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Determination of the critical micelle concentration of short-chain ubiquinones in model systems

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We have investigated the critical micelle concentrations of short-chain ubiquinone and ubiquinol homologs in water, aqueous buffers and ethanol-water mixtures. The physical state of ubiquinones becomes nonmonomeric at definite concentration that can be identified because the aggregation is accompanied by a red shift of λ_{max} and a decrease of the extinction coefficient. Systematic studies following the transition dipole moment against quinone concentration have established that the critical micelle concentration is a function of isoprenoid chain length and state of oxidation of the quinones and of the dielectric constant and ionic strength of the medium. The thermodynamics of micelle formation point out that the quinone ring has a strong tendency to be located in a hydrophobic environment.

Introduction

Exogenous ubiquinone (Q) homologs are often used to study electron transfer in mitochondrial or bacterial membranes, as well as in reconstituted or artificial systems in lipid vesicles [1–10]. A previous investigation on the partition of ubiquinones into lipid vesicles and mitochondrial membranes [11,12] established that under standard conditions similar to those employed for enzymatic studies, a conspicuous amount of ubiquinone is remaining in the water phase in a nonmonomeric organized state.

Although this study has been confirmed by other investigations [13,14], no information is available on the qualitative and quantitative aspects of the organization of the ubiquinone molecules in aqueous solutions.

The potential importance of such an investigation also stems from the uncertainties on the physical state and location of ubiquinone in the lipid bilayer of model and natural membranes [2,12,14–18]; it is largely such an uncertainty to hamper understanding of the mechanisms of electron transfer in the ubiquinone region [19–21].

Materials and Methods

Ubiquinones. Different ubiquinone homologs were kind gifts of Eisai Co., Tokyo, Japan. Their purity was verified by thin layer chromatography, by spectroscopic analysis, and by detection of the melting points. Ubiquinones were stored as solutions in absolute ethanol at −20°C at concentrations ranging between 5 and 35 mM as determined spectrophotometrically at 275 nm using the ex-

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Q_n, ubiquinone with n isoprenoid units; Q_nH_2, ubiquinol with n isoprenoid units.

tinction coefficients typical of each homolog [12,22] and according to Mayer and Isler [23].

Ubiquinones were reduced using the method of Rieske [24] and kept at −20°C in absolute ethanol under slight acidic conditions at concentrations between 5 and 15 mM. Ubiquinol solutions were stable for at least a week under the conditions used.

Solvents. All solvents were pure reagents of Merck and Carlo Erba.

Spectrophotometry. The spectrophotometric determinations of ubiquinone were performed in a Perkin-Elmer 559 UV/VIS spectrophotometer equipped with the accessory for display of the first derivative spectra. All measurements were carried out at 20°C. The ubiquinone aggregates, when present, produce remarkable light scattering; the scattering was corrected and the reliability of the correction was evaluated by the methods of Stoeckenius [25] and Eisenberg [26].

Determination of critical micelle concentration in water. All ubiquinone homologs are characterized by the same spectral properties, having a main absorbance peak in the 270–280 nm region, because of a series of $\pi \rightarrow \pi'$ electronic transitions of the benzoquinone ring. An increase of the polarity of the medium induces a progressive shift to longer wavelengths of the absorbance maximum owing to the stabilization effect of the polar solvent molecules on the transition dipole moment of the quinone chromophore. When quinone is in monomeric form there is a linear dependence [22] of the spectral parameters upon the polarity of the medium expressed as the specific refraction, r:

$$r = \frac{n^2 - 1}{n^2 + 1} \frac{1}{d} \tag{1}$$

where n = refractive index and d = density at 20°C [27]. While Q_1, being a relatively hydrophilic molecule [11,12,17,22,28] exhibits a typical spectrum with a maximum at 278 nm in water, all other ubiquinone homologs aggregate in aqueous media because of their lower water solubility, causing an alteration of the spectral features of the chromophore. In fact a red shift of the λ_{max} can be observed to around 284–286 nm, with reduction of the extinction coefficient [11,12,22].

