nelson, B.D. (1981) in Mitochondria and Microsomes (C.P. Lee, G. Schatz, G. Daliner, eds.) Addison-Wesley Publishing Co., pp. 217-247.

Nicholls, D.G. (1974) Eur. J. Biochem. 132, 417-431.

Nicholls, P. (1965) in Oxidases and Related Redox Systems (eds. King, T.E., Mason, H.S., and Morrison, M.) Vol. 2, pp. 764-783, John Wiley and Sons, N.Y.

Nicholls, P. (1964) Arch. Biochem. Biophys. 106, 25-48.

Nicholls, P. (1974) Biochim. Biophys. Acta 346, 261-310.

Nicholls, P. (1975) in Electron Transfer Chains and Oxidative Phosphorylation (ed. Quagliariello, et al.) North Holland Publishing Co., Amsterdam, pp. 227-232.

Nicholls, P. (1976) Biochim. Biophys. Acta 430, 30-45.

Nobrega, F.G. and Tzagaloff, A. (1980) J. Biol. Chem. 255, 9828-9837.

Ochoa, S. (1940) Nature 146 267.

Ohnishi, T. (1981) in Mitochondria and Microsomes (Lee, C.P., Schatz, G. and Dallner, G., eds.) Addison-Wesley Publishing Co., Massachusetts, pp. 191-216.

Osheroff, N., Brautigen, D.L. and Margoliash, E. (1980) J. Biol. Chem. 255, 8245-8251.

Overfield, R.E., and Wraight, C.A. (1980) Biochemistry 19, 3328-3334.

Papa, S., Capuano, F., Markert, M., and Altamura, N. (1980), FEBS Lett. 111, 243-248.

Parsons, J. and Patton, S. (1967) J. of Lipid Res. 8, 696-698.

Parsons, D.F., Williams, G.R., and Chance, B. (1966) Ann. N.Y. Acad. Sci. 137, 643.

Pennock, J.F. (1965) in Biochemistry of Quinones, (Morton R.A. ed.) Academic Press Inc., pp. 80-87.

Pentilla, T., Saraste, M., and Wikstrom, M. (1979) FEBS Lett. 101, 295-300.

Pentilla, T., and Wikstrom, M. (1981) in Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (eds. F. Palmieri, et al.), pp. 71-80, Elsevier/North Holland Biomedical Press, Amsterdam.

Perkins, S.J., and Weiss, H. (1983) J. Mol. Biol. 168, 847-866.

Poyton, R.O., and Schatz, G. (1975) J. Biol. Chem. 250, 762-766.

Prochaska, L., Bisson, R., Capaldi, R.A. (1980) Biochemistry 19, 3174-3179.

Prochaska, L., Steffins, G.C.M., Buse, G.M., Bisson, R., and Capaldi, R.A. (1981) Biochim. Biophys. Acta 637, 360-373.

Quinn, P.J. and Esfahani, A. (1980) Biochem. J. 185, 715-722.

Ragan, C.I., and Heron, C. (1978) Biochem. J. 174, 783-790.

Ragan, C.I., Smith, S., Early, F.G.P., and Poore, V.M. (1981) in Chemiosmotic Proton Circuits in Biological Membranes, (Skulachev, V.P. and Hinkle, P.C., eds.) Addison-Wesley Publishing Co., Inc., pp. 59-68.

Rascati, R.J. and Parsons, P. (1979) J. Biol. Chem. 254, 1594-1599.

Redfearn, E.R., and Whittaker, P.A. (1962) Biochim. Biophys. Acta 56, 440-444.

Rich, P.R. (1982) Biochem. Soc. Trans. 10, 482-484.

Rich, P.R. (1984) Biochim. Biophys. Acta 768 53-79.

Rieder, R., and Bosshard, H.R. (1978a) J. Biol. Chem. 253, 6045-6053.

Rieder, R., and Bosshard, H.R. (1978b) FEBS Lett. 92, 223-226.

Rieder, R., and Bosshard, H.R. (1980) J. Biol. Chem. 255, 4732-4739.

Rieske, J.S., Zaugg, W.S. and Hansen, R.E. (1964a) J. Biol. Chem. 239, 3017-3022.

Rieske, J.S., Zaugg, W.S. and Hansen, R.E. (1964b) J. Biol. Chem. 239, 3023-3030.

Rieske, J.S., Zaugg, W.S. and Coleman, R. (1964c) Biochem. Biophys. Res. Commun. 15, 338-344.

Roberts, H., and Hess, B. (1977) Biochim. Biophys. Acta 462, 215-234.

Robinson, N.C. and Capaldi, R.A. (1977) Biochemistry 16, 375-381.

