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Major professor

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THE LOCATION AND MOBILITY OF FLUORESCENTLY LABELED UBIQUINONE IN MITOCHONDRIAL MEMBRANES AND UNILAMELLAR PHOSPHOLIPID VESICLES

By

Krishnakumar Rajarathnam

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ABSTRACT

THE LOCATION AND MOBILITY OF FLUORESCENTLY LABELED UBIQUINONE IN MITOCHONDRIAL MEMBRANES AND UNILAMELLAR PHOSPHOLIPID VESICLES

By

Krishnakumar Rajarathnam

The location and mobility of ubiquinone (Q_{10}) in mitochondrial membranes are subjects of controversy. A diffusion coefficient of $3x10^{-9}$ cm^2sec^{-1} was measured by fluorescence redistribution after photobleaching for a ubiquinone analog whereas a value of of $1x10^{-6}$ cm^2sec^{-1} was determined for native ubiquinone by collisional quenching. To assess the influence of the isoprene chain on mobility, we have synthesized a fluorescent derivative of the quinone moiety of native ubiquinone (NBDCOQ) and have measured its diffusion and location in membranes of giant mitochondria and phospholipid vesicles. Lateral diffusion rate for NBDCOQ was 3.1×10^{-9} cm^2sec^{-1} in mitochondria and 1.1×10^{-8} cm^2sec^{-1} in vesicles. Similar rates were observed for head group labeled NBD-phosphatidylethanolamine (NBDPE). However fluorescence emission and quenching studies show that the quinone moiety is in a more hydrophobic environment and is less accessible to quenching

agents than the modified phospholipid head group. These results indicate that ubiquinone is oriented differently than a phospholipid in the membrane, but their diffusion rates are similar.

To Peace and Harmony

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LIST OF ABBREVIATIONS

BSA, bovine serum albumin

CCCP, carbonyl cyanide *m*-chlorophenylhydrazine

CMC, critial micelle concentration

DiI, dioctyl- or dihexyldecylindocarbocyanine

DMAP, dimethylaminopyridine

DMPC, dimyrsitoyl phosphatidylethanolamine

DPPC, dipalmitoyl phosphatidylethanolamine

Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethane-sulfonic acid

IANBD, 4-[N-(iodoacetoxy)ethyl-N-methyl]amino-7-nitrobenz-2-oxa-1,3-diazole

NBDCOQ, 4-[N-(acetoxy)ethyl-N-methyl]amino-7-nitrobenz-2-oxa-1,3-diazole-ubiquinone

NBDPE, N-(7-nitro-2-1,3-benzoxadiazol-4-yl)dipalmitoyl phosphatidylethanolamine

6-NBDPE, 1-acyl-2-[6-{(7-nitro-2-1,3-benzoxadiazol-4-yl) amino}caproyl] phosphatidylethanolamine

12-NBDPE, 1-acyl-2-[12-{(7-nitro-2-1,3-benzoxadiazol-4-yl) amino}dodecanoyl] phosphatidylethanolamine

NMR, nuclear magnetic resonance

T_c, lipid transition temperature

TLC, thin layer chromatography

STRUCTURE, FUNCTION, LOCATION AND MOBILITY OF UBIQUINONE: AN OVERVIEW

Ubiquinone (Q_{10}) is an integral component of the electron transport chain and mediates the transfer of electrons between the dehydrogenases and the cytochromes in mitochondria and participates in the translocation of protons across the membrane. Q_{10} is a hydrophobic molecule with a long isoprenoid side chain of about 50 A^o (Trumpower, 1981) and if it were in an extended form it would span the bilayer (Figures 1, 2B). The isoprenoid chain of Q_{10} is fairly rigid (Trumpower, 1981) due to the presence of the double bonds and the vicinal methyl groups and it is likely that the rigidity of the side chain strongly influences the location of the molecule in the membrane. It has been shown that the orientation of short chain ubiquinones is parallel to a phospholipid with the quinone moiety near the membrane surface (Figure 2A) while long chain ubiquinones appear to be located deeper in the membrane. Studies by Crane (1977) have shown that Q_{10} in the mitochondrial membrane is largely inaccessible to hydrophilic electron donors and acceptors. These and other studies on reconstitution of mitochondrial function led him to suggest a model for orientation of Q_{10} in which the quinone ring and the prenyl side chain are in the center of the bilayer (Figure 2C). This model differs from the more traditional model of Q_{10} in which transmembrane flip-flop

Figure 1. Space filling model of ubiquinone. Reproduced from Trumpower, B.L. (1981) in J. Bioenerg. Biomembr. 13, 1-24.

Figure 1

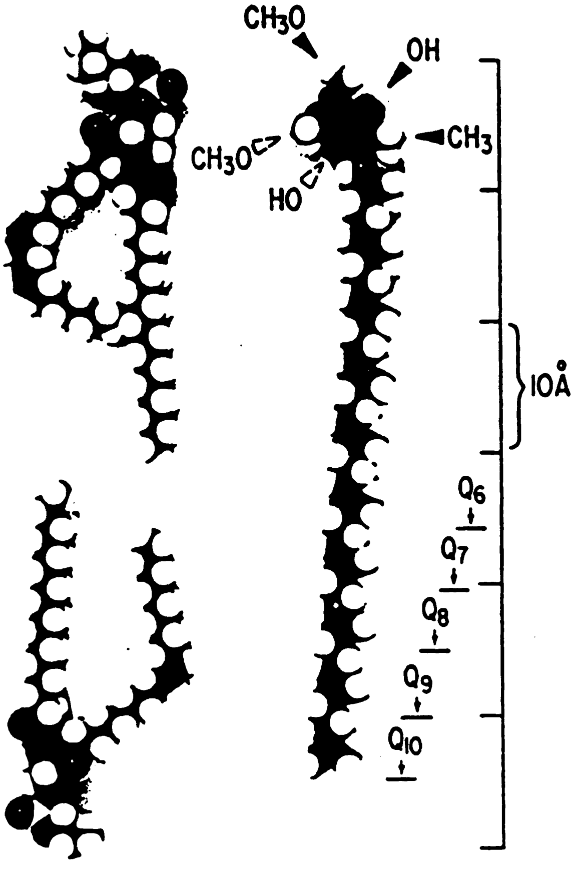


Figure 2. Various possible orientations of short and long chain ubiquinones in a membrane. Reproduced from Siedow, J.N., & Stidham, M.A. (1986) in Biomedical & Clinical Aspects of Coenzyme Q, Volume 5, (page 70).

across the membrane provides the mechanism for electron and proton transport (Mitchell, 1976) (Figure 2D). Existence of Q_{10} as a micelle spanning the bilayer has also been proposed (Ondarroa & Quinn, 1986).

The exact mechanism of energy transduction through Q_{10} remains unclear. Many of the hydrogen carrying substrates of the electron transfer chain such as succinate and NADH are hydrophilic and their function is localized to the aqueous domains on either side of the membrane. Since redox reactions of Q_{10} involve protons, communication with an aqueous environment would appear necessary (Rich, 1982). The membrane bound enzymes that interact with Q_{10} would require their Q-reactive centers to be accessible to the surface of the membranes. Rich (1984) proposes Q and QH_2 are in such an environment that they are essentially unreactive until they collide with an appropriate site on a donor or acceptor, ensuring the biological specificity of the reaction route. One reason, therefore, that the biological quinones possess such long hydrophobic side-chains might be to ensure that they remain unreactive in possible damaging chemical side reactions until they reach their reaction sites.

It has become increasingly evident that diffusion of Q_{10} is important for electron transfer between the dehydrogenases and the cytochromes in mitochondria. Whether its diffusion is rate limiting in electron transfer is not clear. To gain insight into this question it is would be useful to know the diffusion rates of Q_{10}, the E_{act} for the diffusion (Hackenbrock et al., 1986b), the k_m values of the electron carriers for the native quinone in the mitochondria (Fato et al., 1986), and the average distance Q_{10} has to diffuse to accomplish electron transfer. Currently, there is controversy regarding all these values.

Gupte et al. (1984) have measured the diffusion of a fluorescent derivative of a ubiquinone analogue which has an alkyl side chain corresponding in length to only two isoprene units. The lateral diffusion coefficients they determined by fluorescence redistribution after photobleaching (FRAP) was 3 $\times 10^{-9}$ $cm^2 sec^{-1}$ in the mitochondrial membranes and 5.5 $\times 10^{-8}$ $cm^2 sec^{-1}$ in phospholipid vesicles (Hackenbrock et al., 1986a). From diffusion measurements as a function of temperature (Hackenbrock et al., 1986b) and as a function of increased membrane dilution with phospholipids (Schneider et al., 1982), they propose that diffusion of Q_{10} is rate limiting in electron transfer. In contrast, Fato et al. (1986) have measured the diffusion of Q_{10} in small sonicated vesicles and submitochondrial particles by the technique of collisional quenching of fluorescently labeled fatty acids and report a value of 1 $\times 10^{-6}$ $cm^2 sec^{-1}$. They have calculated the E_{act} for diffusion by measuring quenching constants as a function of temperature and the k_m values for Q_{10} with cytochrome bc_1 and NADH dehydrogenase from kinetic measurements. They propose from these studies that diffusion is not rate limiting in electron transfer.

Q_{10} is in excess over its redox partners in the mitochondrial membrane (about 20 fold excess over complex III; Capaldi, 1982) and it was originally proposed by Green (1962) that Q_{10} acts as a mobile electron carrier. The concept of a Q pool is widely accepted but for a few investigators (King & Suzuki,1984., Yu & Yu, 1980) (see discussion on pages 66-70). However, there is conflicting data regarding the homogeneity of the pool (for reviews see Rich, 1984; Ragan & Cottingham, 1985) and the concept that the Q pool is heterogeneous has been postulated by Gutman (1980, 1985). The basic tenet of the concept is that diffusion is not rapid enough to sufficiently randomize the Q pool. It appears that diffusion rate of Q_{10} would affect whether the electron transfer through Q_{10} would follow a simple Q pool behavior or not (Rich, 1984) since it will determine the number of protein molecules a diffusing quinone or quinol molecule could see in its life time.

The major controversies regarding Q_{10} behavior center around how fast it diffuses in the native membrane and where it is located. Two possible experimental problems may have led to the discrepancy observed in the reported diffusion rates: 1) the use of a ubiquinone derivative in lateral diffusion measurements without a native 10 unit isoprene tail; 2) systematic errors in the technique or calculations involved in determining diffusion rates by collisional quenching.

To test the former possibility, we have synthesized a fluorescent derivative of the native ubiquinone (Q_{10}) labeled in the head-group moiety so as to retain an unaltered isoprene tail. We have measured its diffusion in giant mitochondria and cell size phospholipid vesicles. We also report here the experiments performed to locate the position of the fluorescently labeled ubiquinone by fluorescence emission and fluorescence quenching studies. Our results show that the NBDCOQ is located deeper in the membrane than a phospholipid bearing the same fluorescent moiety in the head group but that its diffusion rate is similar.

EXPERIMENTAL METHODS

Materials. The following materials were obtained from the sources indicated. Cuprizone, Aldrich Chemical Co., recrystallized from 95% ethanol according to Bowman & Tedeschi (1983); asolectin, Associated Concentrates, Long Island, NY; NBD-labeled phospholipids, Avanti, Birmingham, AL; IANBD, Molecular Probes, Eugene, OR; preparative TLC plates, Analtech, Newark, DE; DMAP, recrystallized from dichloromethane, ubiquinone(Q_{10}) and CCCP, Sigma Chemical Co.

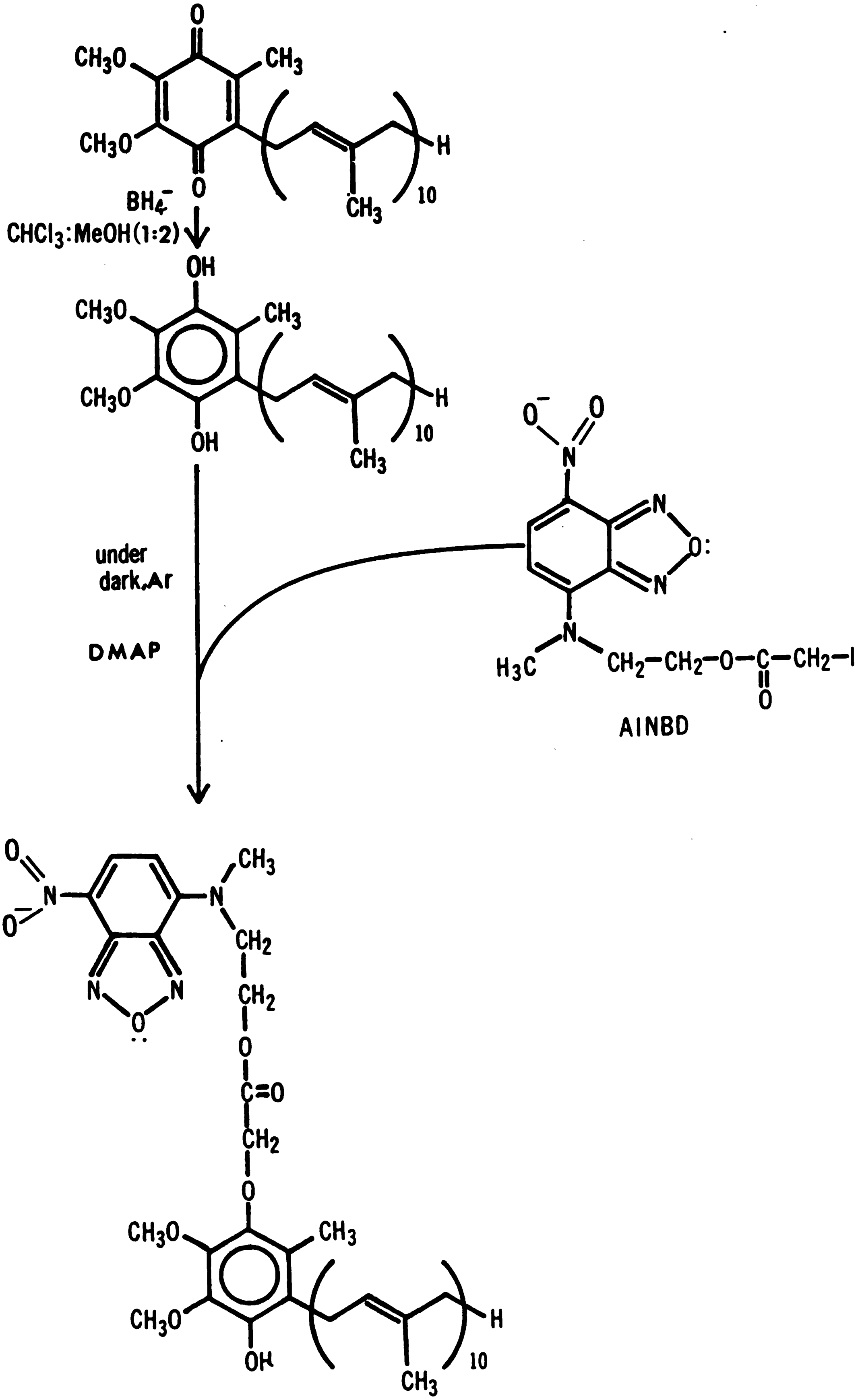
Preparation of giant mitochondria. Fifteen to seventeen day old mice (Swiss Albino ICR) are fed a diet containing 3 grams of cuprizone in 500 grams of rodent chow. Giant mitochondria from these mice are prepared 6 to 12 days after initiating the diet, essentially according to the procedure of Hochman et al. (1985). 3 or 4 livers are taken in 5 ml of isolation buffer (220 mM mannitol, 70 mM sucrose, 2 mM Hepes, 0.05% BSA, pH 7.4 (with Tris)), minced and washed a few times in the same buffer. The minced livers are homogenized twice using a loose fitting glass Dounce homogenizer and the homogenate is centrifuged at 120xg for a minute. The supernatant is saved and the pellet is homogenized and centrifuged again at 120xg for one minute. The supernatants are pooled and layered on 10 ml of 0.5 M sucrose and centrifuged at 730xg for 5 minutes in a swinging bucket bench top centrifuge. The top layer containing smaller mitochondria and a loose bloody pellet at the bottom are discarded and the rest of the 0.5 M sucrose layer and the interface are gently resuspended. The solution is diluted to 0.3 M sucrose with cold distilled water and then spun at 750xg for 5 minutes. The pellet is suspended in 5 ml of isolation buffer, layered on 0.5 M sucrose and centrifuged at 240xg for 3 minutes in a bench top centrifuge and the top layer excluding the interface is collected and pelleted at 750xg for 5 minutes. The giant mitochondria are depleted of outer membrane by first swelling the mitochondria in 5 ml of 5 mM Tris-Phosphate (pH 7.5) for 5 minutes and then adding an equal volume of shrinking buffer (1.8M sucrose, 2mM ATP, 2mM Mg^{2+}) and incubating for another 5 minutes. The mixture is then sonicated for 20 seconds (setting 3, model W-225, Heat Systems Ultrasonics, Inc) and the mitoplasts are pelleted at 1900xg for 10 minutes. The mitoplasts are suspended in 0.1-0.2 ml of 250 mM mannitol, 50 mM Hepes (pH 7.2) buffer and the amount of protein is determined by a modified Lowry assay using bicinchoninic acid (BCA) (Smith et al., 1985).