Ubiquinols have the same behavior as the oxidised forms; in fact it is possible to observe large spectral changes in water due to aggregation phenomena with a red shift of λ_{max} towards 294–296 nm [12]. The spectroscopic properties of ubiquinones have been tested in several solvents [12]. Since the spectra appear of approx. Gaussian shape in all solvents tested, it is possible to describe the absorption curves of oxidised ubiquinones by the following equation [29,30]:

$$|\mu_i^2| = a \frac{\varepsilon_i \Delta_i}{\lambda_i} \tag{2}$$

where μ_i is the transition dipole moment induced by light, ε_i is the millimolar extinction coefficient at the absorption maximum i, λ_i is the wavelength of the absorption maximum in nm, Δ_i is the half-bandwidth at the λ_i/e (Neper's number) in nm, and a is a constant value. Aggregation was monitored by determining the breaks in the plots of $\varepsilon_i \Delta_i / \lambda_i$ vs. Q concentration. The first inflection in the plot was taken as the critical micelle concentration, where micelles start to appear. A second inflection was often present in the plots; the latter depends on the large increase of the ratio micelles/monomers, so that the value of $\varepsilon_i \Delta_i / \lambda_i$ for micelles becomes prevalent on the value for monomers.

Occasionally, aggregation was monitored by plotting either λ_{max} or absorbance as a function of Q concentration and detecting the inflection in the plots either from direct analysis of the spectra or from first derivatives.

Some experiments were performed in an ethanol-water mixture (66 : 34, v/v); in such experiments, micelle formation was diagnosed by the sharp increase of light scattering [31] measured at 90° [32] in a Perkin-Elmer MPF 4 spectrofluorimeter, at a wavelength of 606 nm, for both excitation and emission, to prevent contribution from absorption by Q at lower wavelengths. Either pure ubiquinones or ubiquinone-egg lecithin dispersions were employed in these experiments; to this purpose, egg lecithin was purified from egg yolks according to Robinson [33].

The apparent molecular weight of the micelles was determined in the light scattering experiments by the equation [34]:

$$R = \frac{2\pi^2 n_1^2}{N_L \lambda_0^4} \left(\frac{\Delta n}{\Delta c} \right)^2 \frac{c}{\frac{1}{M} + 2Ac} = \frac{Kc}{\frac{1}{M} + 2Ac} \tag{3}$$

where R = ratio of intensity of eemitted light to incident light; n_1 = refraction index of solvent; $\Delta n/\Delta c$ = increment of refraction index of solute (cm^3/g); λ_0 = wavelength in cm; N_L = Avogadro's number; c = concentration of solution (g/cm^3); M = molecular weight of solute; $K = (2\pi^2 n_1^2/N_L\lambda_0^4)(\Delta n/\Delta c)^2$ and A is an experimental constant.

In a plot of K_c/R as a function of c, the intercept on the ordinate axis gives $1/M$. From the apparent molecular weight of the micelles, the aggregation number (number of molecules per micelle) was readily obtained.

Critical micelle concentration for the very water soluble homologs Q_0 and Q_1 was calculated from the partition coefficients [14,35] according to the Eqn. [36]:

$$PS = 2 \qquad (4)$$

where S is the solubility in mol Q/mol water and P is the partition coefficient in (mol Q/mol PL)/(mol Q/mol water).

Reduction of ferricyanide by ubiquinols. Chemical reduction of ferricyanide by ubiquinol was performed in a medium containing 10 mM Hepes and 1 mM EDTA, at pH 7.5; the ionic strength was varied by additions of NaCl. The concentrations of potassium ferricyanide and ubiquinol were varied as described in the individual experiments. The reaction was followed spectrophotometrically at 420–500 nm in a Sigma ZWS dual-wavelength spectrophotometer equipped with a rapid mixing apparatus of our own design (mixing time, approx. 200 ms); the extinction coefficient used was 1 $mM^{-1} \cdot cm^{-1}$.