Robinson, N.C., and Talbert, L. (1980) Biochem. Biophys. Res. Commun. 95, 90-96.

Rosevear, P., VanAken, T., Baxter, J., and Ferguson-Miller, S. (1980) Biochemistry 19, 4108-4115.

Rubin, M.S. and Tzagoloff, A. (1973) J. Biol. Chem. 248, 4275-4279.

Saraste, M., Pentilla, T., Wikstrom, M. (1981) Eur. J. Biochem. 115, 261-268.

Schnaitman, C., and Greenawalt, J.W. (1968) J. Cell. Biol. 38, 1968.

Schneider, H., Hochli, M., and Hackenbrock, C.R. (1982a) J. Cell. Biol. 94, 387-393.

Schneider, H., Lemasters, J.J., Hochli, M., and Hackenbrock, C.R., (1980) J. Biol. Chem. 255, 3748-3756.

Schneider, H., Lemasters, J.J., and Hackenbrock, C.R., (1982b) J. Biol. Chem. 257, 10789-10793.

Schwerzmann, K., Gazzotti, P., and Carafoli, E. (1976) Biochem. Biophys. Res. Commun. 69, 812-815.

Scott, R.A., and Gray, H.B. (1980) J. Amer. Chem. Soc. 102, 3219-3224.

Seki, S., Hayachi, H., and Oda, T. (1970) Arch. Biochem. Biophys. 138, 110-121.

Siedow, J.N., Power, S., De La Rosa, F.F., and Palmer, G. (1978) J. Biol. Chem. 253, 2392-2399.

Sigel, E., and Carafoli, E. (1980) Eur. J. Biochem. 111, 299-306.

Singer, T.P., Ramsey, R.R. and Paech, C. (1981) in Mitochondria and Microsomes (Lee, C.P., Schatz, G., and Dallner, G., eds.) pp. 155-190.

Sjostrand, F.S. (1979) J. Ultrastruct. Res. 64, 217-245.

Slater, E.C. (1953) Nature 175: 975-978.

Slater, E.C. (1981) in Chemiosmotic Proton Circuits in Biological Membranes (V.P. Skulachev and P.C. Hinkle, eds.) Addison-Wesley Publishing Co., Reading, MA., pp. 69-104.

Smith, M.B., Stonehuerner, F., Ahmed, A.J., Staudenmayer, N., and Millet, J. (1980) Biochim. Biophys. Acta 592, 303-313.

Smith, H.T., Staudenmayer, N. and Millet, F. (1977) Biochemistry 16, 4971-4974.

Snell, J.M. and Wiessberger, A. (1939) J. Am. Chem. Soc. 61, 450-453.

Sone, N. (1981) in Chemiosmotic Proton Circuits in Biological Membrances (Skulachev, V.P. and Hinkle, P.C., eds.) Addison-Wesley Publishing Co., Reading, MA. pp. 197-208.

Sottocasa, G.L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. (1967a) J. Cell. Biol. 32, 415.

Sottocasa, G.L., Kuylenstierna, B., Ernster, L., Bergstrand, A. (1967b) Methods Enzymol. 10, 448-463.

Sowers, A.E. and Hackenbrock, C.R. (1981) Proc. Natl. Acad. Sci. USA 78, 6246-6250.

Speck, S.H., Ferguson-Miller, S., Osheroff, N., and Margoliash, E. (1979) Proc. Natl. Acad. Sci. USA 76, 155-159.

Speck, S.H., Hoppenol, W.H., Dethmer, J.K., Osheroff, N., Margoliash, E., and Rajagopalan, K.U. (1981) J. Biol. Chem. 256, 7394-7400.

Srere, P.A. (1982) Trends in Biochem. Sci. 82, 375-378.

Stynes, H.C. and Ibers, J.A. (1971) Inorg. Chem. 10, 2304-2308.

Suarez, M.D., Revzin, A., Narlock, R., Kempner, E.S., Thompson, D.A. and Ferguson-Miller, S. (1984a) J. Biol. Chem., submitted.

Sutin, N. (1979) in Tunneling in Biological Systems (Chance, B., DeVault, D.C., Frauenfelder, H., Marcus, R.A., Schrieffer, J.R., Sutin, N., eds.) pp. 201-227, Academic Press, New York.

Swanson, M., Speck, S.H., Koppenol, W.H., and Margoliash, E. (1982) in Electron Transport and Oxygen Utilization (Chien Ho, ed.) Elsevier/North Holland, Amsterdam, pp. 51-56.