Preparation of cell size vesicles for FRAP. Unilamellar phospholipid vesicles are prepared essentially according to the procedure of Mueller et al. (1983). Asolectin (6.25 mg), α-tocopherol (0.04 mg) and NBDCOQ (0.125 mg) are dissolved in chloroform in a test tube to give a 1:50 w/w ratio of NBDCOQ to phospholipid. This is made to a final volume of 2 ml in chloroform/ methanol/ 0.5 NaCl (2:2:1.8 v/v) as to make it biphasic. The mixture is vortexed and then spun at room temperature for five minutes in a bench top centrifuge. The upper aqueous layer is discarded and the chloroform layer is filtered through anhydrous $MgSO_4$ into a 25 ml erlenmeyer flask. The filterate is dried under argon and 25 mls of 0.05 mM NaCl solution is added gently into the flask. The flask is wrapped with silver foil and is kept at 4^{o} C. Vesicles form at the bottom of the flask after one to two days and are stable over a period of one to two weeks.

Synthesis of NBD-ubiquinone. The reaction scheme for synthesis of NBDCOQ is given in Figure 3. Ubiquinone (5 mg) is dissolved in one ml of chloroform/methanol (1:2v/v) in a screw top culture test tube and reduced with a few grains of of sodium borohydride. The solution turns colorless and there is a brisk effervesence of hydrogen gas. The solution is stirred until the evolution of hydrogen ceases and then dried using argon. 1.7 millligrams of DMAP (3:4 mol/mol) and 4.7 millligrams of IANBD (2:1 mol/mol) is added to the reduced ubiquinone and the mixture is suspended in 1 ml of dry tetrahydrofuran, flushed with argon and the reaction is carried out over night. The reaction mixture is dried with argon and dissolved in 300 microliters of chloroform/methanol (2:1) and applied to a preparative TLC (uniplate-T, Analtech). The plate is chromatographed in chloroform/hexane/methanol (100:50:2.5 v/v) for fifteen

Figure 3. Reaction scheme for the synthesis of NBD-ubiquinone (NBDCOQ). Ubiquinone is reduced with borohydride (BH_4^-) and reacted with IANBD using dimethylaminopyridine (DMAP) as the catalyst. Tetrahydrofuran (THF) is used as the solvent and the reaction is carried out in the presence of argon (Ar) under dark.

Figure 3



minutes. NBDCOQ (R_f=0.55) runs between IANBD (R_f=0.4) and ubiquinone (R_f=0.85) and is scrapped from the plate and extracted with chloroform/methanol (2:1). The product is filtered, dried using argon, and stored at -20° C until use.

FRAP experiments. Glass slides are washed and rinsed with distilled water and ethanol and then allowed to air dry. For experiments using liposomes, the clean slides are coated with gelatin according to Bowman & Tedeschi (1983). 15 to 20 microliters of labeled mitoplasts or liposomes are applied to a slide and the cover slips are sealed around the edges with paraffin to prevent evaporation.

Incorporation of the fluorescent probe into the mitoplasts. To 100 microliters of mitoplasts (10-15 mg protein/ml) in 250 mM mannitol, 50 mM Hepes buffer (pH 7.2), 3-5 microliters of NBDCOQ (3-5 mg /ml) or 3 microliters of head group NBDPE (1mg/ml) in ethanol, and 5 microliters of BSA (1mg/ml) are added and incubated for 15 minutes at room temperature. The mitoplasts are then swollen with 3 volumes of water over a period of 10 minutes on ice. These conditions, especially the presence of BSA, were important for achieving sufficient incorporation to perform the FRAP experiments. To quantitate the amount of NBDCOQ incorporated into the mitochondrial membranes under the conditions used for FRAP, 50 microliters of NBDCOQ labeled mitoplasts (3 mg protein/ml) were diluted to one ml with 62.5 mM Mannitol, 12.5 mM Hepes (pH 7.2) buffer and centrifuged at 4000xg for 10 minutes. Unincorporated NBDCOQ was extracted from the supernatant by partitioning it into an organic phase by adding chloroform, methanol, and 0.5M NaCl to a ratio 2:2:1.8 (v/v) and spun in a bench top centrifuge for 5 minutes. The upper aqueous layer was discarded and the chloroform layer was filtered through anhydrous $MgSO_4$ and is dried with argon. The pelleted mitochondria were resuspended in 0.1 ml of the Mannitol-Hepes buffer mentioned above and the incorporated NBDCOQ was extracted by adding chloroform, methanol, and 0.5M NaCl to a final volume of 1:2:0.8(v/v). The mixture was spun as before and the supernatant was transferred to another test tube and made biphasic by adding chloroform, methanol, and 0.5M NaCl to a final ratio of 2:2:1.8(v/v) and the extraction was carried out as described for the supernatant. From the absorbance at 457 nm of the mitochondrial and supernatant extracts redissolved in chloroform, the amount of NBDCOQ incorporated into the membrane was calculated. In the case of the liposomes, the final concentration of NBDCOQ or NBDPE is determined from the amount originally added to the phospholipid mixture.

Bleaching conditions and diffusion measurements.

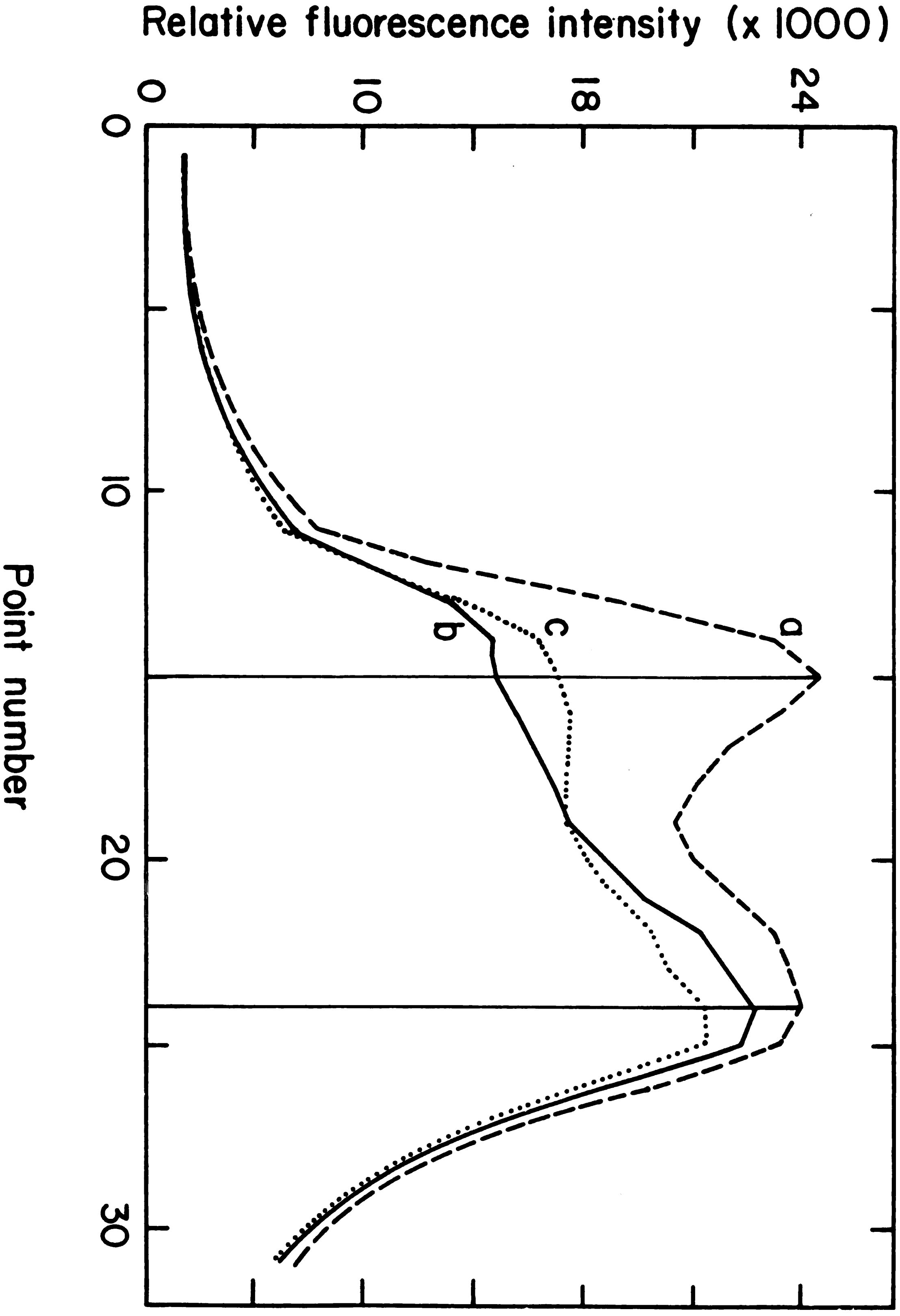
Diffusion measurements were performed by the technique of fluorescence redistribution after photobleaching using the instrumentation and analysis developed by Koppel *et al*.

(Koppel, 1979; Koppel et al., 1980). The low intensity laser beam is scanned across the fluorescently labeled membrane and profiles of the fluorescence emission are recorded. A sample profile for a phospholipid vesicle labeled with NBDCOQ is shown in Fig 4A. The peaks indicate that the fluorescent probe is associated with the membrane, giving a more intense fluorescence at the edges. A higher intensity pulse is applied to one edge of the vesicle resulting in bleaching of the probe in this region (chemical destruction) and giving the profile seen in Figure 4B. The redistribution of the unbleached fluorescent probe (Figure 4C) 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). NBD fluorescence is monitored with an incident wavelength of 477 nm and a combination of Leitz dichroic mirror TK 510 and a barrier filter K 530.

Fluorescence Quenching Measurements. Steady state fluorescence measurements were obtained in a Perkin-Elmer 550-60 spectrofluorimeter. Quenching experiments with $CuSO_4$ was carried out as follows. To a cuvette containing 1 ml of NBDCOQ or NBDPE loaded mitochondrial solution, a 3 mM $CuSO_4$ was titrated in 5 ul aliquots. For the vesicles, a 1 mM solution of $CuSO_4$ was added in 3 or 5 ul aliquots. After each addition, the sample was mixed and then allowed to equilibrate before measuring the fluorescence intensity.

Figure 4. A representative FRAP experiment. Profiles of fluorescence intensity of NBDCOQ in a phospholipid vesicle. Fluorescence distribution before bleaching (t<0 secs) is represented as (-----) and after bleaching (t=4 secs) is represented as (———). The fluorescence recovery after 5 seconds (10 scans) is shown as (..........). The diameter of the vesicle is 8.7 microns.

Figure 4


Relative fluorescence intensity (x 1000)
0
10
18
24
0
10
20
30
Point number
a
b
c

NBD moiety was excited at 468nm and the emission was monitored at 527 nm for NBDCOQ and 536 nm for NBDPE. Slit width was 5 nm for the excitation beam and 10-13 nm for the emission beam.

Effect of NBDCOQ on succinate-oxidase activity.

Succinate oxidase activity of mitochondria was measured on a Gilson polarograph in 1.75 ml of 250 mM mannitol, 50 mM Hepes, (pH 7.2), 13.8 mM succinate, 7.6 uM cytochrome c and 0.55uM CCCP. Turnover numbers (TN) were calculated from the rates of O_2 consumption multiplied by 4 to give the nanomoles of cytochrome c required to reduce 1 nanomole of O_2, and divided by the total nanomoles of cytochrome aa_3 present in the reaction vessel.

Results

Synthesis of NBD-ubiquinone (NBDCOQ)

We are reporting for the first time the synthesis of a fluorescent derivative of Q_{10}, NBDCOQ. There were several considerations involved in deciding on the appropriate labeling method. 1)The only functional group that is sufficiently reactive in ubiquinone to allow modification is the keto group or in reduced form the phenolic group. 2)The fluorescent tag to be attached should be excitable in the visible region so that it can be used for FRAP experiments with our laser. 3)The label should be small and hydrophobic so as not to perturb the hydrophobic character of the quinone. NBD (excitation maximum = 477 nm) and rhodamine (excitation maximum = 531 nm) were two possibilities considered because rhodamine is available as a sulfonyl chloride (Lissamine rhodamine B sulfonyl chloride) and as an alkyl halide derivative (Tetramethyl rhodamine iodoacetamide), and NBD is available as an alkyl halide (IANBD). These compounds can undergo nucleophilic substitution reactions since chloride (in sulfonyl chloride) and iodide are good leaving groups. They are generally used for reactions with sulfhydryl and amino compounds which are fairly strong nucleophiles. Though the hydroxyls (and phenols) are weak nucleophiles, the above compounds can be used to derivatize them, but the conditions have to be more rigorous and the yields are likely to be low. Between rhodamine and NBD, the latter was preferred as it is smaller, less hydrophilic, and likely to perturb the structure of ubiquinone to a lesser extent.

Phenols in general are weak nucleophiles. Q_{10} is a tetra-substituted bisphenol (see fig.3), making it even less reactive. IANBD can be considered as a primary iodide (see fig.3 and the reaction will probably proceed through a SN_2 (second order nucleophilic substitution) rather than a SN_1 (first order nucleophilic substitution) mechanism. The formation of the primary carbocation would not be favorable especially because of the presence of the keto group in the alpha position.

The initial strategy followed was to increase the nucleophilicity of reduced Q_{10} by reacting it with a strong base, potassium *tert*-butoxide to generate a phenoxide ion, a relatively stronger nucleophile. Aprotic polar solvents like dimethylforamide (DMF) and tetrahydrofuran (THF) had to be used to increase the solubility of the reactants and as well as to accommodate the base. THF was favored as it has a low boiling point (b.p.=66^{o} C) and hence can be removed easily. DMF has a b.p.=153^{o} C.

