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^{1}H-NMR STUDY OF THE LOCATION AND MOTION OF UBIQUINONES IN PERDEUTERATED PHOSPHATIDYLCHOLINE BILAYERS

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Summary

Ubiquinones (n = 1,2,3,4,7,9,10) and ubiquinols (n = 1,2,3,4,10) were incorporated into ordinary (protonated) or perdeuterated dimyristoyl phosphatidylcholine vesicles and were found to have significant local molecular motion. The motion of the quinone ring, as judged from the linewidth of the OCH_3 proton resonances, decreased in longer-chain ubiquinones. Minimum values for the transverse mobility (flip-flop rates) of ubiquinones-1,2,3,4,10, measured with the aid of lanthanide shift reagents, suggest that they are all able to function in a protonmotive 'Q cycle' during electron transport. As the length of the side chain increases beyond 1 isoprenoid unit, the quinone/quinol ring tends to be deeper in the outer monolayer of small sonicated vesicles and in both monolayers of larger freeze-thaw vesicles, but little or no change in depth is observed in the inner monolayer of small vesicles. The ubiquinol rings are closer to the membrane surface than are the ubiquinone rings. For side chain n = 9 or 10, a second resonance from the OCH_3 protons of ubiquinones and ubiquinols in vesicles appears in the ^{1}H-NMR spectrum. This is due to the presence of two types of vesicles with different ubiquinone/phospholipid ratios.

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Nomenclature: The term 'ubiquinone' or 'Q_{10}', when used in discussions of electron translocation, refers to all oxidation states involved in the process: quinone, semiquinone, and quinol.

Abbreviations: Q_n (Q_nH_2), oxidized (reduced) ubiquinone with n isoprene units in the side chain; DMPC-d_{72}, perdeuterated dimyristoyl phosphatidylcholine.

Introduction

Ubiquinone (Q_{10}) is an obligatory component of the electron transport chain of oxidative phosphorylation (see reviews [1—3]). It has been suggested that ubiquinone transports protons across the mitochondrial membrane during electron transport by means of a 'protonmotive Q cycle', and similar transmembrane systems have been proposed for Q_{10} in bacteria and for plastoquinone in chloroplasts (see for example Ref. 4).

From the data of Kroger et al. [5], it can be concluded that Q_{10} would have to flip from one surface of the membrane to the other and back again at least four times per second in order to accomplish such vectorial transport if two electrons are transported each time, and eight times per second if only one electron is transported. Some workers have suggested a slow flip-flop of long-chain ubiquinones [1,6], although transport of electrons across artificial membranes has already been demonstrated [7—11].

Hauska and his coworkers [8—10] trapped ferricyanide inside vesicles containing different quinones and measured the rate of reduction of ferricyanide by added dithionite. They concluded that Q_1 and Q_2 were much slower than the longer-chain Q_n in transporting reducing equivalents across phospholipid bilayers and that the flip-flop of the quinone/quinol ring was not the rate-limiting step.

Although studies of the transport abilities of quinones are helpful in understanding the properties and function of quinones in biological membranes, they do not address directly the question of the actual rate of flip-flop in the membrane. If it can be assumed that the transport of reducing equivalents involves transmembrane movement of ubiquinone molecules rather than transfer of electrons to neighboring quinone molecules, then the transport event involves a chemical reaction on each side of the membrane, a flip-flop event, and, if the quinol is added to a preformed membrane, initial insertion of the quinol into the membrane. In each case the observed rate is a measure of the rate-limiting step, which may be different for different quinones, and thus provides a lower limit for the actual flip-flop rate of both the reduced and the oxidized forms. The rates measured by Futami et al. [10] are much faster than phospholipid rates (see for example Refs. 12—15) and are fast enough (for $Q_{n>2}$) to account for proton translocation in biological membranes.

Methods devised for measuring slow flip-flop rates are not suitable for measuring fast rates, e.g. removal or exchange of radioactively labeled molecules [14—16], conductance of asymmetric black lipid membranes [17], chemical reactions [18,19], and certain nuclear magnetic resonance (NMR) methods [12,13,20]. NMR offers the possibility of measuring flip-flop rates directly for many molecules by utilizing the theory of spin (e.g. protons of the OCH_3 groups of ubiquinone) exchange between two sites, for example the two surfaces of the bilayer, with different chemical shifts [21]. The two surfaces of the bilayer can be differentiated by the addition of a shift reagent (for reviews see Refs. 22—24). The resonance from the ubiquinone OCH_3 protons will appear as a single shifted peak if the molecule is in fast exchange between the two surfaces, and will appear as two peaks (one shifted and one unshifted) if the molecule is in slow exchange. In this case 'fast' and 'slow' are relative to the

chemical shift difference between the two environments, which can be as little as a few hertz or as much as several hundred hertz.

The fact that no NMR studies of fast flip-flop rates have been reported is due presumably to interference from the intense phospholipid resonances. This interference can be greatly reduced in ^{1}H-NMR studies by the use of perdeuterated dimyristoyl phosphatidylcholine, DMPC-d_{72} [25—27]. ^{1}H-NMR spectra have been observed from many different molecules, including Q_{10} [26], incorporated into DMPC-d_{72} vesicles. We report here the results of NMR studies of the mobility and location of ubiquinones in sonicated DMPC-d_{72} vesicles.

Materials and Methods

Ubiquinone-10 (Q_{10}), Q_6, and cross-linked Sepharose 4B were from Sigma, St. Louis, MO; Q_0 was from Pfaltz and Bauer, Stamford, CT; other ubiquinones (Q_n) were a gift from Gunter Hauska; lanthanide salts were from Alfa Ventron, Danvers, MA and from Research Chemicals, Phoenix, AZ. Lanthanides were usually obtained as the chlorides or nitrates, though occasionally the oxides were converted to these forms by lyophilization from dilute hydrochloric or nitric acid.

The Q_n were stored as ethanolic solutions at −20°C; only Q_{10} precipitated and had to be redissolved with gently warming. Concentrations were determined, except for Q_0, by the absorbance at 275 nm, assuming an extinction coefficient of 14.3 $mM^{-1} \cdot cm^{-1}$ [28]. Q_0 in ethanol was found to have an absorbance maximum at 265 nm with an extinction coefficient of 13.2 $mM^{-1} \cdot cm^{-1}$ [29].