Results

For the low homologs Q_2 andQ_3, both oxidised and reduced, it is possible to follow the spectral changes due to aggregation in water by monitoring their spectral parameters as a function of concentration (cf. Methods). The ultraviolet band of the oxidised quinones progressively shifts its maximum from 278 nm to $\geqslant$ 282 nm, with a concomitant decrease of the apparent extinction coefficient (cf. Fig. 1 for Q_2); an apparent critical micelle concentration can be extrapolated from the inflection in the plots. The parameter which has been found, however, to best reflect the aggregation phenomenon is $\varepsilon_i\Delta_i/\lambda_i$ as a function of Q concentration (Fig. 2). The results obtained in different aqueous solutions are reported in Table I. It can be observed that the critical micelle concentrations are higher for ubiquinols than for the oxidised forms, in accordance with their more hydrophilic properties; furthermore, the values are inversely proportional to the side chain lengths of the homologs.

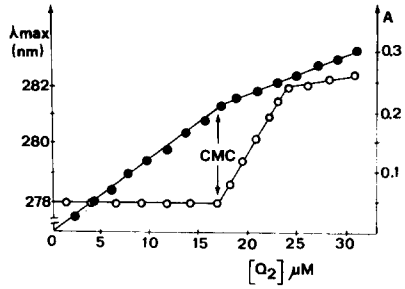

Fig. 1. Effect of Q_2 concentration in aqueous medium on λ_{max} (○) and absorbance at λ_{max} (●). CMC = critical micelle concentration.

The effect of pH could be investigated only for oxidised ubiquinone, but no significant differences were obtained. For example the critical micelle concentration of Q_2 ranged between 11 and 17 μM in potassium phosphate buffer between pH 6 and 8. A detailed pH dependence study of micelle formation could not be performed for ubiquinols,

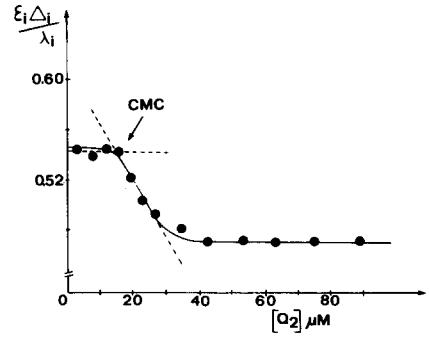

Fig. 2. Effect of Q_2 concentration in aqueous medium on the parameter $\varepsilon_i\Delta_i/\lambda_i$ obtained from spectrophotometric determinations (see text for explanations). CMC = critical micelle concentration.

TABLE I

CRITICAL MICELLE CONCENTRATIONS (μM) OF OXIDISED AND REDUCED SHORT CHAIN UBIQUINONE HOMOLOGS IN TWO DIFFERENT AQUEOUS MEDIA

Homolog	0.5 M potassium acetate (pH 6.0)	25 mM KH_2PO_4/ 1 mM EDTA (pH 7.4)
Q_2	9.2	14
Q_2H_2	12	40
Q_3	≤ 0.65	≤ 0.9
Q_3H_2	1.2	3.3

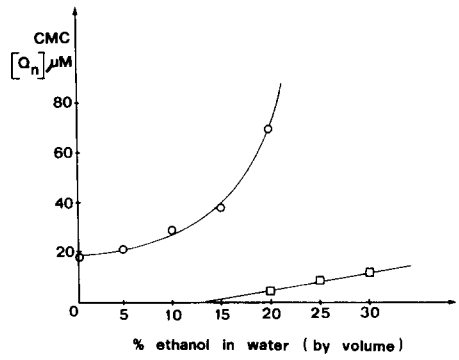

Fig. 4. Effect of ethanol concentration in water on the critical micelle concentration (CMC) of Q_2 (○) and Q_3 (□).

because in most media they were rapidly autooxidised (less than 1 min). Particularly in the very high pH range, where ubiquinols are ionised [12], autooxidation was so fast that no absorption spectrum could be monitored. For this reason, we have consistently determined critical micelle concentrations in pure aqueous media (bidistilled water).