When reduced Q_{10} was reacted with butoxide in THF, the colorless solution turned a intense brown. IANBD was added and the reaction was carried out for 4 hours at the end of which the solution was pale brown in color. NBDCOQ could be purified by preparative TLC. However, the reaction was extremely sensitive and unless conditions were carefully controlled, it was difficult to reproduce. Butoxide is a strong base and hence is sensitive to moisture and the reaction is affected by humidity. It was also observed that if excess butoxide was taken, the reaction did not work and products other than NBDCOQ were obtained.

An alternative strategy of activating IANBD was tried so that phenol rather than the phenoxide would act as a nucleophile. Dimethylaminopyridine (DMAP) was used as a catalyst and we were more successful in obtaining NBDCOQ in a reproducible manner. DMAP is a widely used, highly active acylation catalyst (Hofle _et_ _al_., 1978) and has been shown to be far superior to pyridine. DMAP is not generally used as an alkylation catalyst. In our case, it turned out to be very effective since DMAP is a better leaving group than iodide. The reduced Q_{10} could act as an effective nucleophile to displace DMAP and form a phenoxy bond to give NBDCOQ.

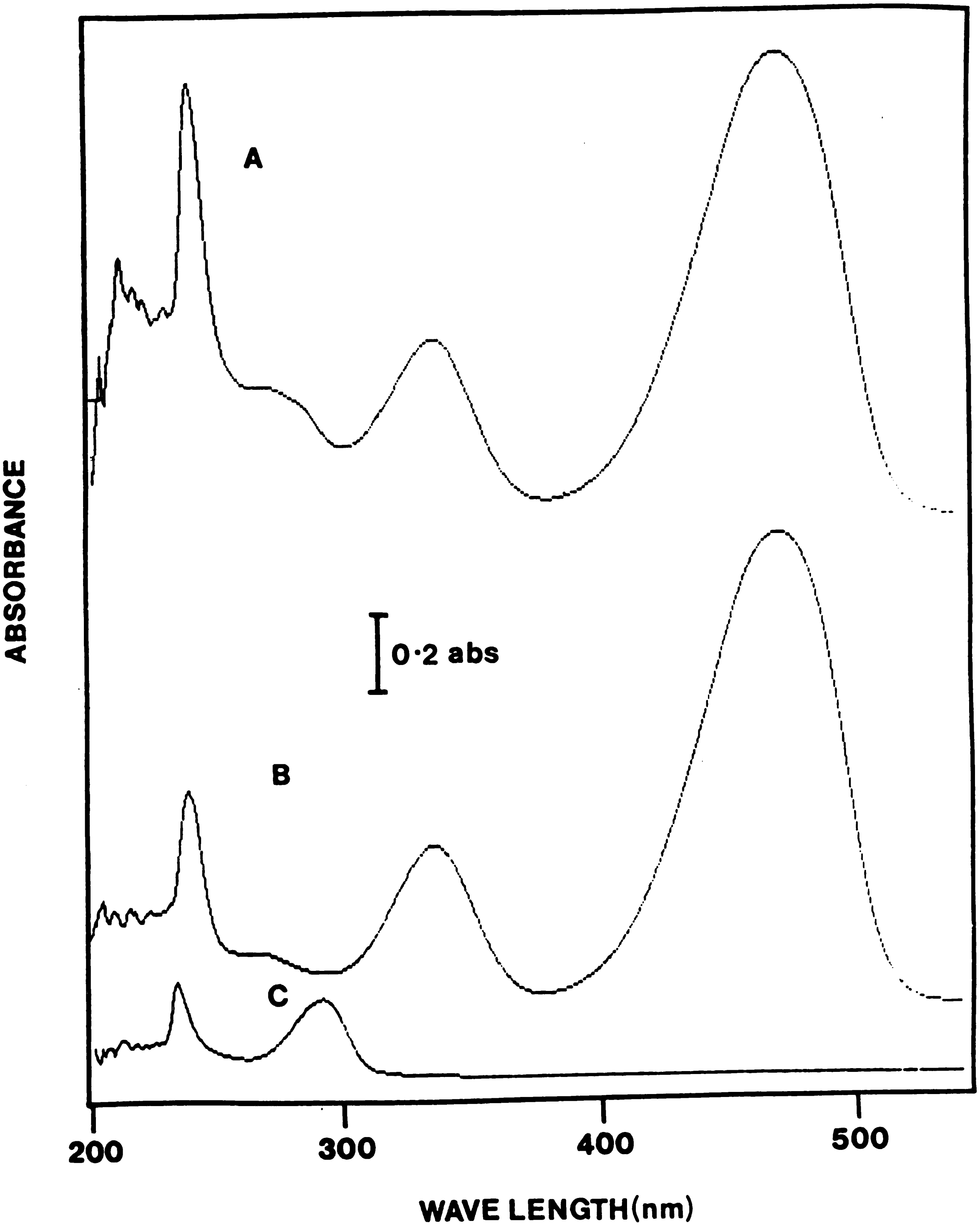
Characterization of NBDCOQ

NBDCOQ has been characterized by various spectroscopic techniques. Absorbance spectra of NBDCOQ (A), IANBD (B), and reduced Q_{10} (C) taken in chloroform are shown in Figure 5. IANBD has absorbance maxima at 463 nm, 332 nm, and 241 nm and reduced Q_{10} at 234 nm and 290 nm. The spectrum of NBDCOQ shows the absorbance characteristics of both IANBD and reduced Q_{10}.

The excitation and emission fluorescence spectra of

Figure 5. Absorbance spectra of NBDCOQ. The absorbance spectra were obtained in a Lambda 4B Perkin-Elmer spectrophotometer using chloroform as the solvent (conc. 56nmol/ml). The spectra of the starting materials, IANBD (B) (36 nmol/ml) and reduced ubiquinone (C) (36 nmol/ml) are also given.

Figure 5



NBDCOQ taken in chloroform are shown in Figure 6. The excitation maximum is at 468 nm and emission maximum is at 518 nm. These spectra were identical to the excitation and emission spectra of IANBD (not shown).

NMR spectra of NBDCOQ(a), Q_{10}(b), and IANBD(c) are shown in Figure 7. Chemical shift values for Q_{10} were assigned as given in Ulrich <u>et</u> <u>al</u>. (1985). Some of the prominent signals are numbered to facilitate comparison. 1.60 ppm, isoprenoid CH_3; 1.68 ppm, trans-terminal CH_3; 1.74 ppm, methyl at C_3'; 1.97 ppm, $CH_2(CH_3)=CH$; 2.01 ppm(1), methyl at C_2; 2.06 ppm(2), $CH_2CH=C$; 3.2 ppm(3), $1'-CH_2$; 3.98 and 3.99 ppms(4), OCH_3; 5.1 ppm(5), vinyl CH; 7.24 ppm, from $CHCl_3$. Chemical shift values for IANBD are: 1.50 ppm, from water impurity; 3.48 ppm(6), $N(CH_3)$; 3.60 ppm(7), $(CH_2)I$; 4.47 ppm(8), a multiplet due to $-CH_2-CH_2-$; 6.17 ppm(9) and 8.44 ppm(10), aromatic peaks with a coupling constant (J)=9 Hz. Signal at 8.44 ppm is from H ortho to the nitro group. 7.24 ppm, from $CHCl_3$. It is seen that NBDCOQ has signals present both in IANBD and Q_{10}. However, some of the signals are shifted either down or up field [example, the methoxy resonance (4)] in NBDCOQ implying that there is some interaction between the NBD moiety and the benzoquinone head group.

There are potentially two reactive groups in reduced Q_{10} and both of them react with the iodoacetoxy group of IANBD to give a mixture of isomers. The two isomers can be separated by preparative TLC. HPLC of the purified products

Figure 6. Fluorescence excitation and emission spectra of NBDCOQ. The spectra of NBDCOQ (conc. 44 nmol/ml) were obtained in a 550-60 Perkin-Elmer spectrofluorimeter using chloroform as a solvent. The slit width for excitation and emission beam was 5 nm.

Figure 6

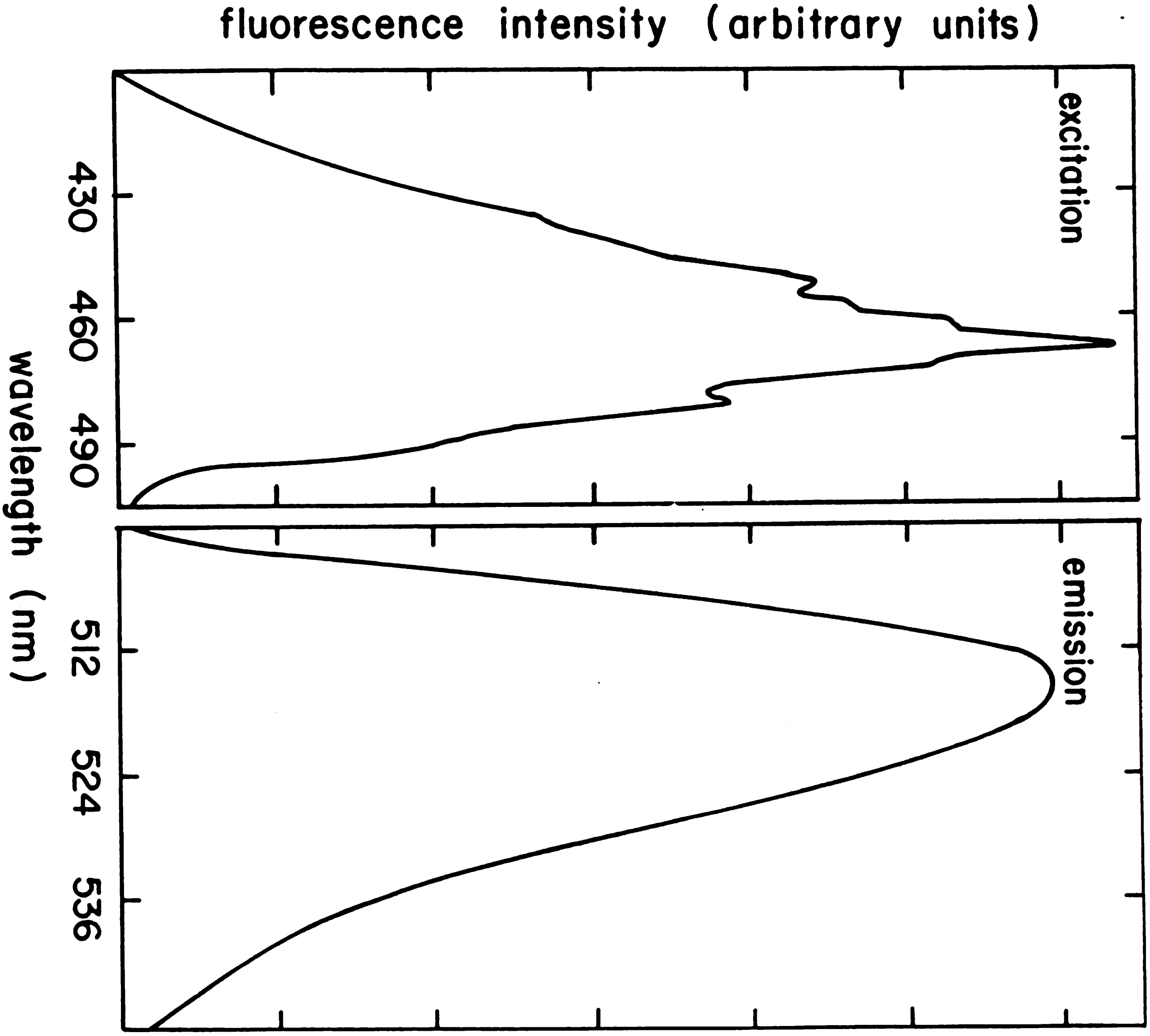
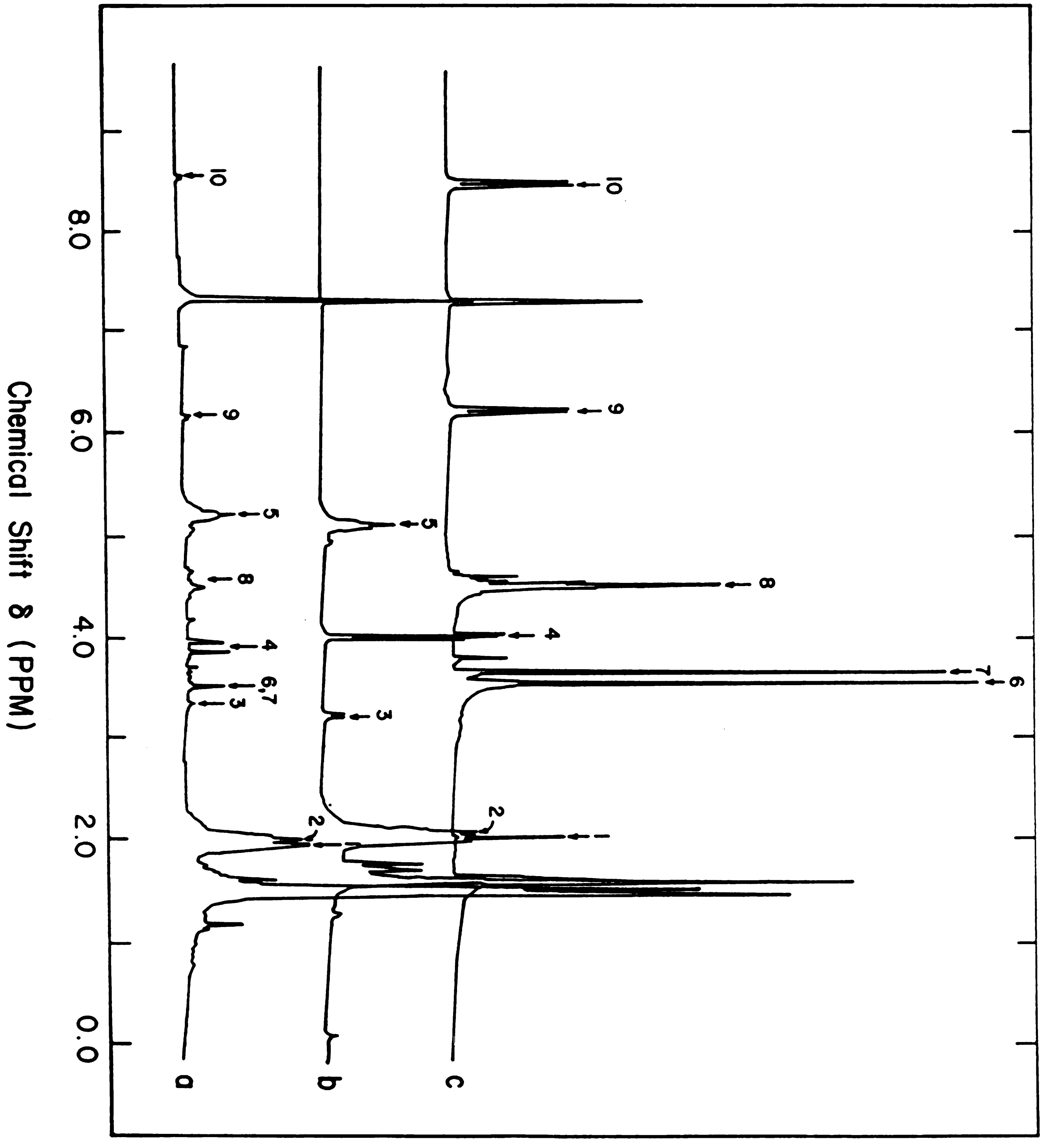

fluorescence intensity (arbitrary units)
excitation
emission
430
460
490
512
524
536
wavelength (nm)

Figure 7. NMR spectra of NBDCOQ. The spectra were taken in a Bruker 250 MHz spectrometer using $CDCl_3$ as a solvent. The spectra of ubiquinone(B) and IANBD (C) are also given for comparison.