Swanson, R., Trus, B.L., Mandel, N., Mandel, G. Kallai, O.B., and Dickerson, R.E. (1977) J. Biol. Chem. 252, 759.

Takano, T., Swanson, R., Kallai, O.B., and Dickerson, R.E. (1973) J. Biol. Chem. 248, 5234.

Tanaka, N., Yamane, T., Tsukihara, T., Ashida, T., and Kakudo, M. (1975) J. Biochem. (Tokyo) 77, 147.

Thompson, D.A. (1984) Ph.D. Thesis.

Thompson, D.A. and Ferguson-Miller, S. (1983) Biochemistry 22, 3178-3187.

Thompson, D.A., Suarez-Villafane, M., and Ferguson-Miller, S. (1982) Biophys. J. 37, 285-292.

Torusso, M., Capuano, F., Boffoli, D., Stefanelli, R. and Papa, S. (1979) Biochem. J. 182, 133-147.

Trumpower, B.L. (1976) Biochem. Biophys. Res. Commun. 70, 73-80.

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

Trumpower, B.L. (1981b) J. Bioenerg. Biomembr. 13, 1-23.

Trumpower, B.L. and Edwards, C.A. (1979) J. Biol. Chem. 254, 8697-8706.

Trumpower, B.L., Edwards, C.A. and Ohnishi, T. (1980) J. Biol. Chem. 255, 7487-7493.

Trumpower, B.L. and Katki, A. (1975) Biochemistry 14, 3635-3642.

Trumpower, B.L. and Katki, A.G. (1979) in Membrane Proteins in Energy Transduction (Capaldi, R.A., ed.), pp. 89-200, Academic Press, New York.

Tsukida, K. (1972) Vitamins (Japan) 46, 1-18.

Tucker, D.F., Begent, R.H.J., and Hogg, N.M. (1978) J. Immunol. 121, 1644-1651.

Utsumi, K., and Packer, L. (1967) Arch. Biochem. Biophys. 121, 633-640.

Vail, W.J. and Riley, R.K. (1974) FEBS Lett. 40, 269-273.

Vanderkooi, G., Senior, A.E., Capaldi, R.A. and Hayashi, H. (1972) Biochim. Biophys. Acta 274, 38-48.

Verheul, F.E.A.M., Boonman, J.C.P., Drayer, J.W., Muijsers, A.O., Borden, D., Jau, G.E. and Margoliash, E. (1979) Biochim. Biophys. Acta 548, 397-416.

Vasington, F.D., Gazotti, P., Tiozzo, R., and Carafoli, E. (1971) Biochim. Biophys. Acta 256, 43-54.

Vinogradov, A.D. and King, T.E. (1979) Methods in Enzymol. Vol. LV (Fliescher, S., and Packer, L., eds.) Academic Press, New York, pp. 118-127.

von Jagow, G., Schagger, H., Riccio, P., Klingenberg, M., and Kolb, H.J. (1977) Biochim. Biophys. Acta 462, 549-558.

Wakabayashi, S., Matsubara, H., Kim, C.H., Kawai, K., and King, T. E. (1980) Biochem. Biophys. Res. Commun. 97, 1548-1554.

Wakabayashi, T., Senior, A.E., Hatase, O., Hayashi, H. and Green, D.E. (1972) Bioenergetics 3, 339-344.

Waring, A., Davis, J.S., Chance, B., and Erecinski, M. (1980) J. Biol. Chem. 255, 6212-6218.

Wei, Y., and King, T.E. (1981) J. Biol. Chem. 256, 10999-11003.

Weiss, H. (1976) Biochim. Biophys. Acta 456, 291-313.

Weiss, H., and Jucks, B. (1978) Eur. J. Biochem. 88, 17-28.

Weiss, H., Jucks, B., and E. Ziganke, B. (1978) Methods in Enzymology 53, 98-112.

Weiss, H., and Kolb, H.J. (1979) Eur. J. Biochem. 99, 139-149.

Weiss, H., and Kolb, H.J. (1979) Eur. J. Biochem. 99, 139-149.

Weiss, H., Wingfield, P. and Leonard, K. (1979) in Membrane Bioenergetics (Lee, C.P., Schatz, G. and Ernster, L., eds.) Addison-Wesley Publishing Co., pp. 119-132.

Weiss, H. and Ziganke, B. (1974) Eur. J. Biochem. 41, 63-71.

Wikstrom, M. (1981) in Mitochondria and Microsomes (eds. C.P. Lee, G. Schatz, and G. Dallner) Addision-Wesley Publishing Co., Reading, MA. pp. 249-269.

Wikstrom, M. and Krab, K. (1980) Curr. Jap. Bioenerg. 10 51-101.