DMPC-d_{72} was prepared as previously described [25] with a few modifications [29].

For vesicle samples (3 mol % Q_n unless otherwise stated) the usual procedure was to place the ethanolic ubiquinone solution in a 15 ml Corex tube and remove the ethanol in a stream of nitrogen. The DMPC-d_{72} in $CHCl_3$ was added and solvents were removed. The sample (10 μmol) was suspended, then lyophilized from 0.5 ml ^2H_2O and resuspended in 0.4 ml ^2H_2O or an appropriate salt solution. Small unilamellar vesicles were prepared by sonication in a bath-type sonicator (Laboratory Supplies Company, Hicksville, NY) for 10—30 min at 30°C or until nearly clear. Since Q_0 was easily lost when a sample was dried either with a stream of nitrogen or under high vacuum, it was added after the lipid was dried. In some cases Q_1 seemed to be lost under high vacuum, so these samples were dried only with a stream of nitrogen.

Freeze-thaw vesicles [30] were prepared from sonicated vesicle samples by freezing in liquid nitrogen for 1 min, thawing for 20 min at ambient temperature, and then sonicating for 45 s. These vesicles have diameters of approx. 500—2000 Å (Caffrey, M., personal communication).

Ubiquinols (Q_nH_2, reduced ubiquinones) were prepared by the addition of one or a few microliters of a freshly prepared, deoxygenated solution of sodium dithionite (sodium hydrosulfite, $Na_2S_2O_4$) to the sonicated ubiquinone sample. The extent of reduction or oxidation was determined from the ^{1}H-NMR spectra (see below).

^{1}H-NMR spectra were obtained on a Varian CFT-20 NMR spectrometer

operating at 79.54 MHz or a Bruker WH-360 NMR spectrometer operating at 360 MHz. Chemical shifts are referenced to internal tetramethylsilane at 0 ppm in C^2HCl_3 and to internal sodium 3-trimethylsilylpropionate-2,2,3,3-d_4 in ^2H_2O. Chemical shifts in the presence of shift reagents were measured relative to the phospholipid acyl resonances when these were resolved, or else relative to the ubiquinone side chain resonances.

The fractions from the Sepharose 4B column were analyzed for lipid using a fluorescence assay [31] and for Q_{10} by measuring A_{275} in 1% sodium deoxycholate.

Results

^{1}H-NMR spectra of ubiquinones in organic solvents

The 360 MHz ^{1}H-NMR spectrum of Q_{10} in C^2HCl_3 is shown in Fig. 1. Assignment of peaks is based on a previously published spectrum [32] and on the 80 MHz spectra of Q_n with shorter side chains [29]. The resonances from the two types of methylene protons on the side chain (an A_2B_2X system) were assigned by decoupling the vinyl protons at −5.12 ppm; the downfield multiplet centered at −2.059 ppm changed from four peaks (typical of A_2B_2X) to three peaks (typical of A_2B_2), in new chemical shift positions, while the upfield triplet centered at −1.974 ppm remained unchanged [29]. The downfield multiplet is thus assigned to the $-CH_2-CH=$ methylene protons, and the upfield multiplet to the $-CH_2-C(CH_3)=$ methylene protons. The resonances from the OCH_3 protons show a solvent-dependent shift, appearing in dilute samples (1—2 mg/ml) at −3.98 and −3.99 ppm in C^2HCl_3, at −3.95 and −3.96 ppm in $C^2H_3O^2H$, and at −3.92 ppm in CCl_4.

In all ^{1}H-NMR spectra of Q_{10} in organic solvents, as in a previously published spectrum [32], there are small resonances near −0.9 and −1.25 ppm not assign-

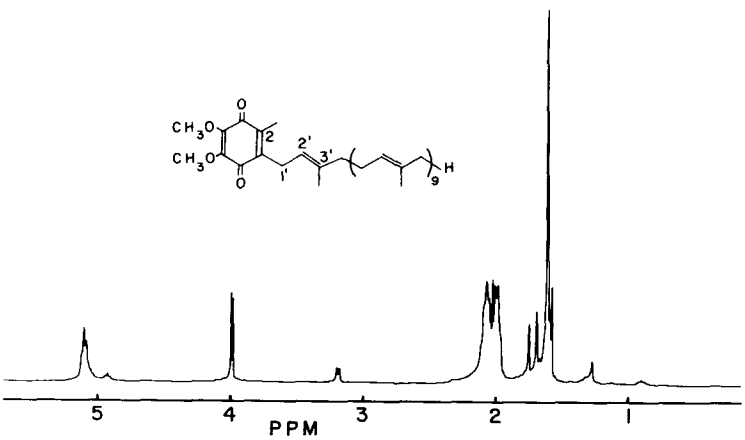

Fig. 1. 360 MHz ^{1}H-NMR spectrum of Q_{10} in C^2HCl_3, 24 transients with a 2-s acquisition time and a sensitivity enhancement giving 0.5 Hz line broadening. Assignment of peaks (see Ref. 32): −1.598 ppm, isoprenoid CH_3; −1.682 ppm, terminal *trans* CH_3; −1.741 ppm, methyl on C3′; −1.974 ppm; CH_2-$C(CH_3)$=CH; −2.014 ppm, methyl on C2; −2.059 ppm, CH_2-CH=C; −3.186 ppm, 1′ CH_2; −3.981 ppm and −3.995 ppm, OCH_3; −5.119 ppm, vinyl CH; −0.9 ppm, −1.3 ppm, and −4.9 ppm, impurities.

able to Q_{10}. The source and nature of this minor impurity are not known. Similar minor impurities were detected in all the other Q_n. The sample of Q_2 contained intense resonances in this region which were not eliminated after silicic acid chromatography, and thus this sample was unsuitable for further study.