The free energy of micelle formation is

$$\Delta G_{mic} = RT \ln X_{CMC} = \Delta H_{mic} - n_1 T \Delta S_{mic} \quad (5)$$

where X_{CMC} is the critical micelle concentration expressed in Moles per mole of water.

In analogy to micelle formation of lipids [37], ΔG_{mic} was assumed to be separable into ΔH_{mic}, the enthalpy of transfer of the quinone ring into an aggregated, more hydrophobic micellar environment and $n_1 T \Delta S_{mic}$, the entropic contribution of the n_1 isoprenoid units. ΔG_{mic} has been plotted as a function of isoprenoid chain length (Fig. 3). The intercept on the ordinate axis gives the free energy of location of the quinone ring in a hydrophobic phase. For the oxidised quinone ring, such a plot extrapolates to -20.8 kJ · mol^{-1}, a value identical to that obtained from the solubility of Q_0 [35], that has a hydrogen atom in place of the isoprenoid sidechain, indicating that the free energy required to remove the quinone ring from a self aggregated, hydrophobic phase into water is rather high. The critical micelle concentration of Q_1 and Q_2 in water have been calculated from their known partition coefficients according to Eqn. 4.

In addition to the determination of critical micelle concentrations in pure water, we have performed experiments in media containing different water-ethanol ratios.

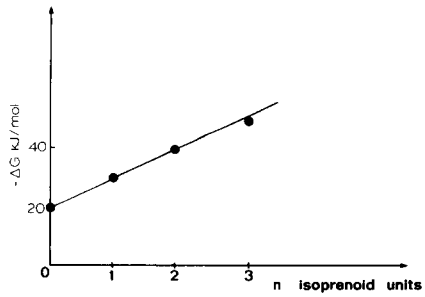

Fig. 3. Relation between the isoprenoid chain length of ubiquinone homologs and the thermodynamics of micelle formation (ΔG_{mic}).

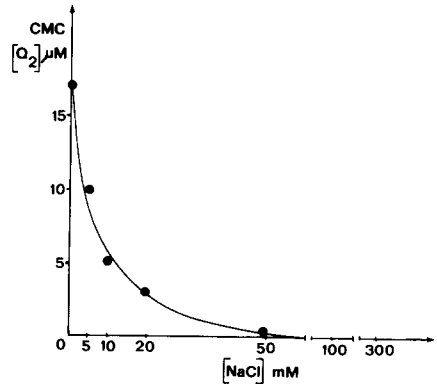

Fig. 5. Effect of ionic strength (NaCl) on the critical micelle concentration (CMC) of Q_2 in bidistilled water.

TABLE II

CRITICAL MICELLE CONCENTRATION (μM) OF DIFFERENT UBIQUINONE HOMOLOGS IN AQUEOUS MEDIA CONTAINING DIFFERENT PROPORTIONS OF ETHANOL

Homolog	% Ethanol in water (by volume)						
	0	5	10	15	20	25	30
Q_2	17	29	38	69.5	–	–	–
Q_3	–	–	–	–	4.23	9.4	10.5
Q_4	–	–	–	–	–	< 0.6 [a]	< 0.6 [b]
Decylbenzoquinone	–	–	4.78	–	7.06	–	–

[a,b] The first inflection in the $\varepsilon_i \Delta_i / \lambda_i$ vs. ubiquinone concentration plots was undetectable; the second inflection was detected at 8 μM [a] and 10 μM [b].