Figure 7



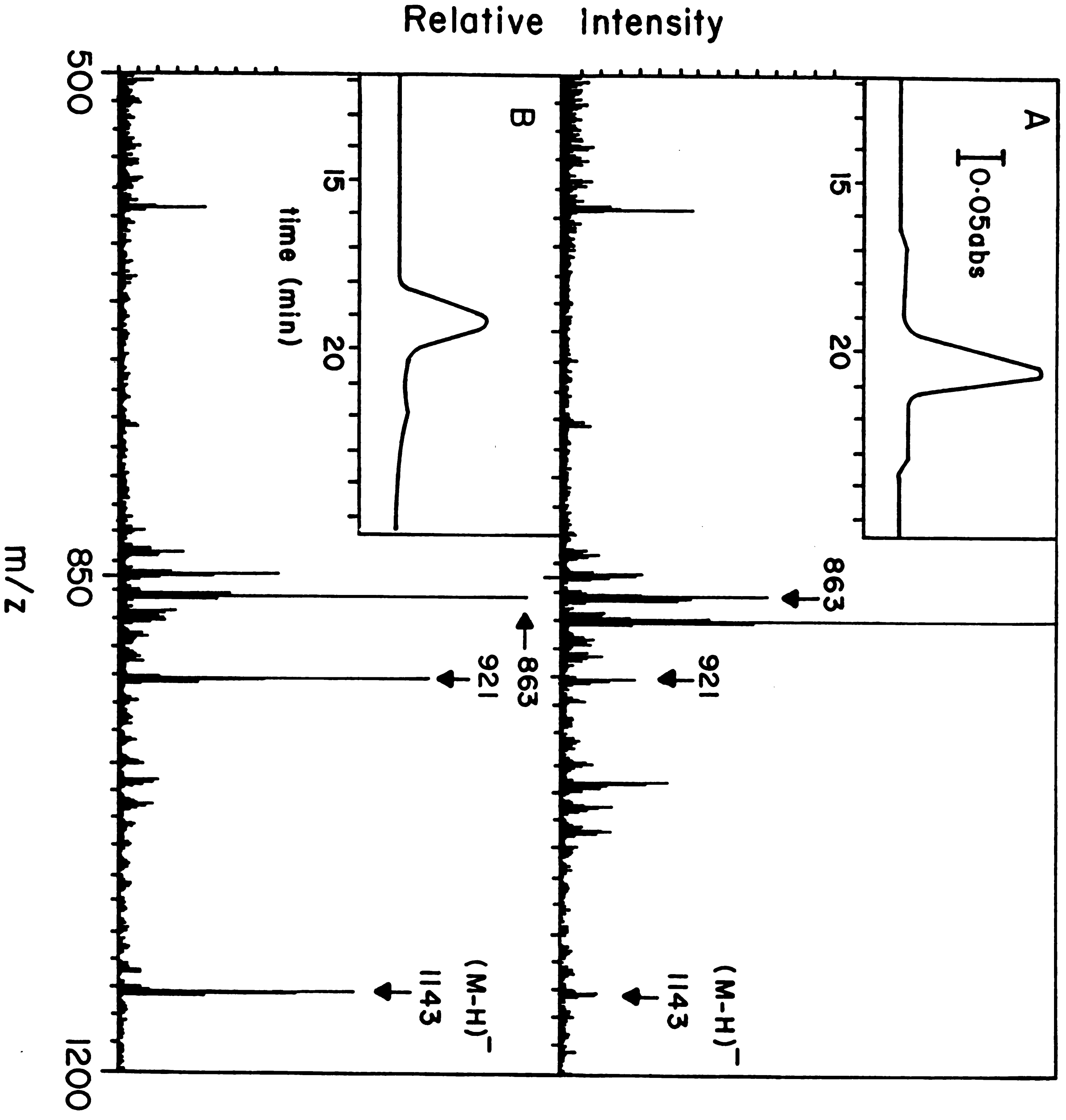
8.0
6.0
4.0
2.0
0.0
Chemical Shift δ (PPM)
a
b
c
10
9
5
8
4
6,7
3
2
10
9
8
4
7
6
3
5
2

performed on a reverse phase column is shown in Figure 8. They had similar absorbance spectra (not shown) and their isomeric character was unequivocally confirmed by mass spectrometry (Figure 8). Both the spectra have peaks corresponding to the molecular weight of NBDCOQ and they have similar fragmentation patterns. Some of the prominent peaks are $[(\mathrm{NBDCOQ}) - \mathrm{H}]^- = 1143$, $[(\mathrm{CoQOCH_2COO}) - \mathrm{H}]^- = 921$, $[(\mathrm{CoQ}) - \mathrm{H}]^- = 863$.

NBDCOQ is purified in a single step by preparative TLC (see methods). Good separation between the starting materials (IANBD, Q_{10}) and NBDCOQ is achieved and when NBDCOQ is rerun on the preparative TLC, the product runs as a single spot. The yield is around 5%. Generally the mixture of both isomers of NBDCOQ was used for the experiments. NBDCOQ is fairly stable when kept in the freezer in the dried form. In solution there is some break down to the starting materials, about 5-10% in 24 hours. NBDCOQ was freshly synthesized whenever needed and once dissolved in ethanol is used within 6 hours. Any contribution of small amounts of hydrolyzed NBD probe to the mobility and quenching measurements would be negligible as it is more water soluble and would partition less into the membrane. Furthermore, the emission maximum of IANBD (and likely of hydrolyzed NBD probe) in membranes is around 540 nm while that of NBDCOQ is 527 nm. So the presence of significant amounts of contaminating NBD would be detectable from an altered emission maximum.

Figure 8. Elution profiles of the two isomers of NBDCOQ from HPLC and their mass spectra. The two isomers of NBDCOQ were separated by preparative TLC under identical conditions used during purification except that the chromatography time was increased to 50 minutes. HPLC of the purified products was performed using a Waters C-18 reverse phase column (3.9 mm x 30 cm). The solvent system used was methanol containing 0.7% $NaClO_4.H_2O$ and 0.1% (v/v) 70% $HClO_4$ (Katayama *et al*., 1980). The flow rate was 1 ml/min and elution was monitored at 254 nm. fast atom bombardment (FAB) mass spectra were obtained on a JEOL HX-110 HF mass spectrometer, operating in the negative mode. The molecular ion is indicated by $(M-H)^-$. The FAB matrix used was triethanol amine. (Mass spectral data were obtained from Michigan State University Mass Spectrometry Facility supported by a grant RR-00480 from NIH).

Figure 8



Relative Intensity
m/z
500
850
1200
A
B
0·05abs
15
20
time (min)
863
921
1143
(M-H)⁻

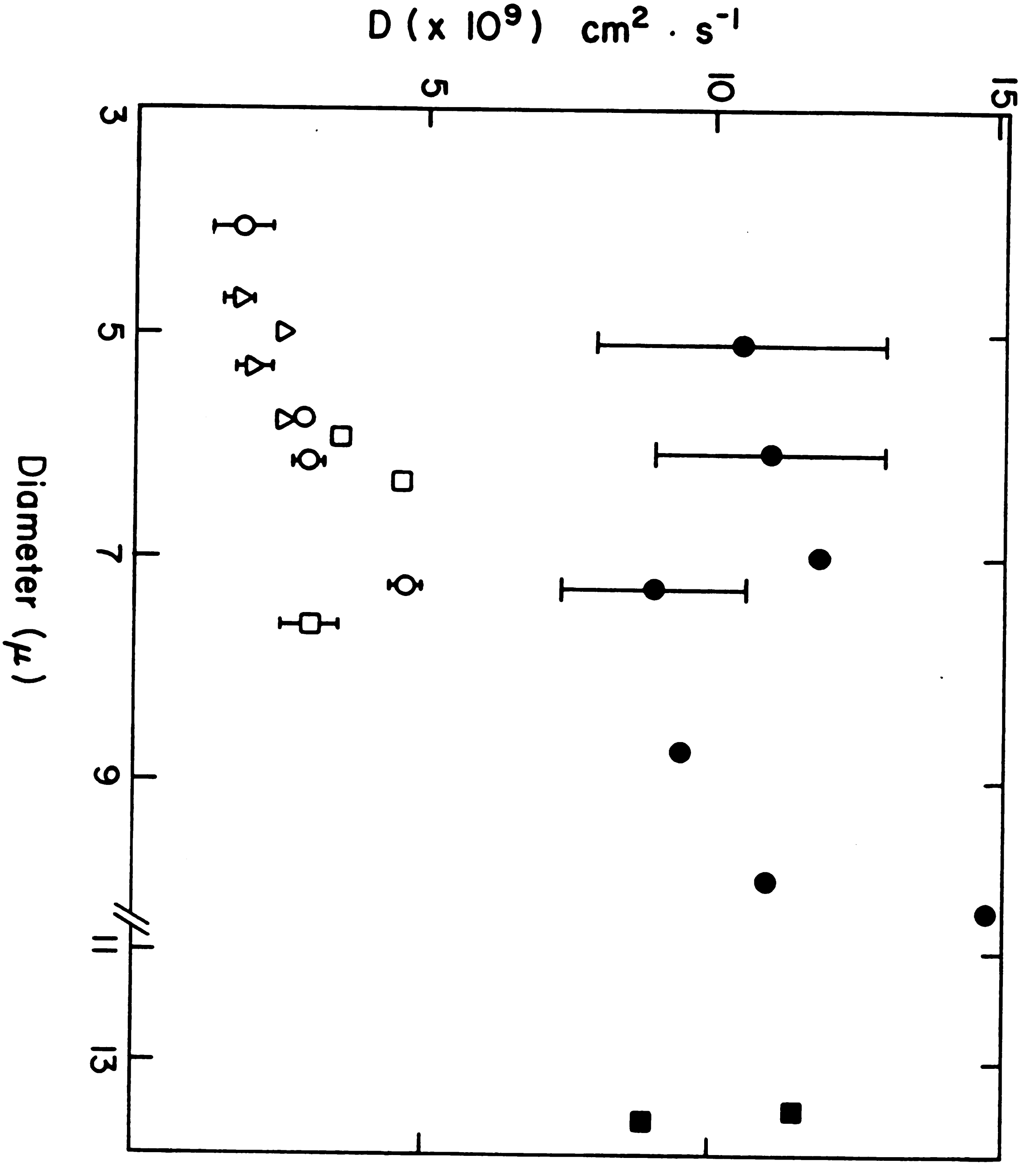
Diffusion measurements of the fluorescent probes in the membranes

Preparation and characterization of giant mitochondria Lateral diffusion coefficients of NBDCOQ and NBDPE were measured in giant mitochondria prepared from cuprizone fed mice and in very large unilamellar vesicles. But for the size, the giant mitochondria have the same characteristics as the normal mitochondria. Their heme content, lipid content (Hochman et al., 1985), electron transfer (Hochman et al., 1982), and respiratory control (Maloff., 1978) are comparable to those found in normal rat liver mitochondria. The mitochondria are used within 10-14 hours after preparation and they are fairly stable in this time frame. NBDCOQ labeled mitochondrial inner membranes on a slide (during FRAP experiments) are used within 15-45 minutes after preparing the slide.

Preparation and characterization of phospholipid vesicles Very large unilamellar vesicles are prepared essentially according to Mueller et al. (1983). As the vesicles were mobile on a glass slide, gelatin coated slides were used to retard the movement of the vesicles. The size of the vesicles was heterogeneous, diameters ranging from 3-20 microns. Diffusion coefficients seemed to show a slight dependence on the diameter of the vesicles (Figure 9), higher diffusion coefficients being obtained at larger diameters. However, only the data obtained from

Figure 9. A plot of the diffusion coefficient values of the fluorescent probes as a function of the diameter of mitochondria (open symbols) and vesicles (closed symbols). (● ○) represent the diffusion coefficients obtained for NBDCOQ; (■ □) head group labeled NBDPE; and (△) acyl chain labeled NBDPE.

Figure 9



vesicles of similar dimensions to the mitochondria were tabulated (Table 1). Since the location of Q_{10} in membranes may be concentration dependent, diffusion measurements were also performed as a function of concentration to see whether it had an affect on diffusion coefficients and nature and extent of recovery. No discernable differences were observed (data not shown) when the experiments were performed over a 8 fold concentration range {1:200 to 1:25 (w/w), NBDCOQ:phospholipid}. The spot bleaching technique was also applied to the large vesicles. The preliminary results gave a diffusion coefficient an order slower than that measured by the edge bleach analysis. The discrepancy could be because the larger vesicles were multilamellar, or that the bleaching conditions were not optimized (overbleaching). Further experiments to determine whether there is actually a difference between the edge and spot bleach analysis in this situation have not been carried out, but extensive analysis in other systems (Swaisgood & Schindler, unpublished observations) indicate that this is normally not the case.

Incorporation of the fluorescent probes into the membrane Incorporation of NBDCOQ or NBDPE was carried out as described in methods. Incorporation of hydrophobic molecules into a membrane seems to be a complex process dependent on a number of parameters such as the structure of the molecule (Struck & Pagano, 1980) and the nature of the membrane system into which the molecule is partitioning

(Arvinte <u>et</u> <u>al</u>., 1986). Extent of incorporation of fluorescently labeled phospholipids is dependent upon whether the fluorescent tag is located in the head or the acyl region (Struck & Pagano, 1980). Acyl labeled phospholipids incorporated more easily than the head group labeled phospholipids. Q_{10} is a hydrophobic molecule and has a very low critical micelle concentration (CMC) ($<0.05 \times 10^{-7}$ M, Lenaz & Degli Esposti, 1985) and does not partition into a membrane easily. A model for the incorporation of Q_{10} into the membranes has been proposed by Lenaz & Degli Esposti (1985) according to which conditions favoring the formation of monomers of Q_{10} such as increase in temperature or sonication results in better incorporation. NBDCOQ, because of the NBD moiety, is somewhat less hydrophobic and hence its CMC may be slightly higher. However, under a variety conditions tested the amount of incorporation was not sufficient to carry out the FRAP experiments. It was not possible to sonicate the system or increase the temperature as it would result in damaging the intactness of the mitochondrial membrane. Sufficient incorporation could be achieved in presence of BSA (see methods) which seemed to act as a carrier protein for delivering NBDCOQ into the mitochondrial membrane. Final concentration of NBDCOQ in the membrane was around 2-6 mol % with respect to lipid (>10 times in excess of endogenous Q_{10}). As the amount of NBDCOQ incorporation was low, the levels to be quantified was limited by the sensitivity of the measurement and there is some error in the measurement as indicated by the range of values obtained and by the fact that the sum of the amounts found in the supernatant and pellet was around 70-80% of the added probe. For the same apparent levels of NBDCOQ in the mitochondria and vesicles, fluorescence intensity was greater in the vesicles (3-4 times). A lower fluorescence intensity of NBDPE in the mitochondria as compared to vesicles was also seen. This seems to indicate that some quenching of NBD is occuring by the mitochondrial proteins.