In the ^{1}H-NMR spectra of ubiquinols in C^2HCl_3, the methoxy resonances are shifted upfield from −3.98 and −3.99 ppm to a single resonance at −3.89 ppm and the 1′ CH_2 resonance is shifted downfield from −3.18 to −3.34 ppm. These differences allow determination of the degree of oxidation or reduction of the quinone.

Location of ubiquinones in aqueous phospholipid systems

In order for ^{1}H-NMR studies to be used in measuring flip-flop rates, the quinone must partition almost exclusively into the phospholipid bilayer rather than into the water or micelles. The partition behavior of each ubiquinone was determined by suspending (without sonication) a mixture of 10 μmol DMPC and 0.3 μmol Q_n in 0.4 ml water and then centrifuging 30 min at 25 000 rev./min at 25°C in a Beckman 50Ti rotor. The A_{275} of the supernatant was measured and converted to Q_n concentration after correcting for light scattering by any phospholipid remaining in the supernatant. The only Q_n with significant levels in the supernatant is Q_0 (40%). The partitioning values for Q_0 and Q_1 are close to the values predicted from the partition coefficients given by Ragan [33]. Other workers [10] have found that none of the Q_n (n = 1,2,3,4,5,7,9, 10) are removed from vesicles on a Sepharose column, confirming the location of the ubiquinones in the lipid bilayer.

Slightly higher concentrations in the water are found for the short-chain ubiquinols (45% for Q_0H_2, 5% for Q_1H_2) prepared by suspending the samples in a dilute dithionite solution. An attempt to reduce the Q_n by addition of dithionite to multilamellar vesicles was unsuccessful except for Q_0, which became colorless almost immediately upon addition of the dithionite, consistent with

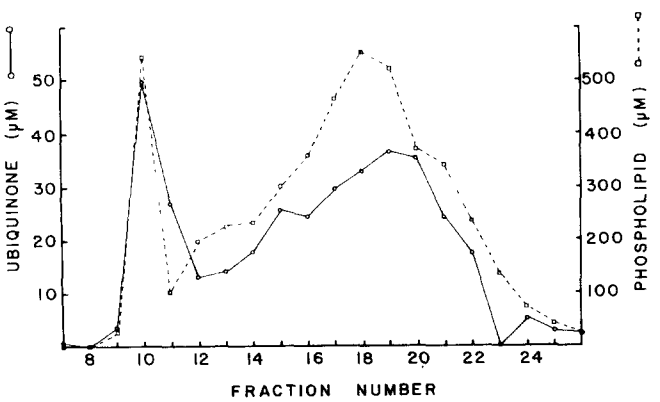

Fig. 2. Elution profile of Q_{10} (○———○) and DMPC (□- - - - -□) on a Sepharose 4B column. A sonicated suspension of 1.25 mM Q_{10} and 25 mM DPMC in 0.3 ml of 0.2 M $NaNO_3$ was loaded onto a 1.5 cm × 15 cm Sepharose 4B column and eluted with 0.2 M $NaNO_3$. The total lipid in each 0.75 ml fraction was measured by a diphenylhexatriene (DPH) fluorescence method [31], and the Q_{10} concentration was determined from the A_{275} in 0.8% potassium deoxycholate, pH 8.

the low water solubility of the other quinones and the high solubility of Q_0.

To confirm that Q_{10} was located in the bilayer rather than in some other form that was also pelleted during centrifugation, a sonicated sample of 5 mol% Q_{10} in DMPC was fractionated on a Sepharose 4B column (Fig. 2). No preference of Q_{10} for either large or smaller vesicles was observed, nor were any Q_{10}-containing particles smaller than vesicles discovered.

NMR spectra of Q_n in phospholipid bilayers

When a ubiquinone is sonicated together with ordinary protonated phosphatidylcholine into small unilamellar vesicles, resonances from the incorporated quinone are detected in the ^{1}H-NMR spectrum, but they are not completely resolved in dipalmitoyl phosphatidylcholine, above or below the phase transition (Fig. 3). Despite the observation of these signals, it has not been possible to determine peak areas because of extensive overlap from phospholipid resonances. Furthermore, the resonances from the ubiquinone OCH_3 protons were not visible after the addition of lanthanide shift reagents due to overlap with the shifted lipid proton resonances.

In contrast, the perdeuterated phospholipid DMPC-d_{72} had less than 1% of the ^{1}H-NMR signal intensity of the ordinary phospholipid at most positions, thereby enabling the accurate measurement of Q peak areas and positions, even in the presence of shift reagents. Q_n (n = 0,1,2,3,4 and 10) all give sharp resonances when incorporated into small unilamellar vesicles of DMPC-d_{72}. The spectrum of Q_4 in these vesicles is shown in Fig. 4. The most interesting signal, from the functional quinone part of the molecule, is the resonance of the six OCH_3 protons near −3.96 ppm. The chemical shifts of this signal for each of the above-mentioned ubiquinones in both the oxidized and reduced forms, and