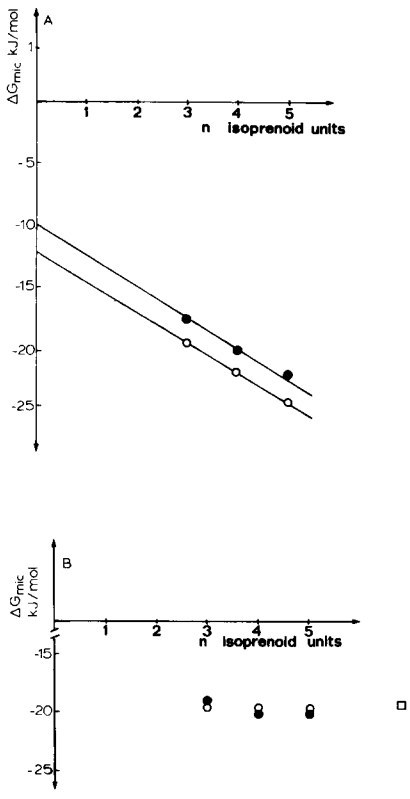

Fig. 6. (A) Thermodynamics of micelle formation in ethanol/water (66:34, v/v) for ubiquinones and ubiquinols. Closed symbols, reduced Q homologs; open symbols, oxidised Q homologs. (B) Thermodynamics of micelle formation in ethanol/water (66:34, v/v) for mixed ubiquinone and ubiquinol dispersions with egg lecithin. Closed symbols, reduced Q homologs; open symbols, oxidised Q homologs. □ stands for ΔG for micelle formation of lecithin alone.

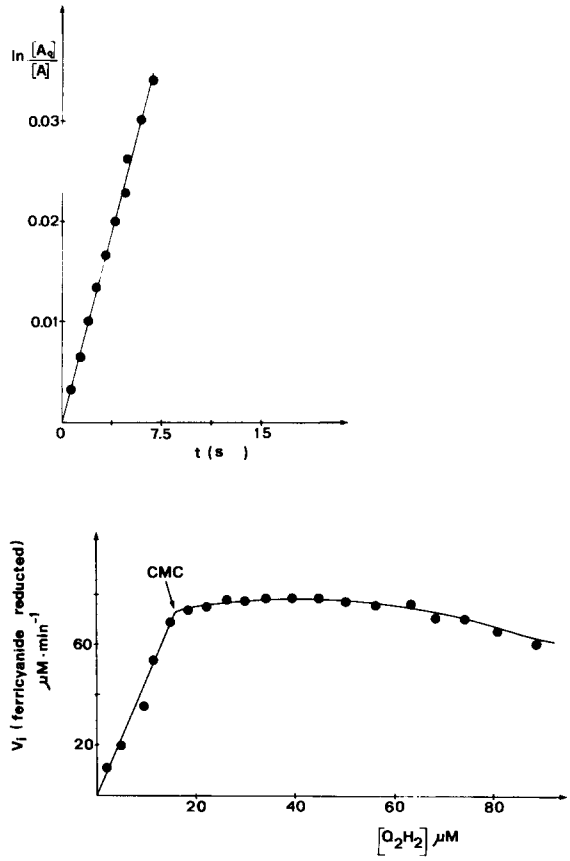

Fig. 7. (A) First-order plot of the reaction of ubiquinol-2 (10 μM) with potassium ferricyanide (250 μM). The medium contained 10 mM Hepes/90 mM NaCl/1 mM EDTA (pH 7.5). (B) Pseudo-first-order rate constants of potassium ferricyanide (250 μM) reduction by different concentrations of ubiquinol-2. CMC = critical micelle concentration.

The addition of ethanol causes an increase of the critical values because of its capability of lowering the dielectric constants of the medium. In this way it is possible to investigate the behavior of the higher homolog Q_4, which aggregates in water at so low concentration to be undetectable with the available instruments. The results are displayed in Fig. 4 and Table II.

It can be noticed that the critical micelle concentrations are proportional to the increase of the percentage of ethanol (i.e., inversely proportional to the polarity of the medium).