Wikstrom, M., Krab, K., Saraste, M. (1981) Cytochrome Oxidase a Synthesis, Academic Press, London.

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

Wikstrom, M.K.F. (1977) Nature 266, 271-273.

Williams, R.J.P. (1983) Trends Biochem. Sci. 8, 48.

Wingfield, P., Arad, T., Leonard, K., and Weiss, H. (1979) Nature 280, 696-697.

Winter, D.B., Bruynincky, W.J., Faulke, F.G., Grinch, N.P., and Mason, H.S. (1980) J. Biol. Chem. 255, 11408-11414.

Wraight, C.A. (1979) Photochem. Photobiol. 30, 767-796.

Yatscoff, R.W., Freeman, K.B., and Vail, W.J. (1977) FEBS Lett. 81, 7-9.

Yee, R., Liebman, P.A. (1978) J. Biol. Chem. 253, 8902-8909.

Yonean, T. (1961) J. Biol. Chem. 236, 1680-1686.

Yu., C.A., and King, T.E. (1977) Biochem. Biophys. Res. Commun. 78, 259-265.

Yu, C.A., Nagoaka, S., Yu, L. and King, T.E. (1978) Biochem. Biophys. Res. Commun. 82, 1070-1078.

Yu, C.A., Nagoaka, S., Yu, L., and King, T.E. (1980) Arch. Biochem. Biophys. 204, 59-70.

Yu, C.A. and Yu, L. (1980) Biochem. Biophys. Res. Commun. 96, 286-292.

Yu, C.A., Yu, L., and King, T.E. (1972) J. Biol. Chem. 250, 1012-1019.

Yu, C.A., Yu, L. and King, T.E. (1974) J. Biol. Chem. 249, 4905-4910.

Yu, C., Yu, L., and King, T.E. (1975) Biochem. Biophys. Res. Commun. 66, 1194-1200.

Yu, C.A., Yu, L., and King, T.E. (1977a) Biochem. Biophys. Res. Commun. 74, 670-676.

Yu, L., Yu, C.A., and King, T.E. (1977b) Biochim. Biophys. Acta 495, 232-247.

Yu, C.A., Yu, L., and King, T.E. (1977c) Biochem. Biophys. Res. Commun. 78, 259-265.

APPENDIX

PUBLICATIONS AND ABSTRACTS

Some of the results presented in this dissertation were obtained through collaborations with other workers and have previously been reported in the publications listed below:

PUBLICATIONS:

Hochman, J.H., Partridge, B., and Ferguson-Miller, S. (1981) An Effective Electron Donor to Cytochrome Oxidase: Purification, identification, and kinetic characterization of a contaminant of ruthenium red, hexaammineruthenium II/III. J. Biol. Chem. 256, 8693-8698.

Hochman, J.H., Schindler, M., Lee, J.G., and Ferguson-Miller, S. (1982) Lateral Mobility of Cytochrome c on Intact Mitochondrial Membranes as Determined by Fluorescence Redistribution after Photobleaching. Proc. Natl. Acad. Sci. USA. 79, 6866-6870.

Hochman, J.H., Schindler, M., Lee, J.G., and Ferguson-Miller, S. (1983) Dynamics of Electron Transfer in Mitochondrial Membranes. In Biochemistry of Metabolic Processes (Ed. F.W. Stratman, D.L.F. Lennon, and R.N. Zalten), Elsevier, N. Holland Press, NY, pp. 441-450.

Hochman, J.H., Ferguson-Miller, S. and Schindler, M. (1984) Mobility in the Mitochondrial Electron Transport Chain. Biochemistry, in press.

ABSTRACTS:

Hochman, J.H., Partridge, B., and Ferguson-Miller, S. (1980) A Contaminant of Ruthenium Red with Electron-Mediating Activity in Mitochondria. Fed. Proc. 39, 2005.

Hochman, J.H., Schindler, M., Lee, J.G., Matia, J., and Ferguson-Miller S. (1982) Mobility of Cytochrome c on Mitochondrial Membranes Measured by Fluorescence Recovery after Photobleaching (FRAP). Biophys. J. 37, Abstract No. W-AM P 133.

Hochman, J.H., Ferguson-Miller, S., Foxall, S., and Schindler, M. (1983) Lateral Mobility of Mitochondrial Membrane Components: Implications for the Mechanism of Electron Transport. Biophys. J. 41, Abstract No. M-AM-A10.

Hochman, J.H., Schindler, M., Ferguson-Miller, S. (1983) Relationship of Lateral Diffusion to Electron Transfer in Mitochondrial Membranes. Fed. Proc. 40, 1776.