Diffusion coefficients of the fluorescence probes in the membranes

Diffusion coefficients of NBDCOQ were 3.1 ($\pm$1.0) x10^{-9} cm^2sec^{-1} in the mitochondria and 1.1 ($\pm$0.2) x10^{-8} cm^2sec^{-1} in the vesicles. The diffusion of NBDPE was similar in both the systems (Table 1). The recovery of the fluorescence after photobleaching of NBDCOQ was monophasic (Figure 10) and was more than 90% both in the mitochondria and vesicles.

Fluorescence emission of the probes in the membranes

Table 2 shows the fluorescence emission maxima of NBDCOQ, NBDPE and IANBD measured in different organic solvents, in phospholipid vesicles and mitochondrial membranes. The emission maximum is often a sensitive indicator of the polarity and structure of the environment surrounding the fluorophore. The fluorescence emission

Table 1: Lateral diffusion of NBDCOQ and NBDPE in mitochondria and phospholipid vesicles

	D ($cm^2 sec^{-1}$)	
	Mitochondria	Vesicles
NBDCOQ	$3.1 \pm 1.0 \times 10^{-9}$ (n=4)	$1.1 \pm 0.2 \times 10^{-8}$ (n=6)
NBDPE	$3.0 \pm 0.4 \times 10^{-9}$ (n=4)	$1.3 \pm 0.3 \times 10^{-8}$ (n=3)

Figure 10. Fluorescence recovery profiles of NBDCOQ in mitochondria (A) and vesicles (B). Diffusion coefficients were measured and calculated using the edge bleach analysis technique as developed by Koppel _et_ _al_. [Biophys. J. _301_, 187,(1980)]. 100 microliters of mitochondria (10-15 mg prot/ml) was incubated with 5 microliters of 1% BSA and 3 microliters of NBDCOQ (3 mg/ml) at room temperature for 15 minutes. 20 microliters of the swollen mitochondria were placed on a glass slide, sealed with wax and used for diffusion measurements. For phospholipid vesicles (2.5 mg lipid/ml) 15 microliters was taken. Each point along the x-axis represents a 0.5 second scan. The symbol (u) represents the proportion of fluorescent probe on the bleached edge of the mitochondria at time t and is defined as the normalized first moment of the unbleached fluorophore concentration distribution.

Figure 10

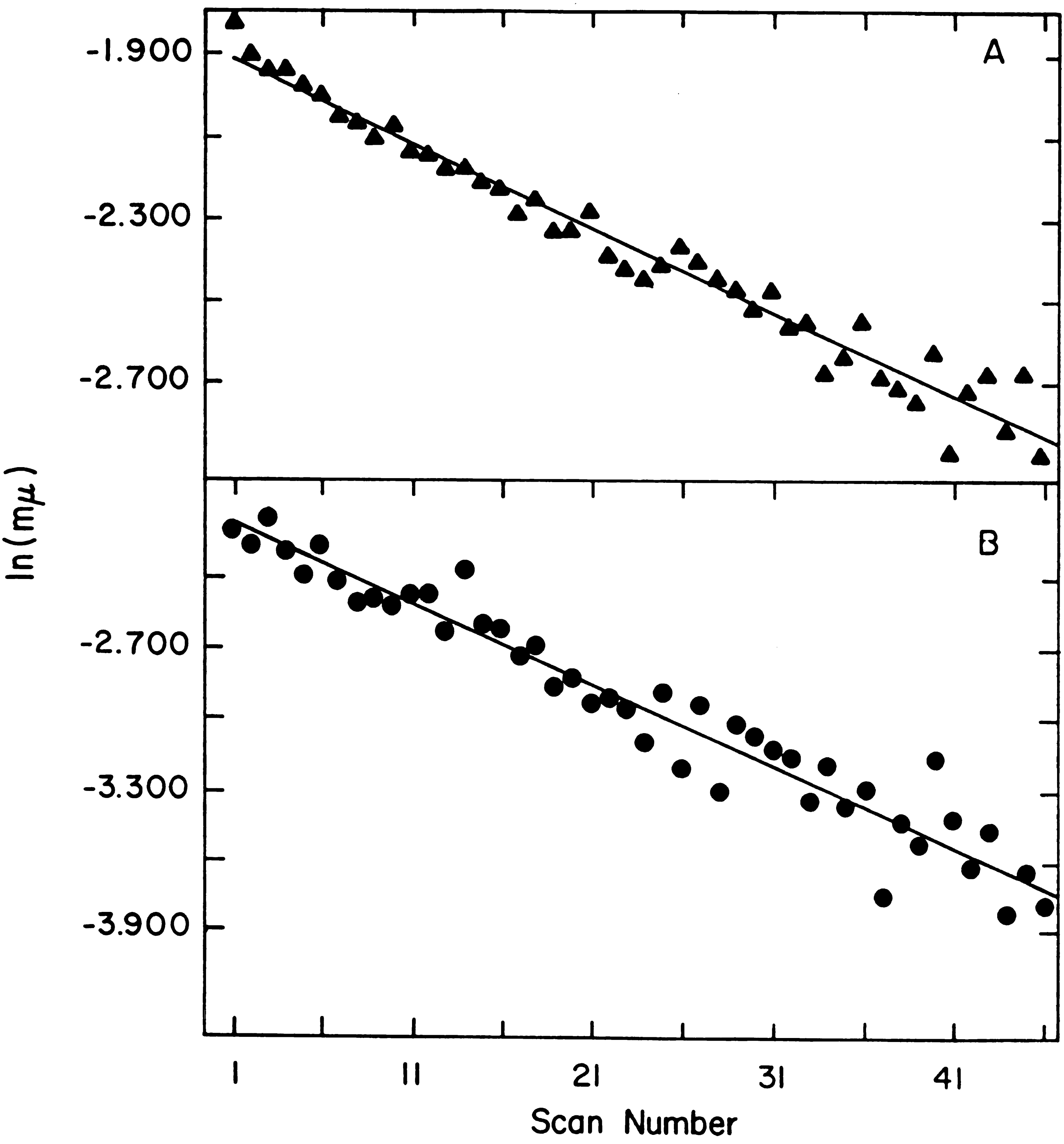


Table 2. Emission maxima of fluorescent probes in organic solvents, phospholipid vesicles, and mitochondrial membranes. (Excitation wavelength :468 nm)

	Emission maximum (nm)		
Solvent	NBDIA	NBDCOQ	NBDPE
CH_2Cl_2	520	521	519
n-Propanol	531	530	530
$CHCl_3$	516	516	519
EtOH	532	531	531
Acetone	530	529	528
MeOH	535	533	533
H_2O	552	560	550
Mitochondria	536-538	527-529	535-537
Vesicles	542-545	526-528	536-537

maxima of the NBD fluorophore attached to these different molecules showed similar changes in response to different solvents. In general, there was a blue shift as the polarity of the solvent decreased. There were exceptions to this: for example the emission maxima of the fluorophores in chloroform were more blue shifted than in dichloromethane and propanol. This implies that the relationship between the emission maxima and the properties of the organic solvents is more complex and could be dependent on parameters such as whether the solvent is protic or aprotic, and its dipole moment. However, when the fluorescent molecules were placed in an anisotropic environment, namely in vesicles and mitochondria, significant differences were observed in the emission maxima obtained. The emission maximum for NBDCOQ was 525-527nm in both the vesicles and mitochondria, significantly blue shifted compared to NBDPE (535-537 nm) and IANBD (542-544 nm), implying that NBDCOQ is in a more hydrophobic environment.

Quenching of the fluorescent probes in the membranes

Quenching of fluorescent molecules has proved useful for establishing the relative position of the fluorescent probes in a membrane. The data is presented in the form of Stern-Volmer plots where the quenching efficiency is related to the total quencher concentrations (Stern & Volmer, 1919).

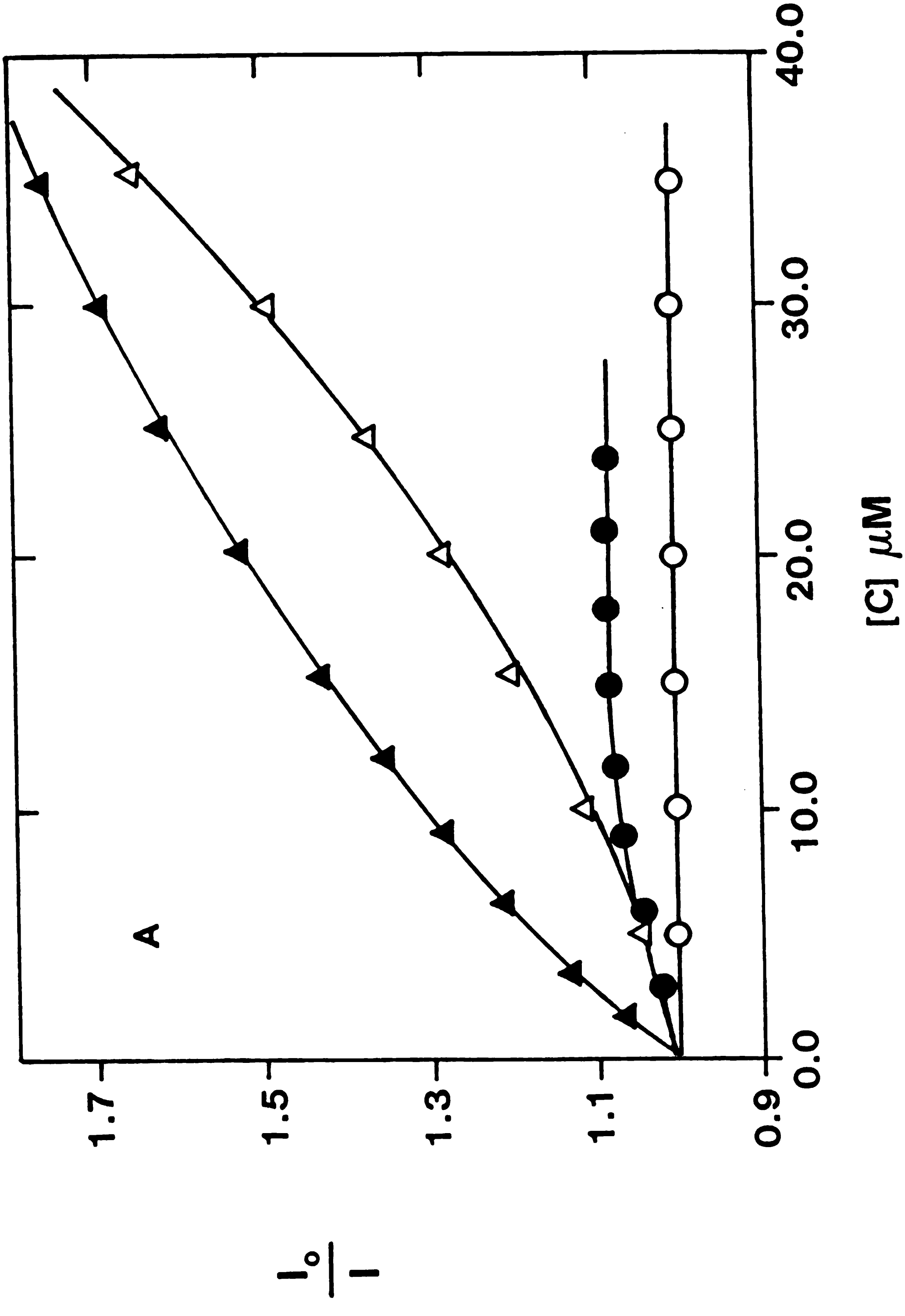
$$\frac{I_o}{I} = 1 + k_q \, t_o \, [Q] \qquad (1)$$

where I_o and I are fluorescence intensities in the absence and presence of the quencher, [Q] is the quencher concentration, k_q is the bimolecular quenching constant, and t_o is the lifetime of the fluorophore in the absence of the quencher. However, the quenching process in the membrane is complicated, depending on the extent to which the quencher binds or partitions into the membrane (Blatt et al., 1986). Also, quenching in the membrane can occur either by dynamic or static mechanism or both. Static quenching occurs when a fluorophore within a spherical volume surrounding the quencher is quenched instantaneously, while fluorophores located outside the active sphere may be quenched by collisional interactions (Blatt et al., 1986). Cu^{2+} is a water soluble paramagnetic quenching agent that has been shown useful for locating the relative depth of fluorophores in a membrane by virtue of its ability to bind to the membrane surface and quench the fluorescence of the probes located on or near the surface (Thulborn & Sawyer, 1978). Thus Cu^{2+} was chosen to investigate the relative position of NBDPE and NBDCOQ in the mitochondrial membranes and vesicles. Stern-Volmer quenching plots are given in Figure 11. As the experiments were performed under nearly identical conditions for both NBDPE and NBDCOQ in the vesicles and mitochondrial membranes, we can assume that the amount of bound and free Cu^{2+} will be the same for both the probes.

In vesicles it is observed that quenching of head group

FIGURE 11. Stern-Volmer plots for quenching of NBDCOQ (● ○) and head group labeled NBDPE (▲ △) in giant mouse liver mitochondria (open symbols) and phospholipid vesicles (closed symbols). Swollen mitochondria (0.3 ml, prepared as described in the legend to Figure 10) incubated with the fluorescent probe were diluted to 1 ml for the fluorescence measurements. Vesicles containing the fluorescent probes (1:50, w/w) were prepared according to Mueller et al. (1983) and were used as such for the fluorescence measurements. Steady-state fluorescence was measured in a Perkin-Elmer 550-60 spectrofluorimeter. Excitation was at 468 nm and the emission was at 527 nm for NBDCOQ and at 536 nm for NBDPE. Slit width was 5 nm for the excitation beam and 10-13 nm for the emission beam.

Figure 11



labeled NBDPE was much higher than NBDCOQ (Figure 11). The plots are non-linear and show apparent saturation at higher Cu^{2+} levels. This behavior is expected in cases where total concentration rather than bound concentration is used on the abscissa and where the bound Cu^{2+} is assumed to be the most effective quencher (Thulborn & Sawyer, 1978). To calculate apparent Stern-Volmer quenching constants, slopes were calculated from the initial changes in fluorescence at low quencher concentrations, since it can be assumed that most of the quenching is occuring by a dynamic mechanism. In Table 3, it can be seen that the apparent k_{sv} values are 5 to 6 fold higher for NBDPE and NBDCOQ. In mitochondria, the difference is even greater (>30x), in fact there is essentially no quenching of NBDCOQ while NBDPE is totally quenched by a mainly static mechanism revealed by the upward slope of the Stern-Volmer plot (Figure 11). It is possible that the binding of Cu^{2+} to the protein of the mitochondrial membranes may contribute to the static quenching observed. Regardless of the different quenching patterns, it is clear from the overall data that NBDCOQ is much less accessible to Cu^{2+} than NBDPE in both the membrane systems.

Extent of quenching can be obtained by using the modified Stern-Volmer equation proposed by Lehrer (1971).

$$\frac{I_o}{I_o-I} = \frac{1}{[Q].fa.K} + \frac{1}{fa}$$

where f_a is the fraction of the fluorophore accessible to the quencher. A plot of I_o/I_o-I vs $1/[Q]$ yields a

Figure 12. Lehrer inverse plots of the quenching of NBDCOQ (●) and NBDPE (▲△) in giant mitochondria (open symbols) and phospholipid vesicles (closed symbols). Data plotted according to the modified Stern-Volmer equation. The x-intercept gives the fraction of the fluorophore accessible to the quencher.