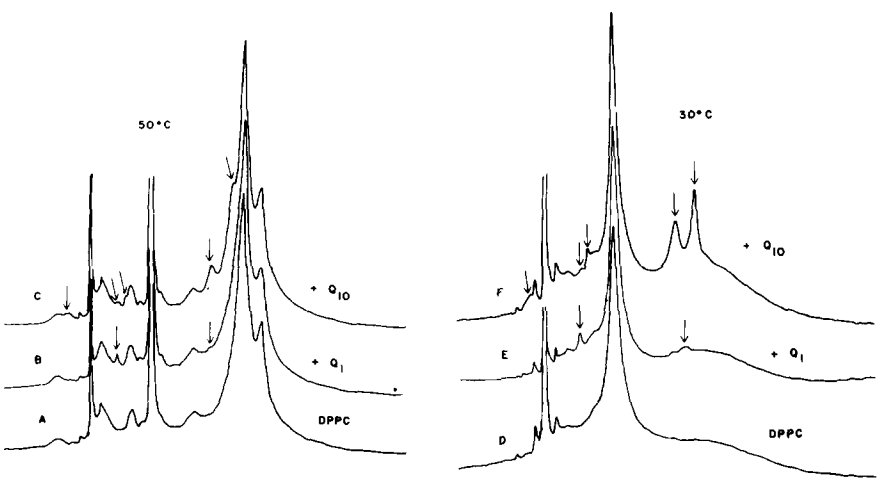

Fig. 3. 80 MHz ^{1}H-NMR spectra of Q_1 and Q_{10} in sonicated dipalmitoyl phosphatidylcholine vesicles, 2000 transients with a 0.5-s acquisition time and a sensitivity enhancement giving 0.6 Hz line broadening. Arrows point to visible Q_n resonances. Resonances from the choline methyl protons at 50°C, and from residual protons in the ^2H_2O at both 30°C and 50°C, have been truncated for purposes of clarity. (A)—(C), 50°C; (D)—(F), 30°C. (A) and (D), dipalmitoyl phosphatidylcholine. (B) and (E), 3 mol% Q_1 in dipalmitoyl phosphatidylcholine vesicles. (C) and (F), 5 mol% Q_{10} in dipalmitoyl phosphatidylcholine vesicles.

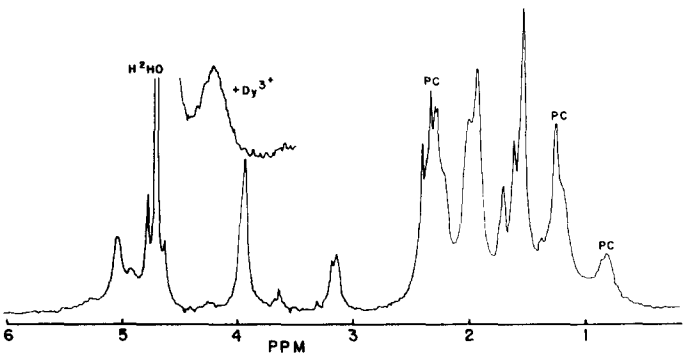

Fig. 4. 360 MHz ^{1}H-NMR spectrum of 3 mol% Q_4 in sonicated DMPC-d_{72} vesicles, 25 mM in lipid, 25°C, 400 transients with a 0.6-s acquisition time and a sensitivity enhancement giving 2 Hz line broadening. Resonances from residual protons on the DMPC-d_{72} are labeled PC. Assignment of Q_4 resonances: —1.55 ppm, isoprenoid CH_3; —1.63 ppm, terminal *trans* CH_3; —1.72 ppm, methyl on C3′; —1.98 ppm, isoprenoid CH_2 plus methyl on C2; —3.18 ppm, 1′ CH_2 and residual choline methyl protons; —3.96 ppm, OCH_3; —5.06 ppm vinyl CH. The inset spectrum, labeled $+Dy^{3+}$, shows the single shifted peak observed when 0.25 mM $Dy(NO_3)_3$ was added and equilibrated (90°C for 40 min), followed by removal of externally bound Dy^{3+} by the addition of 0.56 mM Na_2HPO_4, leaving Dy^{3+} bound only to the inner surface of the vesicles.

the linewidth in the oxidized form, are given in Table I. The minimum linewidth to be expected is about 4—6 Hz, due to the intrinsic chemical shift difference of the two different methoxy groups as observed in organic solvents (0.010—0.015 ppm).

Preliminary T_1 relaxation data, measured by the inversion-recovery method (180°-τ-90°), were not sufficient to determine the T_1 times accurately due to a poor signal-to-noise ratio and, in some cases, problems in accurately determining the baseline of the peak. However, all the T_1 times were between 0.15 and 0.60 s at 25°C at 360 MHz.

Q_{10} differs from the shorter ubiquinones in that two resonances from the OCH_3 protons are clearly visible (Fig. 5). One resonance has the same chemical

TABLE I

CHEMICAL SHIFTS AND LINEWIDTHS OF THE OCH_3 RESONANCES OF UBIQUINONES AND UBIQUINOLS IN DMPC-d_{72} VESICLES

360 MHz ^{1}H-NMR spectra were recorded at 25°C. Chemical shift values were generally reproducible to ±0.01 ppm.

n	Chemical shift		Linewidth (Hz) (oxidized)
	Oxidized	Reduced	
0	3.96	—	9
1	3.98	3.83	11 ± 2
3	3.97	3.83	21 ± 2
4	3.96	3.83	25 ± 3
10 *	3.96	3.83	30 ± 2
10 *	3.80	3.65	9 ± 1

* Two OCH_3 peaks were observed in the spectrum of Q_{10}.

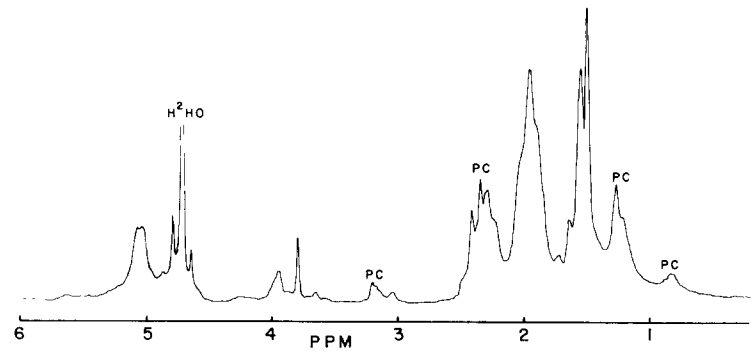

Fig. 5. 360 MHz ^{1}H-NMR spectrum of 2 mol% Q_{10} in sonicated DMPC-d_{72} vesicles, 25°C, 400 transients with a 0.6-s acquisition time and a sensitivity enhancement giving 2 Hz line broadening. Resonances from residual protons on the DMPC-d_{72} are labeled PC. Assignment of peaks: —1.5 to —1.6 ppm, isoprenoid CH_3; —1.96 ppm, isoprenoid CH_2 plus CH_3 on C2; —3.05 ppm, 1′ CH_2; —3.80 ppm and —3.96 ppm, OCH_3; —5.06 ppm, vinyl CH.