The effect of the ionic strength, changed by additions of NaCl, is shown in Fig. 5: increase of salt concentration dramatically decreases the critical micelle concentration, which is already undetectable at 50 mM salt. Similar effects, although with a shift to higher concentrations, are obtained in water-ethanol mixtures. To this purpose we have observed that even Q_1, which has a high water solubility (approx. 1 mM), is aggregated in 800 mM NaCl with a critical micelle concentration of 85 μM.

We have also investigated the properties of different ubiquinone homologs in ethanol/water (66 : 34, v/v) either alone or mixed with lecithin. Under such conditions, the behavior of ubiquinone homologs from one to five isoprenoid units could be investigated.

The critical micelle concentration, investigated by light-scattering measurements, appears to depend on the quinone hydrophobicity and is always higher for the reduced forms than for the oxidized forms (Table III). From the critical micelle concentrations, using Eqn. 5, we have calculated the thermodynamics of removal of the quinone ring from the ethanol/water phase into a hydrophobic medium, and the contribution of the isoprenoid untis (Fig. 6A); it is calculated from the data, by extrapolation to zero isoprenoid units, that the quinone ring in its oxidised form extrapolates to a slightly more negative free energy of transfer from a hydrophobic medium than in its reduced form, in accordance with a slightly higher polarity of the ubiquinols. In mixed lecithin-ubiquinone micelles, the critical micelle concentrations appeared largely independent of the quinone hydrophobicity, contrary to pure Q micelles in the same system, suggesting a non ideal mixing of the two components, especially for longer Q homologs, where a strong decrease in critical micelle concentration would be expected otherwise. The ΔG values for

TABLE III

CRITICAL MICELLE CONCENTRATIONS OF UBIQUINONES AND UBIQUINONE-EGG LECITHIN MIXTURES IN ETHANOL-WATER (63 : 34, v/v) DETERMINED BY LIGHT SCATTERING AT 90°

X_{CMC} = CMC/$n_{A/W}$, where CMC is the critical micelle concentration, and $n_{A/W}$ is the molarity of the alcohol/water mixture (31.5 M). N = aggregation number, calculated from the apparent molecular weight of the micelle (Eqn. 3).

System	CMC (mM)	X_{CMC}	ΔG_{mic} (kJ/mol)	N
Q_3	10.75	$3.41 \cdot 10^{-4}$	−19.7	483
Q_4	4.2	$1.33 \cdot 10^{-4}$	−22.2	–
Q_5	1.63	$0.52 \cdot 10^{-4}$	−24.3	–
Ubiquinol-3	24.0	$7.60 \cdot 10^{-4}$	−17.6	13
Ubiquinol-4	8.38	$2.66 \cdot 10^{-4}$	−20.1	252
Ubiquinol-5	4.2	$1.33 \cdot 10^{-4}$	−22.2	–
Lecithin	10.38	$3.30 \cdot 10^{-4}$	−20.1	39
Q_3-Lecithin (1 : 1)	10.46	$3.32 \cdot 10^{-4}$	−19.7	252
Q_4-Lecithin (1 : 1)	11.0	$3.45 \cdot 10^{-4}$	−19.7	–
Q_5-Lecithin (1 : 1)	9.9	$3.15 \cdot 10^{-4}$	−20.1	–
Ubiquinol-3-lecithin (1 : 1)	13.87	$4.37 \cdot 10^{-4}$	−19.3	89
Ubiquinol-4-lecithin (1 : 1)	9.2	$2.92 \cdot 10^{-4}$	−20.1	–
Ubiquinol-5-lecithin (1 : 1)	9.0	$2.85 \cdot 10^{-4}$	−20.1	–
Q_3-Ubiquinol-3-lecithin (1 : 1 : 2)	13.54	$4.30 \cdot 10^{-4}$	−19.3	428

micelle formation of mixed Q-lecithin systems are almost independent of the redox state and the number of isoprenoid units at least in the range 3–5 experimentally accessible, and equal to that of lecithin alone (Fig. 6B).

From such data, the ΔG_{mic} of the different Q homologs minus ΔG_{mic} for lecithin alone, extrapolated to zero isoprenoid units, would yield about zero.