Figure 12

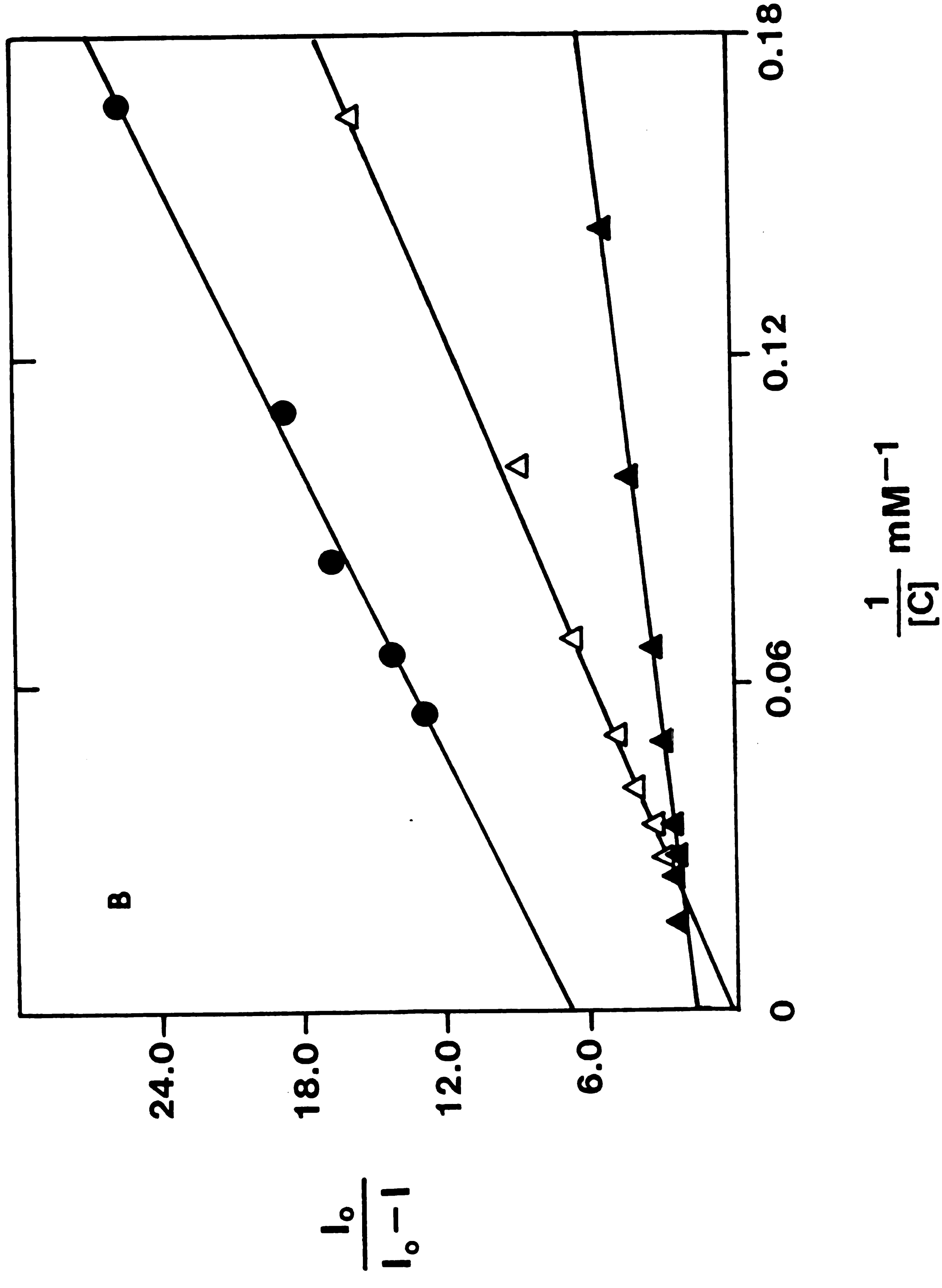


Table 3: Stern-Volmer (k_{sv}) quenching constants $(uM)^{-1}$ of NBDCOQ and NBDPE in the mitochondria and phospholipid vesicles.

	mitochondria		vesicles	
	NBDCOQ	NBDPE	NBDCOQ	NBDPE
k_{sv}	0.3±0.5(n=6)	10.8±1.9(n=7)	7.0±1.1(n=5)	41±4(n=4)
f_a(%)	0	100	17±3	50±9

straight line and the intercept gives the extent of quenching at infinite concentrations of the quencher. Modified S-V plots are given in Figure 12. The intercepts indicate that about 40-50% of NBDPE is quenched in the vesicles and all of it (100%) is quenched in the mitochondria. For NBDCOQ, about 15-20% can be quenched in the vesicles but none is quenched in the mitochondria.

In the vesicles, which were formed after the probe and phospholipid were mixed, about 50% of the probe would be expected to be located in the outer leaflet of the bilayer and thus accessible to the quencher (as observed for NBDPE). In the case of mitochondria, the probe is added exogenously and would be located in the outer leaflet since the flip-flop process for phospholipids is very slow in the natural membranes. In the case of NBDCOQ, it appears to partition into a hydrophobic environment in either membrane system and to be relatively inaccessible to Cu^{2+}. Our results support the idea that it may be predominantly stationed near the mid-plane of the bilayer.

Effect of the fluorescent probes on electron transfer in the mitochondria

The effect of NBDPE and NBDCOQ on uncoupled electron transfer from succinate to oxygen was measured (Table 4). The fluorescent probes had no affect on electron transfer at concentrations similar to those used for mobility measurements. Since the head group of Q_{10} is modified, it would not be expected to bind at the Q-binding sites of the electron transfer and hence would not be able to compete with the endogenous Q_{10}.

Table 4. Effect of the fluorescent probes on succinate oxidase activity in mitochondria

Treatment	Relative Activity (%)	
	5µl[a]	10µl
control[b] (ethanol)	100	100
NBDCOQ[c] (3 mg/ml in ethanol)	105±5 (n=3)	115±5 (n=2)
NBDPE (1 mg/ml in ethanol)	97±7 (n=4)	105±13 (n=4)

[a] 5 and 10 µl are the volumes of ethanol or NBD solution added to mitochondria before measuring activity

[b] Activity obtained with ethanol is taken as 100% activity

[c] Amount of NBDCOQ in the membrane is similar to the levels present during FRAP measurements.

DISCUSSION

Diffusion of NBDCOQ in vesicles and mitochondria

Diffusion of NBDCOQ was 3.1 ± (1.0) $x10^{-9}$ cm^2sec^{-1} in the mitochondrial membranes and 1.1 ± (0.2) $x10^{-8}$ cm^2sec^{-1} in very large unilamellar vesicles. Diffusion of NBDPE was similar. The diffusion coefficient we report is close to that obtained by Hackenbrock's group for a fluorescent derivative of a ubiquinone analog which had an alkyl side chain corresponding to only two isoprene units. It is well established that the short chain ubiquinones orient parallel to the phospholipids near the lipid-water interface. However, evidence suggests that long chain ubiquinones predominantly reside deeper in the membrane. One of the concerns in using the fluorescent derivative of Q_{10} was whether the NBD moiety might cause the quinone moiety to be pulled nearer to the membrane surface. To address this question, we have compared the fluorescence emission characteristics of NBDCOQ, NBDPE, and IANBD. They had similar emission maxima in a variety of organic solvents, but the emission maximum of NBDCOQ in membranes was significantly blue shifted compared to the fluorescent phospholipid, implying it is in a more hydrophobic medium. The quenching of NBDCOQ and NBDPE by a water soluble quenching agent, Cu^{2+}, was also compared in both the membrane systems. The quenching of NBDCOQ was much less implying it is less accessible. These studies indicate that the fluorescent derivative is indeed located deep in the membrane. Similar studies were also carried out with 6-NBDPE and 12-NBDPE (results not shown) but their quenching behavior and fluorescence emission maxima were similar to head group labeled NBDPE, as also observed by Chattopadhyay and London (1987). They postulate that the NBD moiety of 6-NBDPE and 12-NBDPE loops back and preferentially locates itself at the membrane surface, while in their studies with NBD-cholestrol, the NBD moiety is deeply embedded in the membrane, presumably because the rigidity of the cholestrol structure prevents the fluorescent moiety from looping back as observed for 6C-NBDPE and 12C-NBDPE. For NBDCOQ, it appears that any tendency for the NBD moiety to draw the quinone moiety to the membrane surface is counteracted by the hydrophobic and rigid character of the isoprene side chain. Nevertheless, the similarity between NBDCOQ and NBDPE mobilities suggest that the diffusion of Q_{10} is limited by the diffusion of the phospholipid. This seems to be in agreement with the free-volume theory postulated by Cohen and Turnbull (1959), which predicts that a solute can diffuse no faster than its solvent since a diffusive step of the solute is only completed when a solvent molecule moves into the void left by the solute (Vaz et al., 1984). If Q_{10} diffuses at the same rate as a phospholipid, it would imply that Q_{10} does not occupy a completely independent phase but rather that it is solvated to some degree by the phospholipid. It is possible that if Q_{10} is strictly located in the mid-plane of the bilayer, it could diffuse very rapidly (Millner & Barber, 1984) since the resistance would be minimal and such diffusion would not be limited by diffusion of the phospholipids. A model consistent with these data would have the major part of the isoprene chain in the mid-plane while the head group penetrates to some degree among the phospholipid acyl chains. Alternatively, even if totally restricted to the mid-plane, there is no evidence for a fast diffusion rate in such a phase.

Fato _et_ _al_. (1985, 1986) using fluorescent quenching techniques report a diffusion coefficient for Q_{10} as $1\ \mathrm{x}10^{-6}\ \mathrm{cm}^2\mathrm{sec}^{-1}$, which is 2-3 orders of magnitude higher than diffusion values reported by the technique of FRAP. Fluorescence quenching supposedly measures only short range diffusion while FRAP measures long range diffusion and it is argued by Lenaz and co-workers that only short range diffusion is of physiological significance (Fato _et_ _al_., 1986). However long range diffusion measurements in vesicles should be similar to short range diffusion as there are no proteins to retard the movement (Vaz _et_ _al_., 1984) and a diffusion value of $1\ \mathrm{x}10^{-6}\ \mathrm{cm}^2\mathrm{sec}^{-1}$ is still two orders of magnitude higher than what we observe by FRAP in artificial vesicles. Diffusion rates in the order of 10^{-5}-$10^{-8}\ \mathrm{cm}^2\mathrm{sec}^{-1}$ have been measured by the technique of FRAP. Diffusion rates in the order of 10^{-5} -$10^{-7}\ \mathrm{cm}^2\mathrm{sec}^{-1}$ have been measured for a fluorescent fatty acid in phospholipid monolayers (Teissie _et_ _al_., 1978). The authors propose the relative slower diffusion in the bilayer membranes could be explained by each leaflet having a drag effect on the other which brings about slowing down of the translational diffusion of the molecules. In addition, Fahey & Webb (1978) have measured diffusion of dioctadecylindotricarbocyanine (DiI) to be 10^{-7} cm^2sec^{-1} in black lipid membranes and 10^{-8} cm^2sec^{-1} in large bilayer vesicles. These results indicate that the diffusion rates we measure are not limited by the FRAP technique.

With regards to the collisional quenching analysis by Lenaz and coworkers, numerical analysis by Blackwell _et al_. (1987) has shown diffusion-controlled quenching results in a non-linear concentration dependence for diffusion coefficients less than or of the order of 10^{-6} cm^2sec^{-1} and hence lateral diffusion coefficients in membranes are typically overestimated by an order of magnitude or more. They present an alternative empirical method to calculate the diffusion coefficients and have determined the diffusion of plastoquinone and plastoquinol to be 1.3-3.5x 10^{-7} cm^2sec^{-1} in phospholipid membranes. It appears that the very high diffusion coefficients reported by Fato _et al_. are likely to be overestimates for the reasons pointed out by Blackwell _et al_. (1987). They use the modified Smoluchowski's equation (Lakowicz and Hogen, 1980) which assumes the quenching and quenched molecules are spherical and tumbling isotropically in the membrane. The diffusants, Q_{10} and anthroyloxy fatty acids are not spherical and membrane diffusion is likely to be 2-dimensional rather than 3-dimensional, and the orientation is unlikely to be isotropic. In fact, it has been shown that the motion of the anthracene ring of n-(anthroyloxy) fatty acids when incorporated into phospholipids, is hindered and rotational constraints would make its motion highly anisotropic (Badley et al., 1973).

Thus it is reasonable to conclude that the value of 10^{-6} cm^2sec^{-1} originally calculated by Fato et al. (1986) is an overestimate by atleast an order of magnitude (as discussed by Blackwell et al., 1987), but that still leaves an order of magnitude difference between the FRAP analysis and the collisional quenching analysis for Q_{10} diffusion in artificial membranes, which cannot be accounted for by the interference of proteins in long range diffusion.

Location of ubiquinone in the membranes

Fluorescence quenching and emission studies of NBDCOQ in our lab are consistent with a location for NBDCOQ deep in the membrane. The emission maximum of NBDCOQ is blue shifted at least by 10 nm compared to a head group labeled phospholipid in mitochondria and vesicles. In mitochondria, no quenching of NBDCOQ was observed while NBDPE was totally quenched indicating a location for NBDCOQ near the center of the bilayer. However, this does not rule out some transverse motion of the head group as our studies are not capable of locating such motion.

Though a variety of physical techniques have been applied to probe the location of ubiquinone,its location in the membranes remains unclear. Most of the studies suggest that ubiquinone is located in a hydrophobic region but there is little agreement as to orientation, state of aggregation, and the possible existence of more than one population in the membrane. NMR has been used by a number of investigators to locate the position of ubiquinone in unilamellar and multilamellar vesicles and the chemical shift values of the methoxy resonances has proved valuable in these studies. Short chain ubiquinones generally exhibit a single chemical shift (3.96ppm) for the methoxy resonances while the long chain ubiquinones (Q_{9-10}) showed a doublet (3.96,3.80ppm). The chemical shift at 3.96 ppm is similar to the short chain ubiquinones, while the methoxy resonance at 3.80 ppm is up field shifted implying it is in a more hydrophobic medium. The relative intensity of the two peaks seems to be dependent on a number of parameters including the composition of the phospholipid, the diameter of the phospholipid vesicles and whether the phospholipid vesicles were unilamellar or multilamellar. Kingsley and Feigenson (1981) have performed NMR experiments using DMPC perdeuterated unilamellar vesicles. They observe the up field shift of the methoxy resonance only at concentrations >1 mol%. They interpret their results to mean that, the down field peak (3.96ppm) is from ubiquinone dispersed in the phospholipid layer while the up field peak is from ubiquinone in a separate ubiquinone rich phase. Although ubiquinone may exist as a separate phase, it seems to experience considerable local motion since the line widths were narrow. The authors also propose that two types of vesicles may exist with different ubiquinone/phospholipid ratios. Ulrich et al. (1985) make similar observations in their studies using DMPC and DPPC unilamellar vesicles. They also measured the reduction of entrapped ferricyanide in vesicles containing Q_2 and Q_{10} by externally added reductants and they observe that reduction by Q_{10} containing vesicles was relatively less and propose that one essential property of the long chain quinones is their residence in the hydrophobic core. Michaelis and Moore (1985) have performed NMR studies of Q_{10} in egg phosphatidylcholine (having polyunsaturated fatty acids of differing acyl chain lengths) to see if the location of Q_{10} is different than that seen by Kingsley & Feigenson (1980) and Ulrich et al., (1985). After short sonication times (20 minutes), they observe that most of the lipid is in 1000 A^o vesicles and some in vesicles of size of 500-700 A^o and they also observe two methoxy resonances, (3.82, 3.98 ppm) with most of the signal at 3.98 ppm. However, as the sonication time is increased, the relative number of small vesicles increases and after 150 minutes only small vesicles are present (500-700 A^o) and the low field signal (3.98 ppm) almost disappears. They also studied the affect of lanthanide shift reagents on methoxy resonances and they do not see any effect. They conclude that Q_{10} is incorporated at least beyond C-2 of the acyl chain in the membrane and the appearance of the two methoxy resonances is a curvature affect. However, the latter observation may just reflect partitioning of Q_{10} into the membranes as these studies were performed at 20^{o} C while incorporation in the other studies (Kingsley & Feigenson, 1981; Ulrich et al., 1985) was performed at higher temperatures.