shift as the OCH_3 resonance of the other ubiquinones and a linewidth similar to that from $Q_{3,4}$ (Table I). The other resonance is shifted upfield by 0.15 ± 0.01 ppm and its relative intensity increases at very high mole fractions (10—25 mol%) while in DMPC-d_{72} vesicles containing 1 mol% Q_{10} the upfield peak is absent or at least greatly reduced in intensity (Fig. 6). These two peaks are observed whether the sample is sonicated in ^2H_2O or in 0.2 M $NaNO_3$, both above and below the phase transition in dipalmitoyl phosphatidylcholine (Fig. 3), and in membranes made from different phosphatidylcholines such as dilauroyl, distearoyl, and egg phosphatidylcholines [29]. The chemical shift difference between the two peaks is constant (0.15 ± 0.01 ppm), and a single peak in an intermediate position has never been observed. Similarly, the spectra of Q_9

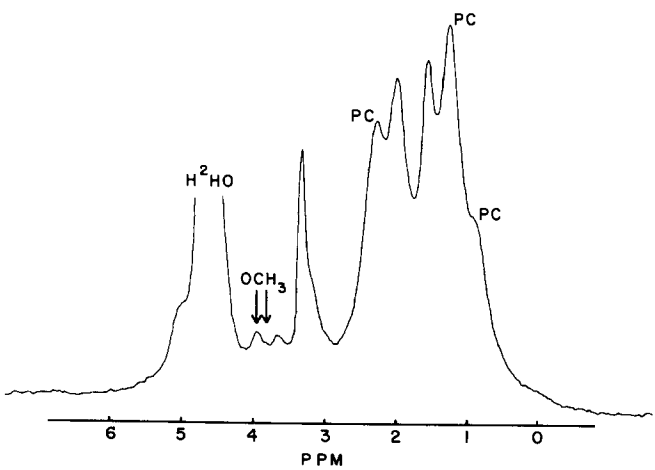

Fig. 6. 80 MHz ^{1}H-NMR spectrum of 1 mol% Q_{10} in sonicated DMPC-d_{72} vesicles, 30°C; 103 673 transients with an acquisition time of 0.5 s and a sensitivity enhancement giving 1.6 Hz line broadening. Resonances from residual protons on the DMPC-d_{72} are labeled PC. The positions of the OCH_3 resonances are indicated. The intense residual H^2HO peak has been truncated for purposes of clarity.

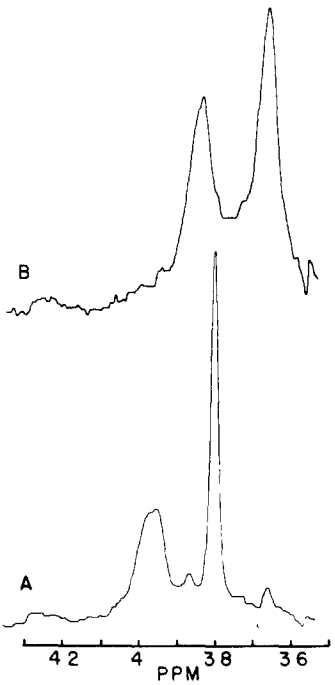

Fig. 7. Methoxy region of the 360 MHz ^{1}H-NMR spectra of 3 mol% Q_{10} and $Q_{10}H_2$ in sonicated DMPC-d_{72} vesicles, 25°C, 400 transients with a 0.6-s acquisition time and a sensitivity enhancement giving 2 Hz line broadening. (A) Q_{10}. (B) Sample A after the addition of enough dithionite to reduce all the Q_{10} to $Q_{10}H_2$.

(data not shown) and $Q_{10}H_2$ (Fig. 7) also display two peaks. In freeze-thaw vesicles, which are significantly larger than small sonicated vesicles, both OCH_3 peaks are also observed and a similar concentration dependence of the relative peak areas is found. In multilayers of DMPC containing 5 mol% Q_{10}, and in distearoyl phosphatidylcholine vesicles with 20 mol% Q_{10} at 30°C, well below the phase transition temperature of 58°C [34], only the upfield OCH_3 resonance is detected. Measurements of peak areas in the multilayer sample suggest that the vinyl CH resonance at −5.12 ppm arises entirely from Q_{10} molecules which are associated with the upfield OCH_3 peak and not from Q_{10} molecules which are associated with the downfield OCH_3 peak. No resonances from any part of the Q_{10} molecules are seen in a 1 mol% Q_{10} multilayer sample. It appears that in bilayers where phospholipid local molecular motion is sufficiently low, as in distearoyl phosphatidylcholine vesicles at low temperatures or in multilayers, resonances from Q_{10} molecules corresponding to the downfield OCH_3 peak are too broad to be detected in high resolution ^{1}H-NMR spectra.

To determine whether the second peak is due to degradation or a chemical modification of the ubiquinone during sample preparation, a sample whose ^{1}H-NMR spectrum displayed both OCH_3 resonances was lyophilized and dissolved in C^2HCl_3. Only the normal OCH_3 resonance was observed, indicating the absence of a chemical change.

The spectrum of a saturated solution of Q_{10} in CCl_4 was compared to the spectrum of a dilute (1—2 mg/ml) sample in order to find out whether a high

concentration of Q_{10} in an organic solvent could lead to a similar upfield shift of the OCH_3 resonance. The increased Q_{10} concentration resulted in a 0.05 ppm upfield shift of the OCH_3 resonance. This change in the chemical shift could result from ring-current effects of nearby quinone and quinol rings (see for example Refs. 35, 36).

To investigate the possibility that the two OCH_3 peaks were from separable vesicle populations, freeze-thaw vesicles were prepared from 0.3 μmol Q_{10} and 2.7 μmol egg phosphatidylcholine in 0.3 ml $^2H_2O/H_2O$ (20% ^2H_2O, v/v) and centrifuged 30 min at 40 000 rev./min (105 000 × *g*) in a 50 Ti rotor at 20°C. The supernatant and the pellet were lyophilized and then dissolved in dry C^2HCl_3, and the relative amounts of Q_{10} and egg phosphatidylcholine were determined from the 80 MHz ^{1}H-NMR spectrum. The pellet appeared to have approximately a 1/1 ratio of Q_{10} to egg phosphatidylcholine, whereas little or no Q_{10} was found in the supernatant (0—3 mol%); interference from the egg phosphatidylcholine resonances prevented a more quantitative determination of small amounts of Q_{10}. Attempts to assign one of the OCH_3 peaks to the pellet fraction and one peak to the supernatant fraction were hindered by the intense H^2HO resonance, even after each fraction was resuspended in ^2H_2O.