An alternative method to measure the critical micelle concentration for reduced ubiquinones was found in the saturation of the chemical reduction of potassium ferricyanide by Q homologs at the onset of aggregation of the quinol. At high ferricyanide concentration the reaction was pseudo-first order (Fig. 7A) with increase of the apparent first-order rate constant at increasing ubiquinol concentration. As shown in Fig. 7B, however, a sudden saturation of the rate constant occurs at a quinol concentration corresponding to the critical micelle concentration measured by direct spectrophotometry. Since the correlation with the critical micelle concentration obtained under different conditions (e.g., varying ionic strength) is very strong, it is concluded that only the quinol monomers are capable of interacting with ferricyanide, whereas the hydroquinone ring in the micelles is not available for reaction.

Discussion

The spectral parameters employed in this study are sensitive enough to yield information on the physical state of the quinones; the results obtained by the shift in absorbance maximum and in extinction coefficient are superimposable; however, the most reproducible data were obtained with the parameter $\varepsilon_i \Delta_i / \lambda_i$, that shares three spectral parameters simultaneously.

The present study on determination of critical micelle concentration for the Q homologs in aqueous media shows that the free energy for transfer of the quinone ring from water to a hydrophobic medium is rather negative, allowing to postulate that, in membranes, it is largely located in the hydrophobic portion. From the micellization properties of the mixed lecithin-ubiquinone dispersions in ethanol-water, the critical micelle concentration appears largely independent of quinone chain length, contrary to pure Q micelles in the same solvent. The results suggest that, if the ubiquinone molecules stack between the phospholipid molecules, they do so by keeping the quinone ring in a nonpolar phase, and the critical length of the isoprenoid chain for stacking is 3–4 units, the remainder of the Q molecules being accommodated in such a way that it does not contribute to the net thermodynamcis of the system. The ultraviolet spectra of Q homologs in lecithin micelles in ethanol-water indicate two environments of different polarity [38], while in lecithin bilayers one environment of nonpolar characteer is indicated [11,12]. It may be inferred from these studies that ubiquinone may be realistically considered to be present with its quinone ring in a nonpolar environment in the membrane. Other studies have reached similar conclusions [13,39] although the location of the quinone ring near the membrane surface was advocated in other studies [14,15].

The lack of interaction of ubiquinol in lipid bilayers with exogenous ferricyanide is taken as a proof for the situation of the hydroquinone ring in the depth of the lipid bilayer [38]; the finding in the present study that ubiquinol micelles do not react with ferricyanide, however, points out that additional reasons make the quinol unreactive with ferricyanide, since it is likely that the Q micelles have the relatively polar quinone group still exposed to the water medium. The stacking of the quinone rings with each other or with the phospholipid molecules (in lipid bilayers) may be responsible for such chemical unreactivity. The chemical reactivity of hydroquinones was studied by Rich and Bendall [40,41], who, however, failed to show saturation of the reaction, due to the use of more hydrophilic quinol derivatives, not showing aggregation under the conditions used.

The present investigation has some consequences of practical character.

Firstly, we have demonstrated that some buffers, usually employed in enzymatic studies, favor rapid autooxidation of ubiquinols; such buffers, therefore, should be used only in case of rapid kinetic studies. Moreover, it is clear from the values reported in Table I, that at the concentrations used in enzymatic studies, only Q_1 and Q_2 have a reasonable chance to be monomeric,

whereas all other homologs are certainly in aggregated form, so that the kinetic values obtained may be of rather doubtful significance. The unreactivity of the micelles with an artificial oxidant, as ferricyanide (Fig. 7B), strongly suggests that similar unreactivity could occur with the natural enzyme systems. The solubility of Q_1 is assumed to be very high, and also Q_2 is believed to be sufficiently water-soluble to be employed without risks in enzymatic assays. It should be noted, however, that at high ionic strengths the critical micelle concentrations fall dramatically, so that an unsuspected aggregation could strongly alter the kinetic data.

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