Quinn's lab (Ondarroa & Quinn, 1986) has studied the location of Q_{10} in multilayer liposomes and they observe only one signal (at low field, 3.80 ppm) for the methoxy resonance up to concentrations of 20 mol %. Their studies (Katsikas & Quinn, 1981) and those of others (Aranda & Gomez-Fernandez, 1985), using differential scanning calorimetry have shown that Q_{10} up to 10 mol % does not effect the T_m of the phospholipid and it has been suggested that Q_{10} is preferentially located between the monolayers of the bilayer. Their NMR studies show some interaction between the Q_{10} molecules in the membrane but they also and others, (Kingsley & Feigenson, 1981; Ulrich et al., 1985) observe high resolution of the methoxy resonances which implies significant local motion. Hence they propose a alternative to the mid-plane model, where micelles or aggregates of Q_{10} can exist, which has a diameter similar to that of the bilayer. However, they agree there is not enough evidence in favor of one or the other.

Using X-ray diffraction, electron microscopy, and especially ^{13}C NMR, Stidham et al. (1984) propose a different model. Their ^{13}C NMR spin-lattice relaxation measurements show a marked effect of the ubiquinone on the lipid hydrocarbon chain atoms near the polar region and no effect on the atoms at the end of the acyl chain, which provides evidence that Q_{10} head group is situated near the polar end of the lipid hydrocarbon chain. This may represent only a fraction of Q_{10}, the remaining Q_{10} existing as a separate phase in the mid-plane of the bilayer and it is conceivable that the perturbation by Q_{10} of the terminal atoms is not sufficient to affect the relaxation times. However Ulrich et al. (1985) observe the perturbation of the terminal proton resonances of the hydrocarbon chain by Q_{10}. These data again raise the issue of two populations of quinone in the membrane. The relaxation measurements of Stidham et al. (1984) may be detecting a small population that may not be measured by other techniques. Indeed, surface pressure studies indicate that only a small percentage of the Q_{10} could intercalate with the phospholipids and be present near the membrane surface (MacDonald, 1987).

MacDonald (1987) discusses partitioning of hydrophobic molecules into a membrane in terms of thermodynamics. He proposes that molecules having the same surface pressure as a phospholipid can freely intercalate with the phospholipids. However if the surface pressure is low, intercalation is not favorable and most of the molecules would partition deeper into the membrane. The surface pressure of Q_{10} is fairly low (12 dynes/cm) compared to the surface pressure of a phospholipid (35 dynes/cm) (Quinn & Esfahani, 1980) and hence it is likely that major portion of Q_{10} partitions deep into the membrane. It is likely that the small fraction of Q_{10} which is located near the surface of the membrane may be of functional significance as the oxidized and reduced quinones seem to have different locations in the membrane. Aranda et al. (1986) observe that the reduced and oxidized Q_{10} behave differently in their studies using DPPC multilamellar vesicles. Using Fourier transform infra red (FT-IR) spectroscopy, they observe that reduced Q_{10} affected the transition temperature of DPPC (T_m=38.5^{o}C) while Q_{10} had no effect (T_m=41.5^{o}C). They (Aranda & Gomez-Fernandez, 1986) also observe that reduced Q_{10} increases the anisotropy of diphenylhexatriene while Q_{10} had no effect. Kingsley & Feigenson (1981) and Ulrich et al. (1985) also observe that the methoxy resonances are down field shifted when Q_{10} is reduced implying that Q_{10} in the reduced state lies closer to the membrane surface.

Schindler (1980) has measured the surface pressure as a function of the diameter of the vesicles. His studies show that surface pressure increases parabolically from diameters of 300 to 800 A^{o}, after which it remains a constant. This implies that the surface pressure of the sonicated vesicles and multilamellar liposomes will differ if the former have diameters<800 A^o. This may account for the appearance of two methoxy resonances in the studies of Kingsley & Feigenson (1980) and Ulrich et al., (1985). They use either DMPC (14:0) or DPPC (16:0) unilamellar vesicles, and these vesicles would tend to be small (diameters $\leq$300-400 A^o) and have low surface pressures allowing more intercalation of Q_{10} between the phospholipids. At a concentration of 1 mol% of Q_{10} in the studies of Kingsley & Feigenson (1980), the up field shift was virtually absent implying that all of the Q_{10} was dispersed in the bilayer. At higher concentrations of Q_{10}, the intensity of the down field signal increases suggesting that most of the Q_{10} is located deep in the membrane. Similarly, Ulrich et al. (1985), observe the high field signal only at concentrations higher than 2 mol%. The appearance of only one methoxy resonance in the studies of Michaelis & Moore (1985) can also be explained. The diameter of the vesicles used in their studies was in the order of 500-700 A^o and would have higher surface pressures and hence would exclude most of Q_{10} leading to the only methoxy resonance at 3.82 ppm.

Q_{10} is present to the extent of 1-2 mol % in mitochondria. The inner membrane of the mitochondria is highly convoluted and hence may exhibit curvature effects and in addition, the inner membrane has a high protein concentration and the presence of Q-binding proteins may affect its orientation and location. The surface pressure of a biological membrane is considered to be the same as that of a planar bilayer but higher values have also been suggested (Conrad & Singer, 1981). The spectrophotometric detection of Q_{10} in native membrane has suggested a location in the hydrophobic region (Chance, 1965). Chatelier and Sawyer (1985) have tried to locate the position of Q_{10} in the native membranes by fluorescence quenching techniques using n-anthroyl fatty acids and they conclude that there are two populations, one near the center and one near the surface of the bilayer. However, for their studies, they measure the quenching of exogenously added Q_{10} and it is known that Q_{10} does not easily partition into the membrane (Degli Esposti _et_ _al_., 1981) and it is possible that the quenching observed by the short chain anthroyl fatty acids could be that of Q_{10} adhering to the surface of the membrane. It is still possible that two populations of Q_{10} are present in the mitochondrial membrane, and a more sophisticated and sensitive analysis should provide a more definite answer.

Although there appears to be a considerable amount of conflicting experimental data regarding the location of Q_{10} when all the potential sources of artifacts are taken into consideration a reasonable picture emerges. The sources of experimental difference have been incomplete incorporation, size of the vesicles and hence the difference in internal pressure, and the limitation and sensitivity of the techniques applied. We postulate that most of the evidence is consistent with majority of Q_{10} occupying a position in the mid-plane of the membrane but in equilibrium with a small portion (especially the reduced form) lying close to the membrane surface and this may be of functional significance in electron and proton transfer.

Electron transfer through Q pool.

Rate of electron transfer through Q segment of the electron transport chain has been observed to follow the simple relationship

$$V_{obsd} = \frac{V_{red} \cdot V_{ox}}{V_{red} + V_{ox}}$$

where V_{obsd} is the overall observed rate, V_{red} is the rate of quinone reduction and V_{ox} is the rate of quinone oxidation. The above expression is known as the homogeneous pool equation. Evidence that Q_{10} acts as a homogeneous quinone pool has been obtained by a number of investigators including Kroger and Klingenberg (1973a, 1973b); Ragan & Heron (1978); Trumpower (1978); Unden & Kroger (1981); and Schneider et al., (1982). A simple Q pool behavior can be explained as follows. In a system in which Q_{10} acts as a freely and rapidly diffusing entity between reductases and oxidases, which are themselves randomly diffusing in the membrane, it follows that electrons donated into the Q pool by any quinone reductase can be withdrawn with equal probability by any quinol oxidase. The quinone redox state is therefore a function of the total activity of the quinone reductases and oxidases, regardless of the numbers of molecules of Q_{10} actually present. Any restrictions on quinone and protein diffusion or a non-random distribution of proteins in the membrane, could lead to deviation from simple Q pool kinetics (Ragan & Reed, 1986).

Kroger & Klingenberg (1973a, 1973b) have measured the redox kinetics of Q_{10} in uncoupled mitochondria and have correlated this to the overall rate of electron transfer. They found that more than 80% of Q_{10} responded to changes in respiratory activity and the response was kinetically homogeneous. Ragan and Heron (1978, Heron et al., 1978) have studied the electron transfer between complex I and complex III in artificial membranes at different protein:phospholipid ratios and at different complex I:complex III ratios. At high protein concentrations, the pool behavior was lost and this was attributed to the formation of associations between complexes I and III at 1:1 molar ratios which permitted direct electron transfer through the bound quinone. When the experiments were performed at lipid:protein ratios representative of the native membrane, the pool behavior was observed. They proposed that electron transfer from NADH to cytochrome c takes place only through complex I-complex III units which, however, are formed and reformed at rates that exceed the rates of electron transfer from complex I to complex III. This proposal accounted for Q pool behavior and flexible stoichiometry of dehydrogenases to cytochromes, but still assigned a special function to the association of complex I and complex III. However, in a later review (Ragan & Reed, 1986), the importance of the experiments performed at high protein concentrations was discounted, since these were non-physiological conditions and might not reflect the organization of the electron transfer components in the native membrane. Pool behavior of Q_{10} is also indicated in experiments of Schneider et al. (1982) where increasing the phospholipid content of the mitochondrial membranes decreased the electron transfer rates from NADH and succinate dehydrogenase to cytochrome c. This effect was reversed when Q_{10} is added along with the phospholipids.

Yu's lab (Yu & Yu, 1980) and King's lab (King & Suzuki, 1984) promote the idea that functionally active ubiquinones are associated with apoquino proteins and therefore all quinone mediated reactions occur through protein bound redox reactions. They rule out any role for freely diffusing Q_{10} in electron transfer. Quinone binding proteins have been identified in complexes I, II and III and and it is proposed that such binding proteins are the actual electron carriers in ubiquinone dependent electron transfer (Yu & Yu, 1980, 1982a, 1982b; Yu et al., 1985; and Suzuki & King, 1983).

The mitochondrial membranes seem to be saturated with the levels of Q normally present in the membrane. Q_{10} is present at a total mole fraction of around 1% with respect to the phospholipid concentration,corresponding to an average volume concentration of around 20 mM in the hydrocarbon region of the membrane (Mitchell & Moyle, 1985). Norling et al. (1974) have systematically studied the effect of depletion and repletion of endogenous Q_{10} in beef heart mitochondria on NADH and succinate oxidase activity. They have devised a procedure for addition of Q_{10}, where all of the added Q_{10} seems to incorporate into the membrane. On depletion, the activity measured was almost zero and on addition of Q_{10} to levels present in the membrane, the activity was restored to 100%. Further incorporation did not result in higher activity though all of the Q_{10} seemed to undergo reduction consistent with the Q pool mechanism. Studies with yeast mutants (Beattie & Clejan, 1986) have shown that succinate-cytochrome c reductase activities can be restored by addition of Q_6 to levels comparable to the wild type. However, further activity could not be stimulated by Q_6 either in the wild type or in the mutants. In contrast, short chain Q analogs could stimulate activity many fold. These studies emphasize the importance of the structural specificities of biological quinones and suggest that studies with Q analogs may be too simplistically interpreted. Zhu et al. (1982) find simple Q-pool behavior even when a large proportion of Q_{10} was extracted. Studies in cultured neuroblastoma cells (Maltese & Aprille, 1985) where the levels of endogenous Q_{10} were reduced nearly to 50% by blocking the *de novo* synthesis of Q_{10}, did not affect the succinate-cytochrome c reductase activity, implying that Q_{10} may not be rate limiting. However, they do observe a decrease in cell cycling and hence they suggest that Q_{10} may play a role in the mitochondrial and cell proliferation other than in energy transduction.

In general, the majority of the available data support a pool behavior of ubiquinone under many conditions, implying that diffusion is reasonably fast ($\geq 10^{-9}$ $cm^2 sec^{-1}$) in agreement with the numbers measured in our experiments. However, deviation from homogeneous pool behavior has been observed in a variety of experimental situations (Gutman, 1985; Ragan & Heron, 1978; Cottingham & Moore, 1983; also see discussion in pages 74-80). The question remains as to whether the diffusion of Q_{10} is always free and random or whether it may be segregated into more than one pool under some conditions. It is also a major issue whether the diffusion of Q_{10} is ever (or always) rate limiting in electron transfer.

Is diffusion of Q_{10} rate limiting in electron transfer?

Hackenbrock's group from their diffusion measurements as a function of membrane dilution with phospholipids (Schneider _et_ _al_., 1982) and as a function of temperature (Hackenbrock _et_ _al_., 1986b), propose that diffusion of Q_{10} is rate limiting in electron transfer. They measured the activation energy (E_{act}) for diffusion of Q_{10} by measuring the temperature dependence of the diffusion of DiI (dioctyl or dihexyldecylcarbocyanine; a fluorescent lipid analog) under the assumption that DiI and ubiquinone will have the same temperature dependence for diffusion. The E_{act} for DiI and complex III were 12.04 kcals/mole and 10.8 kcals/mole, respectively. They have measured E_{act} for uncoupled succinate-cytochrome c reductase activity to be 12.9 kcals/mole. Comparing the temperature dependency of the overall kinetic process to the temperature dependency of the overall diffusion steps, they infer that diffusion of Q_{10} and its redox partners is rate limiting in electron transfer. However, the assumption that temperature dependence of DiI diffusion will be the same as native quinone is questionable since Q_{10} appears to occupy a separate phase in the membrane. Furthermore, if the protein distribution is non-random (see discussion on pages 74-80) quinone may not always be diffusing long distances and hence the E_{act} measurements may not reflect the real picture. Minimum E_{act} for diffusion of a phospholipid is around 7 kcals/mole when measured in various DPPC multilayer liposomes (Vaz & Hallman, 1983). Thus the E_{act} for diffusion of Q_{10} in the membrane may vary from 7-12 kcals/mole.

Another line of evidence presented by Hackenbrock's group (Schneider et al., 1982) for a rate limiting role of Q_{10} diffusion is the observation that electron transfer rates are lowered when mitochondrial membranes are enriched with phospholipids which is reversed when Q_{10} is incorporated into the membrane. Fato et al. (1986) present an alternative explanation for the decrease in electron transfer with membrane dilution. They propose the decrease is due to the concentration of Q_{10} falling below the k_m for the partner enzymes. The concentration of Q_{10} in the mitochondrial membrane is 20 mM (Mitchell & Moyle, 1985). The k_m values of complex III and complex I for Q_{10} in the mitochondrial membrane has been calculated to be 2.4 mM and 32 mM respectively and the authors argue that the known turnovers of complex I and complex III are sufficient to explain the decrease in activity of NADH:cytochrome c reductase due to dilution.