Both OCH_3 peaks from Q_{10} are affected by externally added and by internally trapped ions, both lanthanide shift reagents (see below) and the paramagnetic ions Mn^{2+} and Gd^{3+} [29], which cause an increase in linewidth, showing that both pools of Q_{10} are in some kind of vesicular structure.

Effects of shift reagents on ubiquinone spectra

For most studies with shift reagents, the DMPC-d_{72} vesicles were prepared in 0.2 M $NaNO_3$ in order to increase lanthanide ion binding to the surface of the vesicles [37—39]. The effectiveness of the lanthanides in shifting the external choline methyl resonance was decreased slightly by raising the temperature from 30°C to 50°C (0—5% decrease) and by the presence of 5 mM La^{3+} (5—10% decrease), but the presence of 3 wt.% Q_{10} in the bilayer and the addition of dithionite to vesicles containing Q_{10} had no measurable effect.

The impermeability of these vesicles to lanthanide ions was confirmed in control experiments with ordinary (protonated) DMPC. Using the choline $N(CH_3)_3$ resonances as markers for the inner and outer vesicle surfaces, the lanthanide-induced shift decreased by approx. 20% in 44 h, so leakage during the course of a lanthanide titration (about 4 h at 360 MHz) would be negligible.

After a lanthanide had been added to a vesicle sample, the lanthanide was sometimes equilibrated across the bilayer by heating the sample to 90°C for 40 min [40]. The heating procedure usually led to fusion or aggregation of the vesicles, resulting in much broader resonances. For this reason equilibration was sometimes accomplished by resonication after freezing and thawing the sample several times. Lanthanide ions were removed from the external solution by the addition of Na_2HPO_4, 2 mol per mol of lanthanide.

Pr^{3+} and Eu^{3+} are often used in studies of phospholipid bilayers because they cause very little line broadening [22,23,41]. However, these lanthanides are also very weak shifters [22,23,41]. Dy^{3+} and Tm^{3+} produced much larger shifts in the ubiquinone and ubiquinol OCH_3 resonances, thus extending the range of flip-flop rates which could be measured. Furthermore, because of the small con-

TABLE II

LANTHANIDE-INDUCED SHIFTS OF OCH_3 RESONANCES FROM UBIQUINONES AND UBIQUINOLS IN DMPC-d_{72} VESICLES

$Dy(NO_3)_3$ was added to sonicated dispersions of Q_n (or Q_nH_2) and DMPC-d_{72} in 0.2 M $NaNO_3$, and the induced shift of the OCH_3 resonances was measured in 360 MHz ^{1}H-NMR spectra at 25°C.

n	% Shift	Maximum observed shift	
		Oxidized	Reduced
1	100	354	216
3	33 ± 6	66	150
4	27 ± 4	114	120
10 *	18 ± 4	23 **	24
10 ***	35 ± 5	69	128

* Downfield Q_{10} resonances.

** This maximum value was obtained with $Tm(NO_3)_3$ at 80 MHz instead of $Dy(NO_3)_3$ at 360 MHz. Interference from the upfield OCH_3 peak prevented clear observation of more than a 15 Hz shift with $Dy(NO_3)_3$.

*** Upfield Q_{10} resonance.

centrations of Dy^{3+} or Tm^{3+} that were needed, it was easy to remove these ions from solution by the addition of inorganic phosphate.

When Dy^{3+} is added to a DMPC-d_{72} vesicle preparation containing ubiquinone or ubiquinol, the OCH_3 resonance in the ^{1}H-NMR spectrum is found to shift downfield (Table II) and to broaden. This indicates that this part of each Q_n or Q_nH_2 molecule spends some time near the outer interface of the vesicles.

TABLE III

LANTHANIDE-INDUCED SHIFTS OF Q_n OCH_3 RESONANCES WITH THE LANTHANIDE OUTSIDE, INSIDE, OR ON BOTH SIDES OF SMALL VESICLES

DMPC-d_{72} (25 mM) with 3 mol% Q_n was sonicated in 0.2 M $NaNO_3$ in the presence or absence of the indicated lanthanide nitrate. After the addition of lanthanide to lanthanide-free samples, the ions were equilibrated across the membrane either by heating at 90°C for 40 min (H) or by several freeze-thaw cycles followed by resonication (S). External lanthanide was removed by the addition of Na_2HPO_4, 2 mol per mol of lanthanide. Spectra of Q_{10} were at 80 MHz and 30°C, the others were at 360 MHz and 25°C.

Q_n	L^{3+}	Equilibration method	Induced shift (ppm)		
			Outside	Equilibrated	+Na_2HPO_4
Q_1	0.28 mM Dy	S	0.28	0.59	—
	14 mM Pr	H	0.21	0.28	—
Q_3	0.70 mM Dy	H	0.18	(0.5—1.33) *	—
Q_4	0.26 mM Dy	H	0.06	0.35	0.32
	0.40 mM Dy	S	0.10	0.48	0.31
Q_{10} **	0.8 mM Tm	S	<0.03	0.23	0.13
	2.0 mM Tm ***	S	—	0.26	0.13
Q_{10} §	0.8 mM Tm	S	<0.03	0.30	0.14
	2.0 mM Tm ***	S	—	0.36	0.14

* Obscured by the residual H^2HO peak.

** Downfield Q_{10} peak.

*** 37 mM DMPC-d_{72}.

§ Upfield Q_{10} peak.

In no case did the OCH_3 peak split into two separate peaks, one shifted and one unshifted. Table II also shows that the shift induced by Dy^{3+} on the outer surface of small vesicles depends upon the length of the quinone side chain.

In order to determine whether the presence of trivalent ions somehow affected the location of the ubiquinones in the bilayer, Q_1 and Q_4 samples were prepared in the presence of 5 mM $La(NO_3)_3$, and up to 0.4 mM Dy^{3+} was added to each sample. The presence of La^{3+} had no significant effect on the dysprosium-induced shift of the ubiquinone OCH_3 peak.

When the shift reagent is equilibrated across the membrane either by heating or by sonicating in the presence of the reagent, the induced shift of the OCH_3 peak of each of the ubiquinones is increased (Table III). This occurs despite a significant amount of the total shift reagent binding to the inside surface and a resulting decreased binding of Dy^{3+} to the outer surface as indicated by an approx. 50% decreased shift of the outer choline methyl signal.

In order to determine whether observed shifts are strongly influenced by membrane curvature, Q_4 was examined in small sonicated vesicles and in the larger, freeze-thaw vesicles. Shift reagent concentrations were chosen to give reagent binding in an 'outside only' experiment which was equal to the reagent binding at each interface in an 'inside and outside' experiment. Samples were prepared from DMPC-d_{72} containing 3 mol% Q_4, either in the presence of 1 mM Tm^{3+}, or else 0.5 mM Tm^{3+} was added afterward and 80 MHz ^{1}H-NMR spectra were recorded at 30°C. These two conditions were found to induce identical shifts in the choline methyl resonances in control experiments. The shift induced by external Tm^{3+} on the OCH_3 resonance is similar in freeze-thaw vesicles and in small sonicated vesicles (7 Hz). However, when samples are prepared in the presence of Tm^{3+}, the freeze-thaw vesicles show only about 40% of the shift observed in the small vesicles (21 and 52 Hz, respectively). The depth of the quinone ring of Q_4 in the outer monolayer is thus independent of membrane curvature, while the depth in the inner monolayer decreases as the vesicle size decreases.

Some information about the location of the quinone rings in the bilayer can be obtained by comparing the induced shifts of the phospholipid $N(CH_3)_3$ peak and the ubiquinone OCH_3 peak in samples sonicated in the presence of a lanthanide shift reagent. With 25 mM phospholipid and 0.3—0.4 mM Dy^{3+}, the OCH_3 shift for Q_1 was approximately 50% greater than the $N(CH_3)_3$ shift, indicating that the quinone ring of Q_1 is very close to the membrane surface. The Tm^{3+}-induced shifts of the Q_4 and upfield and downfield Q_{10} methoxy peaks were 60%, 40%, and 30%, respectively, of the Tm^{3+}-induced $N(CH_3)_3$ shift, indicating an increased average depth in the bilayer for the quinone rings of molecules with longer side chains. Because of unknown geometric factors and phospholipid head-group interactions on the surface, a more quantitative estimate of the depth cannot be made.

Resonances from the isoprenoid side chain of shorter ubiquinones and ubiquinols, and from the aromatic methyl group of Q_1 and Q_1H_2, are also shifted by shift reagents (Table IV). These shifts decrease with increasing distance from the quinone or quinol ring, confirming that the ring is, on the average, closer to the membrane surface than is the side chain.

When the ubiquinones are reduced to the ubiquinols by the addition of

TABLE IV

RELATIVE INDUCED SHIFTS OF RESONANCES FROM UBIQUINONES AND UBIQUINOLS IN DMPC-d_{72} VESICLES

Small, sonicated vesicles containing 3 mol% of the indicated Q_n or Q_nH_2 were prepared in 0.2 M $NaNO_3$, $Dy(NO_3)_3$ was added incrementally to each sample, and 360 MHz ^{1}H-NMR spectra were recorded. Chemical shift values are normalized to a value of $OCH_3 \equiv 10$ for each compound.

	OCH_3	CH_3 on C2	CH_3 on C3′	Isoprenoid CH_3	Terminal *trans* CH_3
Q_1	10	7	5	—	5
Q_1H_2	10	7	4	—	4
Q_3	10	—	6	3	3
Q_3H_2	10	6	4	3	2
Q_4	10	—	4	3	1
Q_4H_2	10	—	5	2	2

sodium dithionite, the effect of added Dy^{3+} on the induced shift of the OCH_3 resonance is increased by 50—100%. This effect is observed in Q_1, Q_3, Q_4 and the upfield peak in Q_{10}. Accurate measurements could not be made on the downfield peak in Q_{10} because it quickly became obscured by the upfield peak. This increased shift of the ubiquinol OCH_3 peaks compared to the ubiquinone peaks is not due simply to an increased preference for the outer surface compared to the inner surface, because it is also observed when the sample is sonicated in the presence of Dy^{3+}. For example, a 0.25 ppm induced shift of the OCH_3 resonance from Q_4 in a DMPC-d_{72} bilayer with Dy^{3+} on both sides was increased to 0.38 ppm by the addition of enough dithionite to reduce the Q_4 completely.

Discussion

Two ubiquinone pools

The observation of two distinct OCH_3 resonances from Q_{10} in bilayer vesicles is intriguing. The following explanations have been ruled out by experiments detailed in the Results section: the Q_{10} preparation could have an impurity; Q_{10} might undergo a chemical change during the sample preparation; the two signals could be coming from the two different OCH_3 groups; the two peaks could be from the oxidized and reduced forms of Q_{10}; there might be two pools of Q_{10}, on the inner and outer surfaces of the vesicles, in slow exchange; or Q_{10} might be both in bilayer vesicles and in micelles.

The most plausible explanation for the two OCH_3 peaks in the ^{1}H-NMR spectra of long-chain ubiquinones in phosphatidylcholine vesicles is that the downfield peak is from Q_n dispersed in a phospholipid bilayer, while the upfield peak is from Q_n molecules in a separate Q-rich phase. This assignment of peaks is based on the observation that the downfield signal is similar to the corresponding resonance from the shorter-chain ubiquinones in linewidth and chemical shift (Table I) while the upfield peak is different in these respects.