A rate limiting role for Q_{10} (Crofts & Wraight, 1983) has also been proposed from the studies of chromatophores from *Rhodopseudomonos sphaeroids*. They measured the second order rate constant of the reduction of cytochrome b_{561} by the quinol produced by the photoreduction in the reaction center and calculated a diffusion coefficient of 10^{-10} cm^2 sec^{-1} assuming that diffusion is rate limiting. They argue that the quinol release from the reaction center or its binding to the cytochrome complex are not rate limiting, suggesting that these steps constitute only a minor contribution to the rate constant for the reaction. Consistent with a rate limiting role for diffusion of Q_{10}, they also observed that there was a lag in cytochrome b reduction at low quinol concentrations or when the distance between reaction centers and cytochrome complex was increased by dilution with phospholipids.

In stacked chloroplasts, there is considerable evidence that the PS I and PS II units may be separated by large distances, with PS I (along with cytochrome b_6f) mainly in the non-appressed regions and PS II concentrated in the stacked membranes (Anderson, 1981). Plastoquinone mediates the transfer of electrons between PS II and cytochrome b_6f. These redox complexes appear to be separated over long distances and the distances to be travelled by quinone during one redox cycle may be as high as 200 nm each way (Millner & Barber, 1984). Hence a diffusion value in the order of 10^{-6} cm^2sec^{-1} was postulated as essential for electron transfer and it was argued that plastoquinone can accomplish this if it is in the mid-plane of the bilayer. However, it appears that it may not be necessary to ascribe all of the electron transfer between PS I in the exposed and PS II in the appressed region of the membrane to plastoquinone, as cytochrome bf complex may equally be distributed between stacked and unstacked membranes (Cox & Andersson, 1981; Anderson, 1982).

Lenaz's group (Fato _et_ _al_., 1985, 1986) have measured the diffusion of Q_{10} by collisional quenching and report a value of 1 $x10^{-6}$ cm^2sec^{-1}. They measured diffusion rates as a function of temperature and calculate the E_{act} to be 1.5-2.0 kcal/mole in contrast to values of 12 kcals/mole reported by Hackenbrock _et_ _al_., (1986b). They further calculate the second order rate constants for the reactions of Q_{10} with complex III and complex I, making use of the k_m values measured by other investigators. Comparing the calculated second order rate constant with the bimolecular collision constants determined by fluorescence quenching, they conclude that diffusion cannot be rate limiting because the latter constants are 3 orders of magnitude greater than the former. However, the accuracy and applicability of any of these numbers to electron transfer in mitochondria remain to be determined.

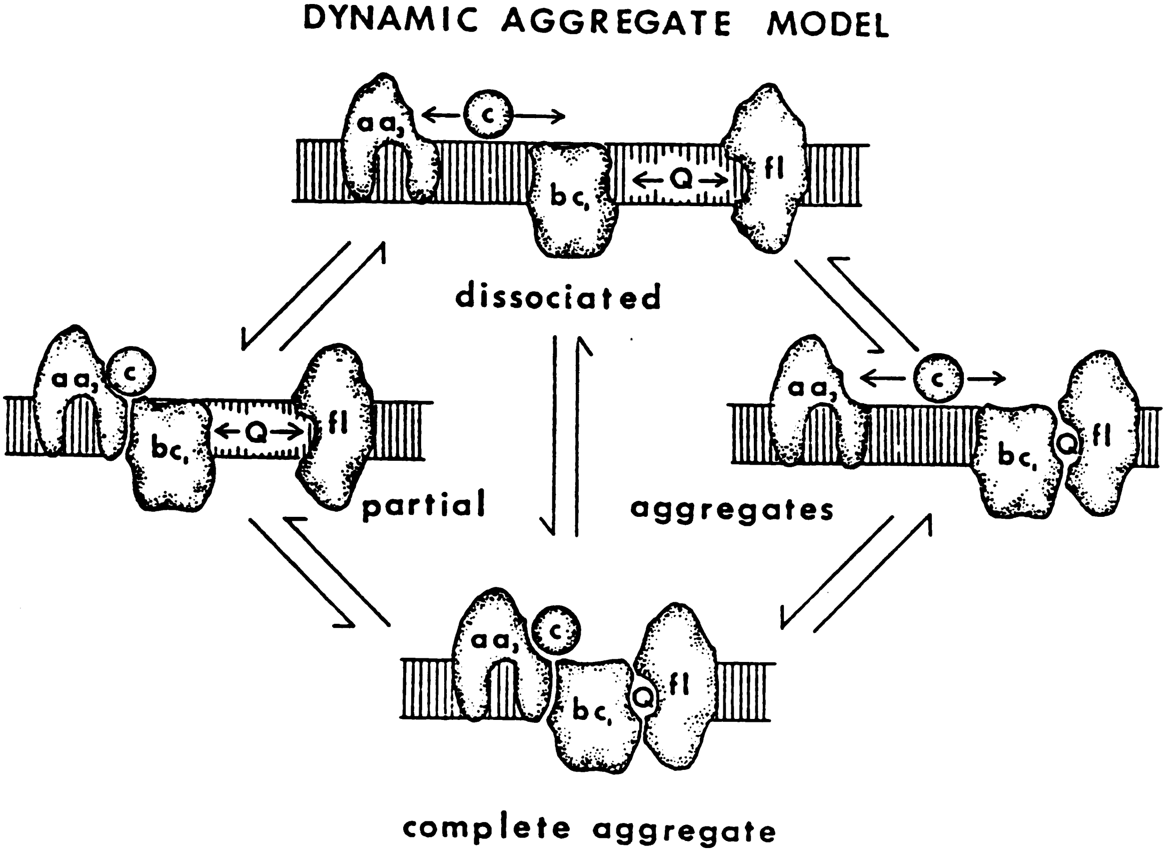
Whether the diffusion of Q_{10} is rate limiting or not is still an open question. Further insight into the ultra structure of the mitochondrial membrane and mechanism of energy transduction should provide a more definite answer.

Heterogeneity of mitochondrial membrane and and its implication in electron transfer through Q_{10}

Hackenbrock's group (Hackenbrock, 1976; Gupte *et al*., 1984; Hackenbrock *et al*., 1986a) propose a random collision model for electron transfer from their ultrastructural observations and diffusion studies of redox components. According to this model, all redox components are independent lateral diffusants, diffusion is long range, electron transfer is diffusion coupled and diffusion of the electron transfer components including that of Q_{10} is rate limiting in electron transfer. From earlier diffusion measurements of cytochrome *c* and cytochrome-c oxidase, we have postulated a dynamic aggregate model for electron transfer in the mitochondrial membrane (Hochman *et al*., 1983, 1985; Ferguson-Miller *et al*., 1986). According to this model, electron transfer can occur by random diffusion (Figure 13, top), but higher rates can be achieved by

Figure 13. The dynamic aggregate model of the mitochondrial respiratory chain. The major electron transfer components are represented in various states of aggregation (aa_3 = cytochrome oxidase; bc_1 = cytochrome $\underline{bc_1}$; c = cytochrome $\underline{c}$; fl = NADH or succinate dehydrogenase). The model assumes that all components are diffusing in or on the membrane, and that aggregates with significant lifetimes are formed between correct redox partners.

Figure 13


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

formation of complexes with life times that are significant compared to electron transfer rates. A crucial distinction between these models is that the former would predict a random distribution of components of the respiratory chain in the mitochondrial inner membrane, while the latter would imply non-random organization, at least in the time scale where multiple electron transfer can occur through the same two components.

It has become increasingly evident that the molecular constituents of biological membranes are not randomly organized within the bilayer matrix and many of the physical and functional properties are sensitive to the particular way in which lipid and protein molecules are distributed in the bilayer (Jain, 1983). Various types of ordered molecular arrangements are possible, ranging from non-specific aggregation or lateral phase separation to highly specific molecular interactions leading to the formation of complex structural patterns. In general, the lateral distribution of molecules in a lipid bilayer is dictated by the energetics of the interactions between the various components.

Non-randomness in the membrane seems to be the rule rather than the exception. Even a simple system of two phospholipids show phase separation (Klausner _et al_., 1980). In the mitochondrial membrane, different types of phospholipids exist at different concentrations and it has been shown that they are asymmetrically distributed between the two monolayers of the membrane (Krebs et al., 1979) and can exist in more than one phase in the mitochondrial membrane (Venetie & de Verkeij, 1982). There is also evidence for segregation of proteins in a number of systems (Friend,1982; Goldstein et al., 1979; De Petris,1979; Bretscher, 1983) including chloroplasts (Anderson, 1981; Staehlin, 1976) and mitochondria (Werner & Neupert, 1972; Silverstein & Rottenberg, 1987; Srere, 1982; Williams, 1983; Sjostrand, 1983).

When rat liver mitochondria were fragmented and separated by density gradient centrifugation, different fractions of the gradient varied in several aspects, especially in protein content and associated enzyme activities (Werner & Neupert, 1972). Time resolved anisotropy measurements in sub-mitochondrial particles have shown that nearly two third of the membrane proteins may exist as dynamic aggregates (Silverstein & Rottenberg, 1987). In the native state, mitochondria have cristae in which the inner membranes appear to be in close apposition under many conditions and separated approximately by 100-200 A^o (Srere, 1982). Intrinsic proteins extend into this space as much as 70 A^o. Observations from electron microscopy (Sjostrand, 1983; Williams, 1983) indicate that proteins are present in larger aggregates than single electron transfer complexes, though accurate size estimates are difficult from freeze-fractured studies. The reconstitution experiments (Ragan & Heron, 1978) have shown that at sufficiently high concentrations of complex I and complex III, there is a stoichiometric association between them. In such a case, no Q pool behavior was observed. Differential scanning calorimetric studies (Gwak et al., 1986) also have shown that there is a preferential interaction between complexes II and III. There is also circumstantial evidence for preferential interactions between specific proteins from co-purification of complexes: succinate dehydrogenase/ cytochrome bc_1 (Yu & Yu, 1980), NADH dehydrogenase/ cytochrome bc_1 (Hatefi et al., 1962), and cytochrome bf/ PS I (Boardman, 1971).

A non-random distribution of enzymes coupled with a limited path for Q_{10} implies that the Q pool would not be expected to behave homogeneously under all conditions since reduced Q_{10} produced by one substrate may not mix with that part of the Q pool associated with the other dehydrogenase before it is oxidized by the local oxidase (Ragan & Reed, 1986). Indeed, when kinetics of electron transfer through Q_{10} is measured in presence of both succinate and NADH, a simple Q pool behavior is no more observed (Gutman, 1985). The data is explained by postulating the presence of domains in which the redox state of the quinones may be different. Electron transfer in plant mitochondria show strong deviations from the Q pool behavior as electron flux between the cyanide sensitive and cyanide insensitive oxidase pathways can be strongly dependent on the nature of the substrate. In *Panicum Miliaceum* bundle sheath mitochondria oxidize exogenous NADH almost exclusively (95%) via the cyanide sensitive oxidase, while malate is almost exclusively (91%) oxidized via the alternative cyanide-insensitive oxidase (Gardestrom & Edwards, 1983). Similar trend has been also observed in mitochondria from spinach (Douce et al., 1977),cassava (Huq & Palmer, 1978), and sweet potato (Cottingham & Moore, 1983). However, in *A.maculatum* mitochondria, exogenous NADH is oxidized primarily by the alternative oxidase (Cottingham & Moore, 1983., Huq & Palmer, 1978) and a direct interaction between the external NADH dehydrogenase and bc_1 complex seems unlikely.

Above observations of non-random distribution of proteins in mitochondria and selective electron transfer through Q_{10} can be interpreted to mean that diffusion of Q_{10} is not random. A dynamic aggregate model and the non-random distribution of proteins would account for the function and location of Q_{10} in the mitochondrial membrane.

SUMMARY

It has become increasingly evident that knowing the correct lateral diffusion rates of ubiquinone is important for understanding its function as a transporter of reducing equivalents (electrons and protons) in the mitochondrial membrane.

The two studies performed so far give very different values. Gupte *et al*. (1984) have measured a diffusion coefficient of 3 $\times 10^{-9}$ $cm^2 sec^{-1}$ for a ubiquinone analog that has an alkyl side chain corresponding to only two isoprene units,using the technique of fluorescence redistribution after photobleaching (FRAP). It is known that short chain quinones orient themselves in a membrane similarly to a phospholipid. In fact, the diffusion rates reported for the ubiquinone analog are about the same as measured for a phospholipid. However, long chain quinones are located deeper in the membrane, probably in the mid-plane of the bilayer, raising the question of whether the quinone analog is giving a diffusion rate representative of native ubiquinone.

Fato *et al*. (1986) have measured the diffusion of native ubiquinone by the technique of collisional quenching using fluorescently labeled fatty acids. They report a diffusion coefficient of 1×10^{-6} $cm^2 sec^{-1}$, several orders of magnitude higher than the values measured by FRAP. Although it has been proposed that ubiquinone can have such high diffusion rates if located in the mid-plane of the bilayer, some of the assumptions made in calculating the diffusion coefficients are questionable, leading to concern that the high rates may not be valid.

To determine whether the long isoprene chain of native Q_{10} has an important influence on the mobility and location of the molecule, we have synthesized a fluorescent derivative of the head group moiety of ubiquinone and have measured its diffusion in giant mitochondria and very large unilamellar vesicles by the technique of FRAP. The diffusion coefficient of NBDCOQ was 3.1×10^{-9} cm^2sec^{-1} in mitochondria and 1.1×10^{-8} cm^2sec^{-1} in the vesicles. Similar diffusion rates were observed for a head group labeled NBD-phosphatidylethanolamine. Fluorescence emission studies carried out in organic solvents of different polarity, and in vesicles and mitochondria indicate that NBDCOQ is located in a more hydrophobic environment compared to NBDPE and IANBD. Fluorescence quenching studies carried out using $CuSO_4$, a water soluble membrane impermeable quenching agent, also indicate that NBDCOQ is located deeper in a membrane, compared to head group labeled NBDPE. These results imply that ubiquinone is oriented differently from a phospholipid in a membrane, but their diffusion rates are similar. This diffusion rate appears to be compatible with a random diffusion (Hackenbrock _et al_., 1986a), or a dynamic aggregate model of electron transfer (Hochman _et al_., 1985). Determining whether diffusion of Q_{10} is actually a rate limiting step in electron transfer will require a more detailed knowledge of the structural and kinetic properties of the electron transfer components.

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APPENDIX

PUBLICATIONS:

Rajarathnam, K., Hochman, J., Schindler, M., & Ferguson-Miller, S. (1987) Mobility and Location of NBD-Ubiquinone (NBDCOQ) in mitochondrial membranes and phospholipid vesicles. (manuscript in preparation, to be submitted to Biochemistry).

Ferguson-Miller, S., Rajarathnam, K., Hochman, J., & Schindler, M. (1988) Is electron transfer mediated by random diffusion alone? (manuscript in preparation, to be published as the proceedings of the `International Conference on Integration of Mitochondrial Function' Plenum, New York.

ABSTRACTS:

Rajarathnam, K., Hochman, J., Hupp, T., Schindler, M., & Ferguson-Miller, S. (1987) Lateral Diffusion of Ubiquinone (CoQ) in Mitochondrial Membranes and Phospholipid Vesicles, Measured by Fluorescence Redistribution After Photobleaching (FRAP). Fed. Proc. 46, 1930.

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Rajarathnam, K., Hochman, J., Hupp, T., Schindler, M., & Ferguson-Miller, S. (1987) Location and Diffusion of Fluorescently Labeled Ubiquinone (CoQ_{10}) in Mitochondrial Membranes & Phospholipid Vesicles Measured by Photobleaching and Fluorescence Quenching Techniques. Presented at the `International Conference on Integration of Mitochondrial Function' Chapel Hill, North Carolina.