The idea of two separate pools of Q_{10} is supported by the finding that the chemical shift of the OCH_3 resonance from Q_{10} in CCl_4 is concentration-dependent, moving upfield at higher concentrations. More definitive evidence for this

hypothesis is the ability to separate on the basis of density a sample of 10 mol% in egg phosphatidylcholine into two fractions with very different ratios of Q_{10} to phosphatidylcholine. The observation of two distinct OCH_3 peaks, indicating slow exchange between the two populations of OCH_3 groups, is also consistent with a physical separation of the Q_{10} pools. In a sonicated sample both populations of Q_{10} molecules seem to be in particles of similar size, because no sign of heterogeneity was found on a Sepharose column (Fig. 2). Both populations are clearly closed vesicles since they are affected by ions in either the inner or external solutions.

The formation of separate Q-rich and phospholipid-rich phases is not surprising in light of the report that ubiquinones (n = 3 or greater) were 'squeezed out' of a phosphatidylcholine monolayer [42], the longer-chain quinones being squeezed out more easily than the medium-chain quinones. Maggio et al. [43] had previously reported that ubiquinones (n = 3,7,9) were only slightly able to penetrate phosphatidylcholine monolayers and seemed to form a separate phase in mixed monolayers.

The detection by NMR in aqueous phosphatidylcholine/Q_{10} systems of two Q_{10} pools, which were not distinguishable by either Sepharose column chromatography or centrifugation in H_2O, suggests that other dispersions of small molecules in phospholipids might also be heterogeneous, even though heterogeneities in such samples may not be detected by ordinary means.

The data presented here may help to explain the report of Futami and Hauska [44] that semiquinone formation was detected with Q_1 but not with Q_9. Under the conditions of their semiquinone experiments (5 mol% Q_n), much of the Q_9 (probably 60—80%) would be in Q_9-rich particles where semiquinones would probably be unstable due to rapid disproportionation. The small amount of semiquinone formed by the remaining Q_9 molecules would not have been detected in their system.

The discovery of two Q_{10} pools in a model system, even at low concentrations (2 mol% Q_{10}, approx. 2 wt.%), has some implications for the function of Q_{10} in biological membranes such as the inner mitochondrial membrane, which contains, based on all membrane components, 0.3—0.5 wt.% Q_{10} [2,5]. Since the inner mitochondrial membrane has 70—80% protein and 20—30 wt.% lipid [45], the effective concentration of Q_{10} in the lipid region is 1—2.5 wt.%. The ubiquinone molecules could form a Q_{10}-rich phase in such membranes, rather than being uniformly distributed, and this increased effective concentration might affect the kinetic properties of ubiquinone and the stability of ubisemiquinone.

Transbilayer flip-flop

When the ubiquinone or ubiquinol populations in small vesicle inner and outer monolayers are distinguished by means of chemical shift reagents, a single OCH_3 resonance is observed for all ubiquinones and ubiquinols studied and for all shift reagent concentrations examined. This means that the minimum transbilayer flip-flop rate is of the order of the lanthanide-induced shift so that the inner and outer magnetic environments be averaged. These rates range from at least 23 s^{-1} for Q_{10} to at least 216 s^{-1} for Q_1 (Table II). We emphasize that these are minimum rates whose values were determined not by motional considera-

tion, but rather by instrumental limitations, e.g. the value for Q_{10} could be much faster than 23 s^{-1}, but resonance overlaps precluded measuring larger shifts.

These minimum flip-flop rates can be compared with the minimum rates measured by Futami et al. [10]. With the assumptions of an inner radius of 100 Å, 4000 phospholipid molecules per vesicle, and two-electron transfers, their minimum flip-flop rates are approximately 12 times the rate constants given in their Table I, or 0.4 s^{-1} for Q_1, 0.7 s^{-1} for Q_2, and 15—22 s^{-1} for Q_3—Q_{10}. The rates measured by NMR thus support the conclusion of Futami et al. [10] that ubiquinone flip-flop was not the rate-limiting step in the transport of electrons from external dithionite to internal ferricyanide. They are also fast enough to allow both short- and long-chain ubiquinones to function in a protonmotive 'Q cycle' during electron transport. The ability of short-chain ubiquinones and ubiquinols to flip rapidly across the bilayer argues against the conclusion of other investigators [11,46] that the shorter-chain ubiquinols are held in one half of the bilayer with a very slow flip-flop rate.

Local molecular motion

The relatively narrow linewidths of the ubiquinone ^{1}H-NMR resonances in sonicated phosphatidylcholine vesicles indicate significant local molecular motion for all the ubiquinones and ubiquinols examined (Table I; Figs. 3—7). The local motion of the quinone ring appears to decrease as the length of the side chain increases. The Q_{10} molecules in Q_{10}-rich phase seem to have greater local motion than those dispersed in the phospholipid-rich phase, both in multilayers and in small unilamellar vesicles (Figs. 3,5,6,7 and Table I).

Location of ubiquinones

Since lanthanide-induced shifts decrease with increasing distance from the lanthanide ion, the relative shifts of the OCH_3 resonances of different ubiquinones, and of a given quinone in vesicles of different sizes, can give a qualitative measure of the location of the quinone rings if other geometric factors are assumed to be the same. The quinone rings of longer-chain ubiquinones are normally buried deeper in the bilayer, but the high curvature on the inside of small vesicles allows the rings to come closer to the surface. This model explains why th einduced shift of the OCH_3 peak by external shift reagents decreases with increasing side-chain length (Tables II and III), why this chain length dependence is diminished when the shift reagent is equilibrated across the membrane, and why in freeze-thaw vesicles the effect of external shift reagents on Q_4 is the same as in small vesicles, whereas internal shift reagents have a decreased effect compared to small vesicles.

Differences between quinones and quinols

The lanthanide-induced shifts for ubiquinol OCH_3 peaks were 50—100% greater than for ubiquinone OCH_3 peaks, whether the lanthanide was only external or was equilibrated across the bilayer. Such a difference in induced shifts might have been caused by higher lanthanide binding by ubiquinols. However, the presence of Q_{10} or $Q_{10}H_2$ does not significantly affect the shifts of the choline methyl resonances by Dy^{3+}, implying negligible specific lantha-

nide binding by either quinones or quinols. It is thus reasonable to conclude that the time-averaged location of the quinol ring is closer to the membrane surface than is the location of the quinone ring.

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